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# Activation of Fas death receptor pathway and Bid in hepatocytes is involved in saikosaponin D induction of hepatotoxicity

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ABSTRACT

Drug-induced liver injury can lead to acute liver failure. Saikosaponin D (SSD) is a major component isolated from the medicinal herb *Bupleurum falcatum*, which has been linked to hepatotoxicity. We previously reported that SSD disrupted PDGF-βR pathway leading to mitochondrial apoptosis in human LO2 hepatocytes. The present study was aimed at further exploring the underlying mechanisms *in vitro* and *in vivo*. We initially determined the concentration range of SSD at up to 2 μM for subsequent apoptosis examinations. SSD significantly upregulated Fas expression, promoted caspase-8 cleavage and activated the pro-apoptotic protein Bid in LO2 cells. Moreover, SSD reduced the abundance of cytochrome c in mitochondria and increased the cleaved-caspase-3 in LO2 cells, but did not apparently affect PI3K/AKT, ERK and STAT3 pathways that are involved in cell fate regulation. Experiments *in vivo* showed that one-week treatment with SSD at 300 mg/kg significantly elevated the liver/body weight ratio and caused histological injury in mouse liver. Furthermore, SSD treatment induced massive hepatocyte apoptosis, and significantly downregulated Bcl-2 but upregulated Bax in mouse liver. Taken together, these results revealed a specific mechanism of activation of extrinsic apoptosis pathway and Bid by SSD, which was involved in SSD-induced mitochondrial apoptosis in hepatocytes and potential hepatotoxicity.



## 1. Introduction

Drug-induced liver injury (DILI) represents a leading cause of acute liver failure. It is also an important cause of discontinuation of drugs in development, or withdrawal or restricted use after marketing. DILI is commonly classified into intrinsic hepatotoxicity and idiosyncratic hepatotoxicity. The former is commonly dose-dependent and predictable above an approximate threshold dose; whereas the latter occurs in an unpredictable fashion and without obvious dose-dependency (Devarbhavi, 2012). Currently, with the prevalence of additional consumption of herbal products in drugs and dietary supplements worldwide, many herbal remedies and natural compounds have been shown to cause liver injury known as herbal hepatotoxicity, which has gained much attention in the field of DILI (Bunchorntavakul and Reddy, 2013).

Hepatocyte apoptotic death is a characteristic of DILI. Mitochondria are thought to play a central role in DILI because mitochondria are the major intracellular organelles mediating the majority of apoptotic pathways in mammalian cells (Pessayre et al., 2012). There are two well-defined pathways of apoptosis: the intrinsic pathway and the extrinsic pathway. The former (also called mitochondrial apoptosis) is mediated by the Bcl-2 family proteins that act as sensors to integrate death and survival signals. Reduction in Bcl-2/Bax ratio leads to release of cytochrome c from mitochondria into cytoplasm and activates caspase cascade culminating in cellular fragmentation (Kuwana and Newmeyer, 2003). The extrinsic apoptotic pathway is activated upon the binding of cytokine ligands (*i.e.*, FasL and TNFα) to the death receptors (*i.e.*, Fas and TNF receptors) (Thorburn, 2004). There is also delicate coordination

*Abbreviations:* DILI, drug-induced liver injury; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HE, hematoxylin–eosin; LDH, lactate dehydrogenase; PDGF-βR, platelet-derived growth factor-β receptor; PI3K, phosphoinositide 3-kinase; SSD, saikosaponin D; STAT3, signal transducers and activators of transcription 3; tBid, C-terminal truncated Bid.

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and crosstalk between the extrinsic and intrinsic pathways, leading to the activation of caspase cascade (Khosravi-Far and Esposti, 2004). Notably, the BH3-only protein Bid serves as the fundamental link between death receptor and mitochondria pathway. Upon cleavage by caspase-8 in response to death receptor signals, the C-terminal truncated Bid (tBid) acquires a strong propensity to bind to mitochondria, where it induces outer membrane permeabilization and promotes cytochrome c release and other mitochondrial apoptotic events (Kaufmann et al., 2012). Increasing evidence has suggested the role of Bid in Fas-mediated hepatocyte apoptosis (Akazawa and Gores, 2007; Yin and Ding, 2003).

*Bupleurum falcatum* is a medicinal herb frequently used in Chinese herbal prescriptions. However, an epidemiological study showed that prescribing Chinese herbal prescriptions containing this herb in hepatitis B virus-infected patients might increase their risk of liver injury (Lee et al., 2011). Saikosaponin D (SSD), a triterpene saponin with steroid-like structure, is a primary component isolated from *Bupleurum falcatum* and responsible for the bioactivity of this herb (Huang et al., 2008). We postulated that SSD might mediate the potential hepatotoxicity of *Bupleurum falcatum*, and have reported that SSD inhibited cell proliferation and stimulated caspase-dependent mitochondrial apoptosis in hepatocytes, which was associated with inhibition of platelet-derived growth factor-β receptor (PDGF-βR) signaling (Chen et al., 2013a). The current study was aimed at further exploring the mechanisms underlying SSD-induced hepatocyte apoptosis highlighting the crosstalk between death receptor and mitochondrial pathway, and to evaluate the SSD hepatotoxicity in mice.

## 2. Materials and methods

### 2.1. Reagents and antibodies

SSD was purchased from Sichuan Victory Biological Technology Co., Ltd. (Chengdu, China). It was dissolved in dimethylsulfoxide (DMSO; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for *in vitro* experiments. Treatment with DMSO alone was used as vehicle control in all *in vitro* experiments in the present study. The following primary antibodies were used in this study: Fas, tBid, and cytochrome c (Signalway Antibody, College Park, MD, USA); cleaved-caspase-8, cleaved-caspase-3, p-PI3K, PI3K, p-AKT, AKT, p-ERK, ERK, p-STAT3, STAT3, Bcl-2, Bax, COX IV, and β-actin (Cell Signaling Technology, Danvers, MA, USA).

### 2.2. Cell culture

Human hepatocytes LO2 cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and was cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Wisent Biotechnology Co., Ltd., Nanjing, China), 100 U/ml penicillin and 100 mg/ml streptomycin, and grown in a 95% air and 5% $CO_2$ humidified atmosphere at 37 °C.

### 2.3. Lactate dehydrogenase (LDH) release assay

LO2 cells were seeded in 96-well plates and incubated in DMEM supplemented with 10% FBS for 24 h, and then were treated with DMSO (0.02%, w/v) or SSD at indicated concentrations (0.1, 0.2, 0.4, 0.8, 1, 2, 4, and 8 μM) for 24 h. LDH activities in culture medium were determined using a LDH release assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the protocols. Results were from triplicate experiments.

### 2.4. Immunofluorescence staining

LO2 cells were seeded in 24-well plates and cultured in DMEM with 10% FBS for 24 h, and then were treated with DMSO (0.02%, w/v) or SSD at 2 μM for 24 h. Immunofluorescent staining with primary antibody against Fas, and in succession with fluorescence-conjugated secondary antibodies (Biogot Biotechnology CO., Ltd., Nanjing, China) was performed. The nucleus was stained with the Hoechst 33342 reagent (Beyotime Institute of Biotechnology, Haimen, China). Images were taken at random fields. Results were from triplicate experiments.

### 2.5. Experimental animal procedures

All experimental procedures were approved by the Institutional and Local Committee on the Care and Use of Animals of Nanjing University of Chinese Medicine (Nanjing, China), and all animals received humane care according to the National Institutes of Health (USA) guidelines. Twenty male ICR mice (18–22 g body weight) were obtained from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China). They were kept in an environmentally controlled room (23 ± 2 °C, 55 ± 10% humidity) with a 12 h light/dark cycle and allowed free access to food and water. After one week adaptive feeding, they were randomly divided into two groups ($n = 10$). Group 1 was the vehicle control in which mice were orally administrated with normal saline of the same volume of SSD suspension. Group 2 was the SSD treatment group in which mice were orally administrated with SSD suspended in normal saline at 300 mg/kg. This dose was based on our pre-experiments for detecting the acute $LD_{50}$ value and the maximum tolerated dose in mice. SSD was given once daily for one week. At the end of experiment, mice were weighted, and sacrificed after anesthetized by intraperitoneal injection with 1% pentobarbital (50 mg/kg), and livers were isolated for calculation of liver/body weight ratio. A small portion of the liver was removed for histopathological analyses by fixation with 10% formalin. The remaining liver was cut in pieces and rapidly frozen with liquid nitrogen for extraction of hepatic proteins.

### 2.6. Hematoxylin–eosin (HE) staining

Liver slices were prepared and stained with HE using standard methods. After washes, sections on the slides were dehydrated in 100% ethanol and in xylene, and then they were mounted in Permount. Photographs were taken in a blinded fashion at random fields. Representative views of liver sections are shown.

### 2.7. TUNEL staining

Liver slices were stained with the TUNEL reagents using a staining kit (Nanjing KeyGen Biotechnology CO., Ltd., Nanjing, China) according to the protocols. Morphology of apoptotic cells was photographed using a fluorescence microscope (Nikon, Tokyo, Japan) in a blinded fashion at random fields. Representative views of liver sections are shown.

### 2.8. Western blot analyses

LO2 cells were treated with DMSO (0.02%, w/v) or SSD at indicated concentrations (0.8, 1, and 2 μM) for 24 h. Whole protein extracts were prepared from treated LO2 cells or liver tissues. In certain experiments, mitochondrial proteins were extracted using a mitochondrial protein extraction kit provided by Nanjing KeyGen Biotechnology Co., Ltd. (Nanjing, China) for detecting the release of cytochrome c. The protein levels were determined using a BCA assay kit (Pierce, USA). Proteins (50 μg/well) were separated by SDS–polyacrylamide gel, transferred to a PVDF membrane