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Hepatoprotective effect and mechanistic insights of deoxyelephantopin, a phyto-sesquiterpene lactone, against fulminant hepatitis☆

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Abstract

Deoxyelephantopin (DET) is an abundant sesquiterpene lactone isolated from an anecdotally hepatoprotective phytomedicine, *Elephantopus scaber*. Our objective in this study was to provide scientific evidence for the *in vivo* efficacy and the underlying mechanisms of action of DET in lipopolysaccharide/D-galactosamine (LPS/D-GalN)-induced fulminant hepatitis. We investigated both the protective effect of pretreatment with DET (10 mg/kg body weight, Pre-DET10) prior to administration of LPS/D-GalN and the therapeutic effect of treatment with 10 mg/kg DET (Post-DET10) or the hepatoprotective drug silymarin (Post-SM10) following the administration of LPS/D-GalN. Our data showed that Pre-DET10 prevented LPS/D-GalN-induced infiltration of F4/80 monocytes/macrophages and an increase of nitrotyrosine and cyclooxygenase-2 protein in liver tissues. Further, Post-DET10 and Psot-SM10 treatments protected against liver cell apoptosis. All three treatments suppressed serum aminotransferase activities, tumor necrosis factor-alpha and interleukin-6 levels, and serum and hepatic matrix metalloproteinase-9 activity. The Pre-DET10 or Post-DET10 and Post-SM10 treatments in combination with inhibition of heme oxygenase-1 expression ultimately decreased protection of mice from LPS/D-GalN-induced mortality, with decreased survival from 75% and 62.5% to 50%, respectively. Results obtained from serial liver scintigraphy with ^{99m}Tc-diisopropyl iminodiacetic acid (DISIDA) on single-photon emission computed tomography analysis showed that both liver uptake and excretion times of DISIDA were significantly delayed in LPS/D-GalN-treated animals and were effectively recovered by DET and silymarin treatment. This report demonstrates that DET functions in the modulating multiple molecular targets or signaling pathways that counteract inflammation during the progression of fulminant hepatitis and may serve as a novel lead compound for future development of anti-inflammatory or hepatoprotective agents.

Keywords: Deoxyelephantopin; Hepatoprotection; Fulminant hepatic failure; Hepatobiliary scintigraphy; DISIDA; *Elephantopus scaber*

1. Introduction

Due to a lack of effective therapy, fulminant hepatic failure (FHF) has an extremely poor prognosis and high mortality, and accumulating evidence indicates that endotoxemia and sepsis occur frequently in patients [1,2]. At present, no specific therapy is available other than liver transplantation [3]. Therefore, there is an urgent need for effective therapies against FHF. Rodents treated with D-galactosamine (D-GalN) show marked sensitization to lipopolysaccharide (LPS) and potentiation of tumor necrosis factor-alpha (TNF-α)-induced hepatocyte apoptosis [4], and therefore, this experimental fulminant hepatitis model is widely used to investigate the underlying mechanisms of clinical FHF and to develop effective therapeutic strategies for endotoxin challenge [5].

Recently, there has been increased interest in the role of improper activation or up-regulation of inducible nitric oxide synthase (iNOS) or cyclooxygenase-2 (COX-2) in the pathogenesis of inflammatory

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; COX-2, cyclooxygenase-2; DAPI, 4,6-diamidino-2-phenylindole; DET, deoxyelephantopin; FHF, fulminant hepatic failure; D-GalN, D-galactosamine; HO-1, heme oxygenase-1; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MMP-9, matrix metalloproteinase-9; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF-κB, nuclear factor-kappaB; Poly (adenosine diphosphate-ribose) polymerase, PARP; SOCS3, suppressor of cytokine signaling-3; SPECT, single-photon emission computed tomography; DISIDA, ^{99m}Tc-diisopropyl iminodiacetic acid; STAT3, signal transducer and activator of transcription 3; TIMP-1, tissue inhibitor of metalloproteinase 1; TNF-α, tumor necrosis factor-alpha; ZnPP, zinc protoporphyrin IX.

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disorders, including toxin-induced liver damage [6]. The iNOS-catalyzed oxidative deamination of L-arginine to produce nitric oxide (NO) after exposure to proinflammatory cytokines (e.g., TNF) or endotoxins (e.g., LPS) has been shown to trigger pathological cellular responses and may result in inflammation, sepsis and various liver injuries such as ischemia–reperfusion injury and FHF [7]. COX-2 is another important inflammatory mediator through its rate-limiting synthesis of the precursors of prostaglandins and thromboxanes [8]. Nonsteroidal anti-inflammatory drugs such as indomethacin, which inhibits COX-2 activity, are associated with reduced incidence of LPS-induced liver injury [9].

Elephantopus scaber L. (Asteraceae) is a popular perennial medicinal plant with anecdotal effectiveness in treating diuresis, infection and hepatitis. Two major germacranolide sesquiterpene lactones isolated from *E. scaber* have been shown to possess anticancer activity. Isodeoxyelephantopin potentiates TNF-α-induced apoptosis in leukemia KBM-5 cells [10], and its isomer, deoxyelephantopin (DET), inhibits the growth of HeLa cells *in vitro* and in xenografted mice [11]. Recently, we elucidated the novel functions of DET in suppressing growth and metastasis of murine mammary adenocarcinoma TS/A cells *in vitro* and *in vivo* by targeting multiple signaling pathways in cancer cells [12,13]. The anti-inflammatory and hepatoprotective effects of *E. scaber* extracts have been investigated [14,15]; however, to our knowledge, no reports describe its bioactive chemical constituents against inflammatory liver damage.

The aims of the current study were (a) to investigate the beneficial effect and the underlying pharmacological mechanisms of the major phytocompound DET from *E. scaber* on fatal, acute liver failure in LPS/D-GalN-induced mice and, in particular, examine the modulation of proinflammatory mediators and inflammatory pathology in liver tissues and (b) to evaluate the feasibility of using liver scintigraphy of single-photon emission computed tomography (SPECT) with ^{99m}Tc-diisopropyl iminodiacetic acid (DISIDA) in a fulminant hepatitis mouse model and determine the feasibility of its application in monitoring the therapeutic potential of the phytoagent DET in the management of fulminant liver failure.

2. Materials and methods

2.1. Reagents and antibodies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), D-GalN, LPS, silymarin (SM), 4,6-diamidino-2-phenylindole (DAPI) and zinc protoporphyrin IX (ZnPP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Silica gel, RP-18 F_{254} TLC plates (Merck, Germany) and RP-18 silica gel (Cosmosil, Japan) were used. All other chemicals and solvents were of reagent or high-performance liquid chromatography grade. Molecular weights in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) experiments were estimated by comparison to a prestained protein ladder (Bioman, Taipei, Taiwan). Primary antibodies against heme oxygenase 1 (HO-1), inhibitor of nuclear factor-κB α isoform (IκBα), c-Jun N-terminal kinase (JNK)1/2, p65, signal transducer and activator of transcription 3 (STAT3) and tissue inhibitor of metalloproteinase 1 (TIMP-1) were obtained from Santa Cruz Biotechnology, while cleaved caspase-3, iNOS, phospho-IκBα, phospho-STAT3, poly(-adenosine diphosphate-ribose) polymerase (PARP), proliferating cell nuclear antigen (PCNA), and suppressor of cytokine signaling-3 (SOCS3) were from Cell Signaling Technology. Additional antibodies used were COX-2 (Cayman Chemical), matrix metalloproteinase (MMP)-9 (Abcam), nitrotyrosine (Upstate), F4/80 (eBioscience) and actin (Chemicon). Recombinant murine TNF-α and enzyme-linked immunosorbent assay (ELISA) kits for murine TNF-α and IL-6 were from R&D Systems, Inc. (Minneapolis, MN, USA). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined by use of commercial kits from Randox Laboratories (UK).

2.2. Isolation and structure elucidation of DET

The molecular weight of DET is 344. The protocols for extraction and identification of DET from the *E. scaber* plant followed the method described by Huang et al. [12]. The compound purity was >98% as judged by liquid chromatography/mass spectrometry and nuclear magnetic resonance spectral data.

2.3. Cell culture

The murine macrophage RAW 264.7 and FL83B hepatocyte cell lines were obtained from the ATCC (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's Medium (Gibco/BRL) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator.

2.4. Measurement of NO production and cell viability

Macrophages were treated with DET for 1 h and then incubated for 24 h with or without LPS. Nitrite levels in cell culture medium were determined by the Griess reaction. Cell viability was examined by MTT assay.

2.5. Immunofluorescent cell staining

Macrophages were seeded on 12-mm glass slips in 24-well plates for 2 h and then treated with vehicle or DET (5 μg/ml) for 1 h, followed by LPS (1 μg/ml) stimulation for an additional 30 min. Cells were fixed, permeabilized with 0.1% Triton X-100 and stained with DAPI (nuclear marker), anti-tubulin antibody (cytoplasmic marker) or anti-p65 antibody, then visualized with goat anti-mouse fluorescein isothiocyanate (FITC)-labeled or goat anti-rabbit Cy3-labeled secondary antibodies (Jackson ImmunoResearch). The nuclear translocation of nuclear factor-kappaB (NF-κB) p65 was visualized at 400× magnification using a fluorescent microscope (Nikon Eclipse E800) equipped with a CCD camera (Nikon DXM1200).

2.6. Structural modeling of the binding of DET to NF-κB p65 (RelA)

Molecular docking was performed with the computer program Discovery Studio Client 2.5 (Accelrys, Inc., San Diego, CA, USA). The core domain in subunit A of the crystal structure of NF-κB p65 (RelA) in complex with DNA adopted from the Protein Data Bank (PDB: 1ram) (http://www.rcsb.org/pdb/explore/explore.do?structureId=1RAM) was used for modeling analysis. Docking experiments were performed using an automated CDOCKER subprogram to fit DET into RelA in a three-dimensional mode. Docking runs were carried out using a standard default setting ("site opening" of 12 Å, "binding site" selected).

2.7. Animals

Male ICR mice (6 weeks old) were supplied from the Laboratory Animal Center (National Taiwan University, Taipei, Taiwan) and given a standard laboratory diet and distilled water *ad libitum* and kept on a 12-h light/dark cycle at 22°C±2°C. This study was conducted according to the institutional guidelines and approved by the Institutional Animal Care and Utilization Committee of Academia Sinica, Taiwan.

2.8. In vivo effects of DET on acute fulminant hepatitis and survival rate of test animals

The *in vivo* therapeutic potential of DET on LPS/D-GalN-induced fulminant hepatitis was investigated and compared with the hepatoprotective agent SM. Twenty-four mice were randomly assigned to four groups (n=6 per group) for treatment: vehicle, LPS/D-GalN, 10 mg/kg SM (Post-SM10) and 10 mg/kg DET (Post-DET10). The mice were treated with 500 ng LPS and 25 mg D-GalN in 250 μl saline, and 1 h later, DET and SM were administered intraperitoneally (ip) as described previously [16]. In parallel, 24 mice were randomly assigned to three groups for treatment (n=8 per group): vehicle, LPS/D-GalN and pretreatment with DET10 (Pre-DET10). Test animals were pretreated ip with DET10 for 3 consecutive days and 1 h before LPS/D-GalN treatment to evaluate the protective effect of DET. Blood samples were collected by retroorbital bleeding 8 h after LPS/D-GalN injection; then all mice were killed, and liver tissues were collected.

2.9. Survival study

FHF is associated with a high mortality rate; therefore, we compared the therapeutic effect of DET and SM on LPS/D-GalN-induced mortality. A total of 72 mice were randomly assigned to eight groups (n=8 per group) for treatment: (1) vehicle, (2) LPS/D-GalN, (3) treatment with DET10 following LPS/D-GalN (Post-DET10), (4) treatment with SM10 following LPS/D-GalN (Post-SM10), (5) pretreatment with DET10 (Pre-DET10), (6) ZnPP+LPS/D-GalN, (7) Pre-DET10+ZnPP, (8) ZnPP+Post-DET10 and (9) ZnPP+Post-SM10. DET10 and SM10 (10 mg/kg each) dissolved in dimethyl sulfoxide were delivered ip 1 h before or after LPS/D-GalN administration. For Pre-DET10+ZnPP treatment, ZnPP (25 mg/kg) was coadministered ip with DET10 30 min before LPS/D-GalN challenge. For ZnPP+Post-DET10 treatment, ZnPP was administered 30 min before LPS/D-GalN challenge and DET10 treatment. Mortality was monitored for 48 h after LPS/D-GalN administration.