Glycyrrhizin protects against acetaminophen-induced acute liver injury via alleviating TNFα-mediated apoptosis

Tingting Yan, Hong Wang, Min Zhao, Tomoki Yagai, Yingying Chai, Kristopher W. Krausz, Cen Xie, Xuefang Cheng, Jun Zhang, Yuan Che, Feiyan Li, Yuzheng Wu, Chad N. Brocker, Frank J. Gonzalez, Guangji Wang, Haiping Hao

State Key Laboratory of Natural Medicines, Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing, Jiangsu 210009, China (T.Y., H.W., M.Z., Y.C., X.C., J.Z., Y.C., F.L., Y.W., G.W., H.H.) and Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892 (T.Y., T.Y., K.W.K., C.X., C.N.B., F.J.G.)
Running Title: Hepatoprotective effect of glycyrrhizin

Corresponding Authors:

Guangji Wang, Ph.D., Prof., State Key Laboratory of Natural Medicines, Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, No.24, Tongjiaxiang, Nanjing 210009, China; E-mail: guangjiwang@hotmail.com; Fax:86-25-83271060; Tel:86-25-83271128

Haiping Hao, Ph.D., Prof., State Key Laboratory of Natural Medicines, Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, No.24, Tongjiaxiang, Nanjing 210009, China; E-mail: haipinghao@cpu.edu.cn; Fax:86-25-83271060; Tel:86-25-83271179.

Document Statistics:

Number of text pages: 41
Number of tables: 0
Number of figures: 10
Number of references: 55
Number of words in the Abstract: 211
Number of words in the Introduction: 749
Number of words in the Discussion: 1499

Abbreviations

APAP, acetaminophen; TCM, traditional Chinese medicine; RIPK1, receptor interacting protein kinase 1; RIPK3, receptor interacting protein 3; CCl₄, carbon Tetrachloride; GSH, glutathione; NAPQI, N-acetyl-p-benzoquinone; CYP450, cytochrome P450; TNFα, tumor
necrosis factor α; TNFR1, TNFα receptor 1; ALT, Alanine aminotransferase; AST, aspartate aminotransferase; GL, glycyrrhizin; GL50, glycyrrhizin 50 mg/kg; GL100, glycyrrhizin 100 mg/kg; GA, glycyrrhetinic acid; GA 50, GA 50 mg/kg; 18β-GA, 18 β-glycyrrhetinic acid; NEC-1, necrostatin-1; HMGB1, high-mobility group box-1; H & E, hematoxylin and eosin; IHC, immunohistochemistry; PK-PD, pharmacokinetics-pharmacodynamics; NAPQI-GSH, 3-glutathionyl-APAP; NAC-APAP, N-acetylcysteinyl-APAP; KET, ketoconazole; DDC, ammonium diethyldithiocarbamate; zVAD, pan-caspases inhibitor, ZVAD-fmk. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; FACS, fluorescence-activated cell sorting; qRT-PCR, quantitative real-time polymerase chain reaction; I.P., intraperitoneal injection. I.V., intravenous injection; P.O., oral intake; NAC, N-acetyl cysteine; SEM, standard error of mean.
Abstract

Acetaminophen (APAP) overdose is the leading cause of drug-induced acute liver failure in western countries. Glycyrrhizin (GL), a potent hepatoprotective constituent extracted from the traditional Chinese medicine liquorice, has potential clinical use in treating APAP-induced liver failure. The present study aims to determine the hepatoprotective effects and underlying mechanisms of action of GL and its active metabolite glycyrrhetinic acid (GA). Various administration routes and pharmacokinetics-pharmacodynamics (PK-PD) analyses were used to differentiate the effects of GL and GA on APAP toxicity in mice. CYP2E1 or RIPK3-deficient mice and their relative wild-type littermates were subjected to histological and biochemical analyses to determine potential mechanisms. TNFα/caspase-mediated hepatocyte death was analyzed by use of human liver-derived LO2 cells. PK-PD analysis using various administration routes revealed that GL, but not GA, potently attenuated APAP-induced liver injury. The protective effect of GL was found only with intraperitoneal and intravenous administration and not with gastric administration. CYP2E1-mediated metabolic activation and RIPK3-mediated necroptosis were unrelated to GL’s protective effect. However, GL inhibited hepatocyte apoptosis via interfering with TNFα-induced apoptotic hepatocyte death. These results demonstrate that GL rapidly attenuates APAP-induced liver injury by directly inhibiting TNFα-induced hepatocyte apoptosis. The protective effect against APAP-induced liver toxicity by GL in mice, suggest the therapeutic potential of GL for the treatment of APAP overdose.
Introduction

Acetaminophen (APAP) overdose is a leading cause of drug-induced acute liver failure in the United States (Blieden et al., 2014). Because of its prevalence and severity, the FDA decided to limit and monitor the exposure of high-dose APAP (McCarthy, 2014; Mitka, 2014). Cytochromes P450 enzymes (CYP450s) including CYP2E1, CYP3A11, and CYP1A2, are mainly responsible for the metabolic activation of APAP into its toxic intermediate, \( N\)-acetyl-\( p\)-benzoquinone imine (NAPQI) (Lee et al., 1996; Manyike et al., 2000; Cheung et al., 2005). High NAPQI production depletes glutathione (GSH), produces NAPQI-protein adducts, triggers mitochondrial oxidative stress, and ultimately initiates hepatocellular apoptosis and/or necrosis with acute liver inflammation. The therapeutic options for treating APAP hepatotoxicity are limited. The only clinical choice is administration of \( N\)-acetyl cysteine (NAC). However, it has a narrow therapeutic time window after the onset APAP overdosing, and thus new antidotes are warranted (Lancaster et al., 2015).

In recent years, studies have revealed that some traditional Chinese medicines (TCMs) have therapeutic effects, and the TCMs are recognized as potential drugs for ameliorating liver (Zhang and Schuppan, 2014) and other diseases (Chan et al., 2015; Xiong, 2015; Zheng et al., 2015). Notably, glycyrrhizin (GL), also known as glycyrrhizic acid, is one of the most effective medicines extracted from the TCM liquorice, for treating liver diseases. GL has potent hepatoprotective (Li et al., 2014b), anti-inflammatory (Honda et al., 2012; Fu et al., 2014; Kim et al., 2015), and neuroprotective effects (Ni et al., 2013; Barakat et al., 2014). GL has been formulated as a drug widely used for treating chronic liver diseases in Asian countries (Li et al., 2014b). Structurally, GL is a glycoside that is rapidly hydrolyzed to...
glycyrrhetinic acid (GA) by intestinal bacteria (Akao et al., 1994; Takeda et al., 1996). In a previous study, single intraperitoneal injection of GL was used as an inhibitor of HMGB1 to confirm the pathophysiological roles of HMGB1 in APAP toxicity (Wang et al., 2013). Pretreatment of GL (400 mg/kg) for 7 consecutive days attenuated APAP-induced hepatotoxicity via reversing fatty acid metabolism (Yu et al., 2014). GL’s hydrolyzed metabolite, GA, was demonstrated to directly protect against several types of liver injury induced by exposure to CCl₄ (Jeong et al., 2002; Chen et al., 2013), free fatty acids (Wu et al., 2008), and toxic bile acids (Gumpricht et al., 2005). However, it remains unknown whether GL’s hepatoprotective effect is derived from GL or its putative active metabolite GA, and how they protect against APAP-induced cell death of hepatocytes.

The type and mechanism of APAP-triggered death of hepatocytes are still in debate. Protein complex of receptor interacting protein kinase-1 (RIPK1) and RIPK3, also known as "necrosome" carries out necroptosis (Li et al., 2012). The RIPK1 inhibitor, necrostatin-1 (NEC-1), and RIPK3 inhibitor, dabrafenib, were reported to protect against APAP-induced hepatotoxicity both in vivo and in vitro, suggesting that necroptosis plays a role in the protection (Li et al., 2014a; Takemoto et al., 2014). Two studies used gene knockout mice to elucidate the roles of "necrosome" signaling genes including Ripk1, Ripk3 and Mlkl in mediating APAP-induced toxicity. However, these two studies reported different results about the role of RIPK3 in APAP-induced toxicity. One study showed that RIPK3 was an early mediator of APAP-induced toxicity (Ramachandran et al., 2013), while the other study showed no difference in sensitivity to APAP between Ripk3-null mice and their wild-type littermates (Dara et al., 2015). Thus, the role of RIPK3-mediated necroptosis in...
APAP-induced hepatocyte death remains controversial.

APAP induces a severe inflammatory response in liver (Lawson et al., 2000) and significant GSH depletion (Matsumaru et al., 2003). Tumor necrosis factor-α (TNFα) potentiates apoptotic hepatocyte death under conditions of low cellular GSH (Colell et al., 1998; Matsumaru et al., 2003). These studies suggest possible synergy with pro-inflammatory cytokines in mediating hepatocyte damage. Some studies suggested that GL prevented the release of pro-inflammatory cytokines from immune cells (Fu et al., 2014; Kim et al., 2015). Therefore, GL is a potential immunoregulator. However, the crosstalk among TNFα, APAP and the effects of GL on this process, especially in hepatocytes, is still poorly understood.

In this study, the hepatoprotective effects of GL and GA against APAP-induced hepatotoxicity were examined to uncover the potential mechanisms involved in the protective effects. The protective effect of GL on APAP-induced liver injury is not from the metabolic inhibition of APAP activation and unlikely related to RIPK3/necroptosis. Intraperitoneal or intravenous injection of GL, but neither intraperitoneal injection of GA nor intragastric administration of GL, had potent hepatocyte protective effect via interfering with TNFα-induced hepatocyte apoptosis.
Materials and Methods

Chemicals and Reagents

Glycyrrhizin (GL; 97%) and glycyrrhetinic acid (GA; 99.5%) were purchased from TCI (Shanghai, China). 18β-Glycyrrhetinic acid (18β-GA; 97%), acetaminophen (APAP), necrostatin-1 (NEC-1), ammonium diethyldithiocarbamate (DDC), ketoconazole (KET), formic acid, dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT), chloropropamide were purchased from Sigma-Aldrich (St. Louis, MO). Acetaminophen glutathione disodium salt (Cat# A161223, NAPQI-GSH) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). NAC-APAP standard was kindly provided by Professor Bernhard Lauterburg, University of Bern, Switzerland. Recombinant human TNFα was purchased from Peprotech (London, UK). ZVAD-fmk (zVAD) and GSH/GSSG assay kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). High-performance liquid chromatography (HPLC)–grade methanol and acetonitrile were obtained from Merck (Damstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore, Billerica, MA). ELISA kits for mouse TNFα were purchased from Excel (Shanghai, China). Annexin V FITC apoptosis detection kit was purchased from BD Biosciences (San Diego, USA) and FACS analysis was performed on BD Accuri C6 flow cytometer (BD Biosciences, USA). In Situ Cell Death Detection Kit, AP (Cat# 11684809910) was purchased from Roche (Indianapolis, USA).

Experimental Animals and Treatments

Male C57BL/6 wild-type mice (6- to 8-weeks-old) were obtained from the Shanghai SLAC Laboratory Animal Center (Shanghai, China) and allowed free access to food and
water until experimental use. The animal room was maintained at 23±1°C with a 12 h light-dark cycle and 55 ± 5% humidity. The animal studies were approved by the Animal Ethics Committee of China Pharmaceutical University and carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Ripk3-null mice and wild-type mice on a C57BL/6N background were provided by Vishva Dixit (Genentech, Inc.), and Cyp2e1-null mice on a 129/CV genetic background were maintained in the National Cancer Institute, with handling in accordance with animal study protocols approved by the National Cancer Institute Animal Care and Use Committee. Before intraperitoneal dosing of APAP, the mice were fasted overnight (14 h) with free access to water. Mice were administered 600 mg/kg of APAP for inducing hepatotoxicity in Cyp2e1-null mice, 300 mg/kg of APAP for inducing hepatotoxicity in wild-type C57bl/6 mice and 200 mg/kg of APAP for inducing hepatotoxicity in wild-type 129/CV mice. For single GL/GA treatments, GL by intragastrical administration was performed at 1h before APAP dosing, GL or GA by intraperitoneal injection was performed at 30 min before APAP dosing, and GL by intravenous injection was co-administrated with APAP dosing. A dose of 50 mg/kg or 100 mg/kg of GL and 50 mg/kg of GA or 30 mg/kg of 18β-GA were injected to mice, and the mice killed at 0 h, 0.5 h, 2 h, 4 h, 6 h, 8 h and 24 h and serum and livers collected. For multiple GL injection, mice were pretreated with 50 mg/kg of GL for 7 consecutive days, subjected to APAP overdosing 30 min after final GL injection, and sacrificed at 24 h after APAP challenge. A portion of liver was fixed in 10% formalin solution and the remaining liver was flash frozen in liquid nitrogen and stored at -80°C for further use. APAP was freshly dissolved in warm saline at 55°C and cooled to 37°C before use. GL was
freshly dissolved in saline with the pH adjusted to 7.0-7.2. GA or 18β-GA was freshly dissolved in saline containing 5% Tween 80.

In vitro Studies

Human non-tumor hepatic LO2 cells were obtained from the Chinese Academy of Sciences (Shanghai, China). For testing the protective effect of GL or GA against APAP-induced LO2 cell death, LO2 cells were seeded in 96-well plates for MTT assays or 6-well plates for FACS assays and then grown to 70-90% confluence before use. GL, GA or 18β-GA was dissolved in DMSO as stock solution and diluted 1000 times, while APAP was dissolved in DMEM and 0.1% DMSO was set as control when used. LO2 cells were pretreated with control DMSO, or GL, GA, 18β-GA 30 min before adding APAP. For cell viability assays, MTT was added after 18-24 h of APAP treatment; and for FACS assays, samples were collected at 10 h after APAP treatment and analyzed according to the instruction manual protocol for the Annexin V FITC apoptosis detection kit.

Serum Aminotransferase Analysis

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were quantified using a standard clinical automatic analyzer or a commercial ALT or AST assay kit (Catachem, Bridgeport, CT).

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total tissue RNA extraction was performed by using the RNAiso Plus reagent (Takara, Bio-Tech Co., Ltd, Dalian, China) according to the manufacturer’s protocol. Purified total RNA was reverse-transcribed using the Prime Script RT Reagent Kit (Takara Biotechnology Co., Ltd.) according to the manufacturer’s protocol. Real-time PCR was performed by using
DMD#69419

the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Bedford, MA) and SyBr Green reagent kit (Takara Biotechnology Co., Ltd.). Values were normalized to GAPDH. Sequences for primers are listed in supplementary data (Supplementary Table 1).

**Hematoxylin and Eosin (H&E) Staining and Terminal Deoxynucleotidyl Transferase-mediated Deoxyuridine Triphosphate Nick-end Labeling (TUNEL)**

Formalin-fixed liver tissues were embedded in paraffin and 5 μm thick sections were cut for H&E staining and TUNEL staining according to the manual protocols. Data represent n=3 in each group for all the analysis.

**Mass Spectrometry (LC-MS/MS) Analysis of GL and GA**

Chromatographic separation was obtained on a Waters ACQUITY I Class UPLC system composing of a binary solvent manager, a FTN auto sampler, and column manager using a Waters ACQUITY BEH C-18 2.1x50 mm column. A liquid chromatography/tandem mass spectrometry (LC–MS/MS) method using multiple reaction monitoring (MRM) in positive mode for monitoring 823.7→453.5 for GL, 471.5→149.1 for GA and 277→110.9 for the internal standard chlorpropamide with a slight modification of the mobile phase as previously described (Li et al., 2013).

**Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry (LC/Q-TOF/MS) Analysis of NAPQI-GSH**

The oxidized APAP intermediate NAPQI, formed in mice microsomal incubation system, was trapped by reduced glutathione (GSH) with a slight modification of the in vitro APAP incubation system described previously (Fan et al., 2014; Jiang et al., 2015), and NAPQI-GSH was determined by a LC/Q-TOF/MS method. Briefly, mouse liver microsomes
DMD#69419

(MLMs, final concentration is 1 mg/mL protein) were incubated with 500 μM APAP alone and with GL (10 μM, 50 μM, 100 μM, 200 μM and 500 μM), or GA/18β-GA (2 μM, 5μM, 10 μM, and 20 μM). The reaction was initiated by adding NAPDH, trapped by 5 mM GSH and quenched by adding cold acetonitrile. NAPQI-GSH was identified through accurate mass measurement, compared with authentic standards and monitored at m/z 457.1393, and internal standard chlorpropamide was monitored at m/z 277.0414 in positive mode. Chromatographic separation was obtained on a Waters Synapt-HDMS Q-TOF mass spectrometer running in ESI+ mode. The capillary and cone voltage were 3.0 kV and 30 V, respectively. The source and desolvation temperature were 150°C and 400°C. The desolvation and cone gas (nitrogen) were 850 L/h and 50 L/h. Sulfadimethoxine (100 pg/μL) was used for lock-Spray mass and infused at 70 μL/min. Mobile phases: A, water with 0.1% formic acid and B, acetonitrile with 0.1% formic acid. Flow rate was maintained at 0.4 ml/min. Gradient is as follows. Initial conditions: 98% A for 0.5 min, to 80% A at 4 min, to 5% A at 8.0 min, held for 1 min returning to initial conditions and holding for 2 min for column equilibration with a total running time of 11 min.

Statistical Analysis

Data are presented as means ± SEM. Differences between the control and experimental groups were determined by a two-tailed Student’s t-test in GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA). P value less than 0.05 was considered statistically significant.
Results

Intraperitoneal Administration of GL Reduces APAP-induced Hepatotoxicity

To determine whether GL attenuates APAP-mediated damage in vivo, a non-lethal dose of APAP (300 mg/kg) was administered to wild-type mice and serum liver enzyme levels and histology were measured to determine hepatocellular toxicity after 24 hours. Serum ALT and AST were sharply increased in the vehicle/APAP-treated group, while they were markedly decreased in the GL/APAP-treated group (Figure 1, A and B). Necrotic areas were markedly decreased in liver from GL-treated mice (Figure 1C). Since APAP-induced liver toxicity is associated with increased inflammation (Lawson et al., 2000; Liu et al., 2004), the APAP-induced inflammatory response was analyzed. Secreted TNFα in serum was assessed in the APAP-administrated mice treated with or without GL. Serum TNFα levels were significantly reduced by GL-treatment (Figure 1D). Moreover, expression of mRNAs encoding pro-inflammatory cytokines, TNFα, IL-6, and IL-1β were normalized in liver by pretreatment with GL (Figure 1, E-G). Taken together, these results suggest that GL attenuated both liver toxicity and inflammation induced by APAP administration.

Intravenous, but not Oral Administration of GL Inhibits APAP-induced Hepatotoxicity

In the clinic, GL is administrated orally or intravenously. Therefore, effect of GL by intragastric administration or intravenous injection in APAP-caused liver injury was determined. A single gavage administration of GL at 50 mg/kg and 100 mg/kg showed no effect in attenuating APAP-induced liver injury at 24 h after APAP dosing (Figure 2, A-C). GL was not detected, and GA was significantly detected in both serum and liver at 24 h after APAP challenge (Figure 2, D and E), while GL administered at 50 mg/kg by intraperitoneal
injection was used as a positive control. Indeed, it is known that GL is hydrolyzed to GA by intestinal bacteria (Akao et al., 1994; Takeda et al., 1996). In contrast, intravenous injection of GL (50 mg/kg and 100 mg/kg) decreased APAP-induced hepatocyte death (Figure 3A) and markedly attenuated the increased serum ALT and AST levels (Figure 3, B and C). By intravenous injection, the serum concentrations of GA were less than 1 μM in GL alone-injected mice and GL/APAP-treated mice, respectively, while approximately a 700-fold increase of GL was detected in serum of GL/APAP-treated mice (Supplemental Figure 1A and Figure 3D). In liver, the concentration of GA was 2 ng/mg, while a 50-fold increase of GL to approximately 100 ng/mg was detected (Supplemental Figure 1B and Figure 3E). Similarly, after intravenous administration of GL at 100 mg/kg, serum and liver GA levels were markedly lower than GL (Figure 3, D and E). These data indicate that GL attenuates liver injury only after intraperitoneal and intravenous administration and not gastric administration because GL was hydrolyzed in the gut into GA after oral administration.

GL, not GA, Attenuates APAP-induced Liver Damage

The observation that intravenous/intraperitoneal injection, but not oral administration of GL showed a protective effect suggest that GL itself, and not the metabolite GA, directly contributed to GL’s protective effect. To determine the compound responsible for GL’s hepatoprotective effect, pharmacokinetics and pharmacodynamics (PK-PD) of injected GL were investigated after APAP dosing. Mice were pretreated with a single intraperitoneal injection of GL at dose of 100 mg/kg 30 min before APAP (300 mg/kg) challenge, and killed at 2, 4, 8 and 24 h after APAP (Figure 4A). GL and GA distribution in liver and serum were then determined. During the time course of GL/APAP challenge, GL markedly attenuated...
APAP-caused liver injury. Intriguingly, the potent protective effect was observed even at the earliest time point of 2h, suggesting that GL could affect the initiation of APAP-induced toxicity and rapidly inhibit liver injury (Figure 4, B and C). Moreover, GA levels peaked at 8 h in liver and 4 h in serum, while GL levels were time-dependently decreased in both serum and liver in GL/APAP-treated mice (Figure 4, D and E) and in GL/saline-treated control mice (Supplemental Figure 1, C and D). Given that GA attenuates liver injury, ALT and AST would be expected to be decreased time-dependently. However, ALT and AST were markedly increased from 8 hours after APAP administration in the GL/APAP challenged group (Figure 4, B and C). The potent protective effect of GL and increase of liver damage commencing at 8h were also confirmed by histology (Supplemental Figure 1E). These data suggested that GL, and not GA, directly attenuated APAP hepatotoxicity.

To examine the direct effect of GA in combating APAP-induced liver injury, GA (50 mg/kg, approximately equimolar to GL 100 mg/kg) was injected to wild-type mice by a single intraperitoneal injection before APAP (300 mg/kg) challenge. All mice were killed at 24 hours after APAP administration and subjected to histological and serum analysis. In serum, GA was only detected in GA-treated mice, and not detected in GL-treated mice (Figure 5A). In liver, approximately a 10-fold increase of GA was detected in GA-treated mice compared with GL-treated mice (Figure 5B). Thus, GA was efficiently absorbed and detected in both serum and liver. Histology revealed that the necrotic areas that developed after APAP in the GA-treated group was not decreased as compared with the non-treated group, while the areas were significantly decreased in the GL-treated group (Supplemental Figure 2A). In agreement with this result, ALT and AST were also decreased in the
GL/APAP-treated group. In contrast, these markers in the GA-treated group were moderately but not significantly decreased (Figure 5, C and D). Furthermore, the effect of 18β-GA showed no significant effect in preventing APAP-induced liver injury (Supplemental Figure 2, B-D), while 18β-GA was efficiently absorbed in both serum and liver (Supplemental Figure 2, E and F). These data demonstrate that the effects of GA derived from hydrolyzed GL in the GL-treated group in vivo could be excluded.

The direct effect of GL and the hydrolyzed form GA were next analyzed in vitro by use of human hepatic-derived LO2 cells. Cell survival rates were determined after APAP treatment together with GL, GA or 18β-GA. GL was found to significantly inhibit APAP-induced cell death in vitro (Supplemental Figure 3A), while neither GA nor 18β-GA showed protective potency against APAP (10 mM)-induced cytotoxicity (Supplemental Figure 3, B and C). This protective effect was more remarkable at higher dose of APAP (20 mM). GL significantly rescued the survival rate of APAP-treated cells from approximately 10% to 40%, even at the low concentration of 5 μM (Figure 5E), while both GA and 18β-GA showed no significant protective effect at various concentrations (Figure 5, F and G). Both concentrations of GA and 18β-GA used included the maximum non-toxic concentration in the hepatic cells (Supplemental Figure 3, D-F). These data demonstrate that GL and not GA directly prevent APAP-caused liver injury by inhibiting hepatocyte damage.

**Hepatoprotective Effect of GL is unrelated to metabolic activation of APAP**

To clarify possible mechanisms, we first asked whether the hepatoprotective effect of GL against APAP toxicity was from the direct interference of APAP metabolic activation. We found treatment with a single or multiple injections of GL alone to mice had no significant
effect in the expression of Cyp2e1, Cyp3a11, and Cyp1a2, although both treatments significantly attenuated APAP-induced downregulation of such enzymes (Figure 6, A-C). Similar results were obtained from the in vitro mouse primary hepatocytes study; no significant change of Cyp2e1, Cyp3a11 and Cyp1a2 mRNA level was observed by GL, GA or 18β-GA (Supplemental 4, A-F). APAP hepatotoxicity is mainly induced by NAPQI, which subsequently binds with hepatic GSH and forms 3-glutathionyl-APAP (NAPQI-GSH). To test whether GL and/or GA directly inhibited APAP bio-activation, APAP/MLMs incubation system was used. A peak at the same retention time (RT=1.9 min) with NAPQI-GSH authentic standard was found in in vitro APAP incubation system, while no peak was found when we removed NAPDH from the APAP incubation system (Supplemental Figure 5A). In this system, we found that GL, GA and 18β-GA did not significantly influence APAP metabolic activation in vitro, while positive CYP3A11 inhibitor (KET) and CYP2E1 inhibitor (DDC) significantly inhibited the formation of NAPQI-GSH (Figure 6, D-F).

In mice, hepatotoxic NAPQI produced APAP metabolic activation is very unstable and known to subsequently form NAPQI-GSH and N-acetylcysteinyl-APAP (NAC-APAP) (Chen et al., 2008). To determine the extent of APAP metabolic activation in vivo, serum level of NAPQI-GSH and NAC-APAP at 3 h after APAP injection were not significantly changed by both single GL injection and multiple GL injection (Figure 6, G-H). Meanwhile, the GSH/GSSG ratio in APAP-administrated and GL-treated liver was analyzed. In time course experiments, 300 mg/kg of APAP administration resulted in a rapid depletion of GSH in liver at 2 and 4 h after administration; GL pre-treatment did not alter the liver GSH/GSSG ratios at both time points (Supplemental Figure 5B).
CYP2E1 has a significant role in the metabolic activation of APAP, as Cyp2e1-null mice showed resistance to APAP toxicity (Lee et al., 1996). Analysis of relationships between the protective effect of GL and CYP2E1-mediated APAP activation revealed that 200 mg/kg of APAP administration to wild-type 129/CV mice or high dose (600 mg/kg) of APAP administration to Cyp2e1-null mice induced liver injury as revealed by increased serum ALT and AST as well as severe hepatocytes death. In contrast, the injury markers were robustly inhibited by intraperitoneal injection of GL at dose of 50 mg/kg both 6 hours and 24 hours after APAP challenge in both wild-type (Figure 7, A and B) and Cyp2e1-null mice (Figure 7, C and D). Histology showed a similar attenuation of APAP-induced toxicity by GL in wild-type (Supplemental Figure 6A) and Cyp2e1-null mice (Supplemental Figure 6B). These results revealed that GL’s protective effect did not depend on the metabolic activation of APAP and CYP2E1 expression. Together, these results strongly support that the hepatoprotective effect of GL is unrelated to the metabolic activation of APAP.

**RIPK3 is not involved in APAP Toxicity and Hepatoprotective Effect of GL**

Recent studies revealed a type of necrosis involved in programmed cell death called as "necroptosis" (Li et al., 2012). RIPK1 and RIPK3 are both central players in TNFα-induced necroptosis (Zhang et al., 2009; Li et al., 2012), with the involvement of its downstream cellular signaling, mixed lineage kinase domain-like protein (MLKL) (Sun et al., 2012; Zhao et al., 2012). In particular, RIPK3 was confirmed to have an essential role in ameliorating APAP-induced liver injury (Ramachandran et al., 2013). To confirm whether APAP exposure influenced RIPK3 expression, Ripk3mRNA levels were measured. APAP induced a 2- to 3-fold increase of Ripk3 mRNA as early as 2 h (Supplemental Figure 7A). Upstream signaling
of RIPK3, \(Tnf\alpha\), and downstream \(Mlkl\) mRNA were also elevated (Supplemental Figure 7, B and C). GL-treatment significantly attenuated both of the \(Ripk3\) and \(Tnf\alpha\) mRNA levels, while no significant decrease of \(Mlkl\) mRNA by GL-treatment was noted (Supplemental Figure 7, A-E). These results suggest that RIPK3-mediated necroptosis is possibly involved in APAP toxicity and GL's protective effect. This was further investigated by use of \(Ripk3\)-null mice. Histological observation (Figure 8, A and B) as well as levels of serum ALT and AST (Figure 8, C and D) were not different between \(Ripk3\)-null mice and their wild-type littermates at 6 h after APAP challenge. Furthermore, GL robustly and significantly attenuated APAP toxicity in both genotypes. These results suggested no involvement of RIPK3 in APAP-induced liver toxicity and GL's protective effect against APAP toxicity was not through RIPK3/necroptosis. Conversely, these data suggest that RIPK3 upregulation is possibly only a consequence, RIPK3-mediated necroptosis is not a mediator of APAP-induced hepatocyte death and that GL's protective effect does not depend on RIPK3.

**GL Protects Against TNF\(\alpha\)-mediated Apoptotic Hepatocyte Death**

Since RIPK3-mediated necroptosis was demonstrated as unlikely to be a major player in APAP-induced liver injury in as noted above current study and in a recent publication (Dara et al., 2015), the possible role of apoptotic hepatocyte death was investigated. The extent of hepatic apoptosis was investigated by using TUNEL assay. Mouse livers exposed to APAP revealed that TUNEL-positive cells emerged at the early phase (2 h) after APAP dosing, while few TUNEL-positive cells were observed in GL/APAP-treated mice during the time course of APAP challenge (Figure 9). Analysis of mRNA encoding BCL2a1c, which is an apoptosis marker, also showed a 2- to 3-fold increase in APAP-challenged livers, whereas the
Factors that initiate apoptotic hepatocyte death after APAP challenge remain largely unknown. TNFα, one of the apoptosis-inducible factors (Colell et al., 1998; Matsumaru et al., 2003), may mediate APAP-induced hepatocyte death. In vivo, APAP-administration induced significant TNFα release in serum as early as 2 hours after administration (Figure 10A). GL (100 mg/kg) treatment markedly decreased TNFα secretion at 4 hours after APAP injection (Figure 10B). Considering that the time course of TNFα release coincides well with that of the APAP toxicity, the involvement of TNFα in potentiating APAP-induced hepatocyte apoptosis needs to be considered. To validate this assumption, a study was performed for testing the potentially synergistic role of TNFα with APAP toxicity in vitro. TNFα aggravated APAP-induced cytotoxicity in a dose-dependent manner in LO2 cells, although TNFα alone showed no significant effect on the cell viability (Figure 10C). Interestingly, pan-caspases inhibitor, zVAD, but not the small necrosis inhibitor, NEC-1, significantly inhibited TNFα/APAP-induced cytotoxicity (Figure 10, C and D). The protective effect of zVAD was also observed in APAP-induced cytotoxicity (Figure 10E). These results indicate that caspases-dependent apoptosis is a critical factor in both APAP and TNFα/APAP-induced cytotoxicity. Furthermore, addition of GL, GA and 18β-GA to culture medium revealed that GL (Figure 10D and Supplemental Figure 3G), but not GA or 18β-GA (Supplemental Figure 3, H and I), attenuated TNFα/APAP-induced cell death. FACS analysis further indicated that, although TNFα (20 ng/ml) alone did not induce apoptosis, TNFα/APAP markedly induced apoptotic cell death. In addition, apoptosis was potently inhibited by GL addition (Figure 10F). These data demonstrated that GL, but not GA, attenuated APAP-induced hepatocyte
DMD#69419

damage via inhibiting the release of TNFα and its downstream caspase-dependent apoptotic signaling.
Discussion

In this study, GL, but not its active metabolite GA, was found to prevent APAP-induced liver injury via inhibiting TNFα/caspase-mediated apoptotic hepatocyte death. The hepatoprotective effect of GL was unlikely from the direct interference of APAP metabolic activation and not through RIPK3-mediated necroptosis. Intraperitoneal and intravenous injection, but not oral administration of GL, had a potent protective effect against APAP-induced liver toxicity. Moreover, with GL as a chemical probe, this study revealed that the release of TNFα upon APAP challenge might be an important factor in APAP-induced hepatotoxicity via potentiating APAP-induced apoptotic cell death of hepatocytes.

One of the major challenges in ascertaining the hepatoprotective effects of GL is how to differentiate the role of GL and its bioactive metabolite GA. It is widely claimed that the hepatoprotective effect of GL is due to its metabolite GA, considering that GL is rapidly metabolized to GA by the gut bacteria and thus GL was previously assumed as to be a prodrug. However, in the present study, evidence is provided that GL itself and not its bioactive metabolite GA, contributes to the hepatoprotective effect against APAP toxicity. The transformation of GL to GA mainly happens in intestine and thus the differential effects of GL via different routes of administration would be helpful for differentiating the role of GL and GA. We found, unexpectedly, that intravenous/intraperitoneal injection of GL, rather than intragastrical administration of GL, exerted a significant protective effect. Second, a PK-PD correlation analysis was performed demonstrating that GL, rather than GA, contributes to the hepatoprotective effect of GL, proving the potential application of “reverse pharmacokinetics” in the mechanistic studies of natural compounds (Hao et al., 2014). Finally,
in vitro studies further revealed that GL, rather than GA, protects against APAP-induced hepatocyte damage. All of these data strongly suggest that GL and not its metabolite GA, possesses hepatoprotective activity against APAP-induced liver injury. Given the fact that GA shows a potent protective effect in liver injury caused by other toxicants, including CCl₄, bile acids, and free fatty acids both in vivo and in vitro (Jeong et al., 2002; Gumpricht et al., 2005; Wu et al., 2008; Chen et al., 2013), GL and GA are considered to exert hepatoprotective effects in different mechanisms. Because formulations of GL for both intravenous and oral administration are available in the clinic (Li et al., 2014b), our data suggest that, in the therapy of APAP induced liver injury, intravenous but not oral formulations of GL could be used.

CYP2E1-mediated activation of APAP is mainly responsible for initiating the cascade of APAP hepatotoxicity through formation of NAPQI (Lee et al., 1996). However, a single dose of GL alone did not change the CYP2E1 expression in the current study and showed no inhibition of CYP2E1 activity in a previous study (Paolini et al., 1999). Conversely, GL significantly increased APAP-induced CYP2E1 down-regulation. The results in Cyp2e1-null mice further provide direct evidence that GL’s protective effect is not dependent on CYP2E1 activity. Moreover, GL and GA have little effect on the metabolism of APAP and the enzymes involved in APAP metabolic activation. All these evidence strongly support that GL prevents APAP-induced liver injury independent of CYP2E1 and not through direct inhibition of APAP metabolic activation.

The role of RIPK3/necroptosis in APAP-induced toxicity is highly controversial (Ramachandran et al., 2013; Zhang et al., 2014). Another study suggested RIPK3 was an
early mediator in APAP toxicity and RIPK3 protein, though absent at baseline, was induced in liver at the early phase of APAP toxicity (An et al., 2013; Ramachandran et al., 2013; Zhang et al., 2014). Ripk3-null mice were reported to be protective at 6 h after APAP overdosing, while the protective effect was lost at 24 h after APAP challenge (Ramachandran et al., 2013). In the current study, APAP also induces RIPK3 expression, at least at the mRNA level, which partially supports a previous study (Ramachandran et al., 2013). Unexpectedly, when further tested in Ripk3-null mice, no significant difference of sensitivity to APAP is found between Ripk3-null mice and their wild-type littermates, while GL markedly attenuates APAP-induced liver injury in both genotypes, suggesting no involvement of RIPK3 in APAP toxicity. The present data on RIPK3/APAP toxicity are in agreement with a recent study suggesting no involvement of RIPK3 in mediating APAP toxicity (Dara et al., 2015). The present work also revealed that APAP significantly induced Mlkl mRNA, a downstream target of RIPK3, and GL did not decrease APAP-induced Mlkl mRNA upregulation. Therefore, although RIPK3 upregulation was observed upon APAP toxicity, it may not be an initiating factor in APAP toxicity. The RIPK1 inhibitor, NEC-1, was shown to inhibit APAP toxicity both in vivo and in primary hepatocytes in vitro (An et al., 2013; Ramachandran et al., 2013; Takemoto et al., 2014). In LO2 cells, GL, as well as the pan-caspase inhibitor, zVAD, but not NEC-1, prevented APAP-induced cell death, suggesting that GL may act through a caspase-dependent apoptosis pathway, rather than RIPK1/RIPK3-dependent necroptosis in modulating APAP-induced hepatotoxicity.

Additional studies focused on elucidating how GL protects against apoptotic cell death of hepatocytes. TUNEL positive staining and apoptotic hepatocyte death (which can be
potentiated by TNFα) in vitro suggest the possible involvement of hepatic apoptosis in APAP toxicity. Previous reports on the involvement of apoptotic cell death in the APAP model are largely controversial (Boulares et al., 2002; Matsumaru et al., 2003). It is widely believed that necrosis represents the exclusive cell death in APAP-overdosed livers with no detection of caspase activation (Jaeschke et al., 2006; Jaeschke et al., 2011). However, positive TUNEL staining was found at 2 h, 4 h, and 8 h post APAP treatment, while the positive staining was sharply decreased at 24 h. Since the necrotic areas were significantly increased across the time course, the transfer from apoptosis to late necrosis possibly explains the current observation (Possamai et al., 2013). Therefore, caspase-dependent apoptosis may be dominant in the early phase of APAP-induced hepatocyte death, which can be aggravated by APAP-induced release of TNFα, but may be rapidly transferred to typical necrosis or necroptosis through an unknown mechanism. Thus, GL may block caspase-dependent apoptotic cell death at the very early stage, but not the latter necrotic phase, of APAP toxicity.

TNF-deficient or TNF receptor type 1 (TNFR1)-deficient mice are not protected against APAP toxicity (Nagai et al., 2002; Chiu et al., 2003; James et al., 2005), indicating that TNFα/TNFR1 signaling is probably not an important factor in mediating APAP toxicity. However, TNFα, as a pleiotropic cytokine, can antagonize liver injury by facilitating liver regeneration, especially at low concentration, while over release of TNF-α potentially aggravates hepatotoxicity. Thus, the results of TNF or TNFR1 knockout mice possibly represent an integrated effect of the two roles. The role of TNFα over release in aggravating APAP toxicity is still highly possible. In the current study, a direct role of TNFα, in potentiating APAP-caused hepatocyte apoptosis in vitro was demonstrated in LO2 cells.
These results agree with previous studies in primary hepatocytes (Colell et al., 1998; Matsumaru et al., 2003; Gandhi et al., 2010). GL shows a direct effect against TNFα/APAP-induced apoptotic cell death in LO2 cells, supporting that GL has a direct anti-apoptotic effect. Of interest, APAP treatment induced an increase in the release of TNFα in serum and GL repressed APAP-triggered release of TNFα. Therefore, the prevailing evidence suggests that APAP-induced TNFα over release at the very early phase of toxicity can potentially enhance APAP-induced hepatocyte death. Since GL is also an immunoregulator (Honda et al., 2012; Fu et al., 2014; Kim et al., 2015; Wu et al., 2015), it seems that GL could block both APAP-triggered TNFα release and its downstream apoptotic cascade.

In conclusion, the present study provides evidence that GL rather than its metabolite GA, contributes to GL’s protective effect in APAP-induced toxicity. GL’s protective effect is independent of CYP2E1 and not through interfering with APAP metabolic activation as well as RIPK3-mediated necroptosis. APAP-induced caspases-dependent apoptosis can be potentiated by TNFα and inhibited by GL. GL can suppress the APAP-induced inflammatory response and decrease TNFα release. Collectively, GL, not GA, potently prevents APAP toxicity through TNFα/caspase-mediated apoptotic cell death. Translationally, our study suggests that GL, via intravenous injection, but not oral intake, is a potential option for clinical application. However, it is important to note that when GL is intravenously injected at 2 h post APAP overdosing when the liver was severely damaged, no significant hepatoprotective effect was observed in our study, although both multiple injections of GL and single injection of GL showed a potent protective effect as a positive control.
(Supplemental Figure 8, A and B), suggesting that GL alone may have limited clinical efficacy for combating APAP induced liver injury. FXR agonists could combat APAP toxicity via the promotion of liver regeneration, which is a latter phase of APAP intoxicity (Xie et al., 2016). It would be of interest to determine whether the timely administration of GL in the early phase and FXR agonists in the latter phase would confer a synergy in combating against APAP toxicity.
Acknowledgements:

We thank Dr. Vishva Dixit (Genentech, Inc) for providing Ripk3-null mice.

Authorship Contributions:

Participate in the design: Hao, G. Wang, Yan.

Conducted the experiments: Yan, H. Wang, Zhao, Yagai, Chai, Krausz, Xie, Cheng, Zhang, Che, Li, Wu, Brocker.

Performed data analysis: Yan, Hao, H. Wang, Yagai.

Wrote or contributed to the writing of the manuscript: Yan, Yagai, Gonzalez, Hao, G. Wang.
References:


Fu YH, Zhou ES, Wei ZK, Song XJ, Liu ZC, Wang TC, Wang W, Zhang NS, Liu GW, and


DMD#69419


Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, and Han JH (2009). RIP3,


Footnotes:

This work was supported by National Natural Science Foundation of China [No.81430091, 81325025, and 81273586], the National Cancer Institute Intramural Research Program and China Scholarship Council (No.201407060024). The authors have declared that there is no conflict of interest.
Legends for Figures:

Figure 1. GL pretreatment reduces serum transaminases, improves liver histology and normalizes inflammation. (A and B), Serum ALT and AST levels. (C), H & E-staining of liver sections, original magnification: 20×, and black scale bar, 50 µm. (D), Serum TNFα levels; (E, F and G), Relative mRNA level of TNFα, IL-1β, and IL-6. n=6-8 in each group. Control, saline-treated control mice; GL100, only GL 100 mg/kg-treated mice; APAP, saline/APAP-treated mice; GL50+APAP, GL 50 mg/kg/APAP-treated mice; GL100+APAP, GL 100 mg/kg /APAP-treated mice. Data are expressed as the mean ± SEM. #P<0.05, ##P<0.01 and ###P<0.001 versus control mice. *P<0.05, **P<0.01 and ***P<0.001 versus APAP-overdosed mice.

Figure 2. Intragastric administration of GL fails to prevent APAP-induced hepatotoxicity. (A), H & E-stained liver sections 24 h after APAP challenge, original magnification, 20×, and black scale bar, 50 µm. (B and C), serum ALT and AST levels. (D and E), GL and GA level in serum and liver. Control, saline-treated control mice; APAP, saline/APAP-treated mice; GL50 (po) + APAP, GL50 mg/kg (po)/APAP-treated mice; GL100 (po) + APAP, GL100 mg/kg (po)/APAP-treated mice; GL50 (ip)+APAP, GL50 mg/kg (ip)/APAP-treated mice; Data are expressed as the mean ± SEM, n=4-5 in each group. ###P<0.001 versus control mice. **P<0.01 versus APAP-overdosed mice.

Figure 3. Intravenous injection of GL inhibits APAP-induced hepatotoxicity. (A) H & E-stained liver sections 4 h after APAP challenge, original magnification, 20X, and black scale bar, 50 µm. (B and C), serum ALT and AST levels. (D and E), GL and GA levels in serum and liver. Data are expressed as the mean ± SEM, n=5-8 in each group for all analysis.
Control, saline-treated control mice; APAP, saline/APAP-treated mice; GL50 (iv) + APAP, GL50 mg/kg (iv)/APAP-treated mice; GL100 (iv) + APAP, GL100 mg/kg (iv)/APAP-treated mice; GL100 (iv), only GL100 mg/kg (iv)-treated mice; ###, $P<0.001$ versus control mice. ***$P<0.001$ versus APAP-overdosed mice.

**Figure 4.** Intraperitoneal injection of GL decreases serum ALT and AST level at the early stage after APAP challenge. (A), Mouse experiment procedure scheme. (B and C), Time course of serum ALT and AST levels after APAP challenge. (D and E), Pharmacokinetic distribution of GL and GA in serum (D) and in liver (E) in GL100 (ip)/APAP-treated mice. Data are expressed as the mean ± SEM, n=3 in each group for control mice and n=6 for other groups. #*$P<0.05$ and ##*$P<0.01$ versus control mice. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus APAP-overdosed mice.

**Figure 5.** GL, rather than GA, prevents APAP-induced hepatocyte damage both in APAP-overdosed mice and APAP-treated LO2 cells. (A and B), GA exposure in serum (A) and in liver (B) in GA/APAP-treated mice and GL/APAP-treated mice. (C and D), Serum ALT and AST levels. (E), Cell viability of GL/APAP 20 mM-treated LO2 cells. (F), Cell viability of GA/APAP 20 mM-treated LO2 cells; (G) Cell viability of 18β-GA/APAP 20 mM-treated LO2 cells. Data are expressed as the mean ± SEM, n=5-6 for both animal experiments and LO2 cell experiments. Control, saline-treated mice; GA, only GA 50 mg/kg-treated mice; GL, only GL 50 mg/kg-treated mice; APAP, saline/APAP-treated mice; GA+APAP, GA 50 mg/kg/APAP-treated mice; GL+APAP, GL 50 mg/kg/APAP-treated mice; ##*$P<0.01$ and ###*$P<0.001$ versus control mice or 0.1% DMSO-treated control LO2 cells. **$P<0.01$ and ***$P<0.001$ versus APAP-overdosed mice or 0.1% DMSO/APAP-treated LO2
cells.

Figure 6. GL shows no significant effect in mRNA level of Cyp2e1, Cyp3a11, and Cyp1a2 and modifying APAP metabolic activation. (A, B and C), Effect of GL and/or APAP injection in the mRNA level of Cyp2e1 (A), Cyp3a11 (B), and Cyp1a2 (C). (D, E and F), Effects of GL (D), GA (E), and 18βGA (F) in production of NAPQI-GSH in APAP/MLMs incubation system. Control, saline-treated mice; Single GL50, only single GL 50 mg/kg-treated mice; APAP, saline/APAP-treated mice; Single GL50+APAP, single injection of GL 50 mg/kg (ip)/APAP-treated mice. Multiple GL50+APAP, multiple injections of GL 50 mg/kg (ip)/APAP-treated mice. Data are expressed as the mean ± SEM, n=5 mice in each group. #P<0.05, ##P<0.01 and ###P<0.001 versus control mice. *P<0.05, **P<0.01 and ***P<0.001 versus APAP-overdosed mice.

Figure 7. GL markedly attenuates serum ALT and AST levels in both wild-type and Cyp2e1-null mice. (A and B), Serum ALT and AST levels in wild-type mice. (C and D), Serum ALT and AST levels in Cyp2e1 null mice. Control, saline-treated mice; GL 50, only GL 50 mg/kg-treated mice; APAP, saline/APAP-treated mice; GL50+APAP, GL 50 mg/kg (ip)/APAP-treated mice. Data are expressed as the mean ± SEM, n=5 mice in each group. #P<0.05, ##P<0.01 and ###P<0.001 versus control mice. *P<0.05, **P<0.01 and ***P<0.001 versus APAP-overdosed mice.

Figure 8. GL combats APAP-induced hepatotoxicity in both Ripk3-null mice and their wild-type littermates. (A and B), H & E-stained liver sections of Ripk3 +/+ mice (A) and Ripk3 -/- mice (B), original magnification, 20×, and black scale bar, 50 µm. (C and D), serum ALT and AST levels. Data are expressed as the mean ± SEM, n=5-6 mice for APAP and GL +
APAP group and n=3 for control group. Control, saline-treated control mice; APAP, saline/APAP-treated mice; GL50+APAP, GL 50 mg/kg (ip)/APAP-treated mice. All experiments in Ripk3-null mice and their wild-type littermates were repeated twice. #P<0.05; ##, P<0.01 and ###, P<0.001 versus control mice. *P<0.05, **P<0.01 and ***P<0.001 versus APAP-overdosed mice.

Figure 9. GL blocks TUNEL-positive staining at the early stage after APAP challenge.
TUNEL-staining analysis of paraffin-embed livers, original magnification, 20×. Control, saline-treated mice; GL, only GL 100 mg/kg-treated mice; APAP, saline/APAP-treated mice; GL+APAP, GL 100 mg/kg/APAP-treated mice.

Figure 10. GL attenuates APAP-caused TNFα release, and TNFα/caspase-mediated apoptotic hepatocyte damage. (A), Time course of APAP-induced TNFα release in serum. (B), Effect of GL in serum TNFα level at 4 h after APAP treatment. (C), Effect of TNFα in APAP-induced LO2 cell death and effect of zVAD-fmk in TNFα/APAP-induced LO2 cell death. (D), Effects of GL and NEC-1 in TNFα/APAP-induced LO2 cell death. (E), Cell viability of APAP-treated LO2 cells pretreated with control DMSO, zVAD-fmk 20 µM or NEC-1 20 µM. (F), Statistic analysis of FACS analyses. n=6 in each group for experiments in vivo in mice and in vitro in LO2 cells. Data are expressed as the mean ± SEM. #P<0.05, ##P<0.01 and ###P<0.001 versus control mice or 0.1% DMSO-treated control LO2 cells. *P<0.05, **P<0.01 and ***P<0.001 versus APAP-overdosed mice or 0.1% DMSO/APAP-treated LO2 cells. φφφP<0.001 versus TNFα/APAP-treated LO2 cells.
Figure 1
Figure 2

[Image showing histological sections and bar graphs for ALT, AST, Serum GA, and Liver GA levels with different treatment groups and comparisons.]

A. Histological sections showing liver tissue with various treatments.

B. Bar graph showing ALT levels (IU/L) for different groups: Control, APAP, GL50(po)+APAP, GL100(po)+APAP, GL50(ip)+APAP, and GL100(ip)+APAP. Significance levels are indicated: # for p<0.001, ** for p<0.01.

C. Bar graph showing AST levels (IU/L) for different groups: Control, APAP, GL50(po)+APAP, GL100(po)+APAP, GL50(ip)+APAP, and GL100(ip)+APAP. Significance levels are indicated: # for p<0.001, ** for p<0.01.

D. Bar graph showing Serum GA (µM) levels for different treatments: GL50(ip)+APAP, GL50(po)+APAP, GL100(po)+APAP, GL50(po)+APAP, and GL100(po)+APAP.

E. Bar graph showing Liver GA (ng/mg) levels for different treatments: GL50(ip)+APAP, GL50(po)+APAP, GL100(po)+APAP, GL50(po)+APAP, and GL100(po)+APAP.
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10