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Glycyrrhizin and glycyrrhetic acid inhibits alpha-naphthyl isothiocyanate-induced liver injury and bile acid cycle disruption

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Abstract

Alpha-naphthyl isothiocyanate (ANIT) is a common hepatotoxicant experimentally used to reproduce the pathologies of drug-induced liver injury in humans, but the mechanism of its toxicity remains unclear. To determine the metabolic alterations following ANIT exposure, metabolomic analyses was performed by use of liquid chromatography-mass spectrometry. Partial least squares discriminant analysis (PLS-DA) of liver, serum, bile, ileum, and cecum of vehicle- and ANIT-treated mice revealed significant alterations of individual bile acids, including increased tauroursodeoxycholic acid, taurohydrodeoxycholic acid, taurochenodeoxycholic acid, and taurodeoxycholic acid, and decreased ω -, β - and tauro- α/β -murideoxycholic acid, cholic acid, and taurocholic acid in the ANIT-treated groups. In accordance with these changes, ANIT treatment altered the expression of mRNAs encoded by genes responsible for the metabolism and transport of bile acids and cholesterol. Pre-treatment of glycyrrhizin (GL) and glycyrrhetic acid (GA) prevented ANIT-induced liver damage and reversed the alteration of bile acid metabolites and *Cyp7a1*, *Npc1l1*, *Mttp*, and *Acat2* mRNAs encoding bile acid transport and metabolism proteins. These results suggested that GL/GA could prevent drug-induced liver injury and ensuing disruption of bile acid metabolism in humans.

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Conflict of interest
None.

Keywords

alpha-naphthyl isothiocyanate; glycyrrhizin; glycyrrhetic acid; bile acid cycle; metabolomics

1. Introduction

Alpha-naphthyl isothiocyanate (ANIT) is a hepatotoxicant used experimentally in rodents to model human intrahepatic cholestasis. Drug-induced liver injury and cholestasis in humans can be modeled by exposure of rodents to ANIT (Kossor et al. 1993). Determining the mechanism of ANIT-induced liver injury would be of great value for understanding the pathogenesis of drug-induced liver injury and the development of efficient therapeutics. However, the mechanism of ANIT-induced liver injury has not been elucidated. Previous studies have yielded clues to potential mechanisms of action. ANIT-induced liver damage is mediated by an ANIT-GSH conjugate which can dissociate upon crossing the canalicular membrane to yield free GSH and ANIT in the bile (Dietrich et al. 2001). ANIT damages the bile duct epithelium leading to cholestasis. Earlier studies revealed that inflammation also contributes to ANIT-induced hepatotoxicity (Faiola et al. 2010; Kodali et al. 2006).

Bile acids (BAs), the major end-products of cholesterol metabolism that serve as detergent molecules that enhance the absorption of vitamins and lipids from the diet, are also signaling molecules and metabolic regulators that activate nuclear receptors and a G protein linked membrane-type receptor (M-BAR or TGR5) (Makishima et al. 1999; Parks et al. 1999; Wang et al. 1999) involved in cell signaling pathways in the liver and intestine, such as c-Jun N-terminal kinase (JNK) 1/2 to regulate hepatic lipid, glucose, and energy homeostasis, and maintain metabolic homeostasis (Kawamata et al. 2003; Maruyama et al. 2002). BAs also influence intestinal bacterial growth in the small intestine (Inagaki et al. 2006). BAs are synthesized in liver via the classic and alternative pathways (Chiang 2013). The primary BAs in mice are cholic acid (CA), and α - and β -muricholic acid (MCA) (Elliott and Hyde 1971), which are synthesized from cholesterol. Primary bile acid metabolites are conjugated with taurine (in mice and rats) or glycine (in humans) in the liver before being transported into the bile and where gut bacterial enzymes generate secondary BAs through dehydroxylation and deconjugation reactions. Both primary and secondary BAs are subjected to enterohepatic circulation in which over 90% of BA produced in the liver are reabsorbed to the circulation. Disorders in BA metabolism may cause cholestatic liver diseases, and contribute to dyslipidemia, fatty liver diseases, cardiovascular diseases, and insulin resistance. The total levels of BAs in liver and serum have been widely used for determining the extent of ANIT-induced cholestasis (Yang et al. 2013). Individual BAs (*e.g.*, CA, α -MCA, β -MCA, *etc.*) have also been used for phenotyping the severity of cholestasis (Cui et al. 2009). However, the mechanism of BA disruption caused by ANIT remains undetermined.

Glycyrrhizin (GL), the major pentacyclic triterpene isolated from licorice, is known for its detoxifying and hepatoprotective properties, as well as many other pharmacological activities (Armanini et al. 2002; Isbrucker and Burdock 2006). GL is hydrolyzed by glucuronidase expressed by intestinal bacteria to its active principle aglycone, 18 β -

glycyrrhetic acid (GA). GL and GA possess several pharmacological activities, such as anti-inflammatory (Finney and Somers 1958), hepatoprotective (Kiso et al. 1984; Nose et al. 1994; Zhai et al. 2007), anti-tumor (Lee et al. 2008), and anti-viral ones (Mahato et al. 1992). GL and GA have been used for more than 20 years in the treatment and prevention of hepatitis, chronic bronchitis, gastritis, tumor growth and immunological disorders (Wan et al. 2009). The known anti-inflammatory and hepatoprotective effects of GL and GA warrant investigation as a potential therapy for chemically-induced cholestasis.

In the present study, BA metabolomics was used to profile BAs in liver, serum, bile, ileum, and cecum, to determine how ANIT influences whole-body BA metabolism. Furthermore, the protective role of GL and GA towards ANIT-induced cholestasis was examined from the perspective of BA metabolism and transport.

2. Materials and methods

2.1. Chemicals

ANIT (purity=95%), GL and GA were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). BAs were ordered from Steraloids, Inc. (Newport, RI) and Sigma-Aldrich. HPLC grade solvents were obtained from Fisher Scientific (Hampton, NH). All the other chemicals and solvents were of the highest grade commercially available.

2.2. Animal experiments and sample collections

Six- to 8-week-old male 129/Sv mice were maintained in the NCI animal facility under a standard 12 h light/12 h dark cycle with food and water *ad libitum*. The NCI Animal Care and Use Committee reviewed and approved all mouse experiments. Mice were randomly divided into four groups: untreated control group (n=5), GL- or GA-treated group (n=5), ANIT-treated group (n=4 to 5) and GL+ or GA+ANIT-treated group (n=5). GL and GA dissolved in corn oil were administered by intraperitoneal injection (*i.p.*) at a dosage of 50 mg/kg body weight seven times with an interval of 24 h; the control groups were treated by *i.p.* with corn oil. The ANIT groups were administered corn oil by *i.p.* every 24 h for the first four days. On day 5, the ANIT and GL- or GA/ANIT-treated groups were gavaged with ANIT (75 mg/kg, dissolved in corn oil). On day 6, the ANIT group, GL- or GA/ANIT-treated groups were administered corn oil, GL and GA by *i.p.*, respectively, as in the first four days. The mice were killed 48 h after administration of ANIT (on the 7th day). Serum samples were collected and the liver, bile, ileum and cecum harvested. A portion of the liver was collected in 10% buffered formalin for histological assessment and the remaining tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80 °C for further use.

2.3 Histological and biochemical assessments

Liver tissues were immediately fixed in 10% neutral formalin, dehydrated in graded ethanol/xylene, and embedded in paraffin. The tissues were cut to 4- μ m thickness, stained with hematoxylin and eosin (H&E), and examined using an Olympus BX41 microscope. Serum alanine and aspartate aminotransferase (ALT and AST, respectively), and alkaline phosphatase (ALP) activities were evaluated using VetSpec Kits (Catachem, Bridgeport, CT)

following the manufacturer's protocols. Plasma levels of total cholesterol in serum were measured using assay kits from Wako Diagnostics (Richmond, VA).

2.4. Metabolomics analysis of bile acids

Serum samples were thawed and 10 μ L added to a microcentrifuge tube containing 190 μ L 66% aqueous acetonitrile and 5 μ M chlorpropamide, used as the internal standard. One μ L of bile was added to a microcentrifuge tube containing 199 μ L 50% aqueous acetonitrile (5 μ M of chlorpropamide). Liver, ileum and cecum samples (about 20 mg) were homogenized with 50% acetonitrile containing 5 μ M of chlorpropamide (1:20). The samples were vortexed for 30 s and centrifuged at 14,000 rpm for 15 min at 4 $^{\circ}$ C to remove particulates and precipitate proteins. The supernatant was transferred to an autosampler plate for analysis. A previously published UPLC-MS method was modified to determine the contents of individual BAs (Li et al. 2013; Qi et al. 2015).

2.5. Data processing and multivariate data analysis

Centroided and integrated chromatographic data were processed using MarkerLynx software (Waters Corp., Milford, MA) to generate a data matrix consisting of peak areas corresponding to a unique m/z and retention time (RT) without normalization. The multivariate data matrix was imported into SIMCA-P 13.0 (Umetrics, Kinnelon, NJ) for partial least-squares discriminant analysis (PLS-DA).

2.6. Quantitative polymerase chain reaction (qPCR) mRNA analysis

Total mRNA was extracted from approximately 20 mg of frozen liver tissues and 50 mg of ileum tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) and quantified with NanoDrop (Thermo Scientific, Rockford, IL). Complementary DNA (cDNA) was synthesized from 1 μ g of total RNA with a SuperScript II Reverse Transcriptase kit and random oligonucleotides (Invitrogen). All the qPCR primer sequences used in this study were designed using qPrimerDepot and have been previously reported (Fang et al. 2013; Tanaka et al. 2012). qPCR reactions contained 25 ng cDNA, 150 nM of each primer, and 5 μ L of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a total volume of 10 μ L. All reactions were performed on an ABI-Prism 7900HT Sequence Detection System (Applied Biosystems). Relative mRNA levels were calculated by the comparative threshold cycle method using 18S rRNA as the internal control.

2.7 Western blot analysis

Protein extracted from liver tissue were obtained using Radio Immunoprecipitation Assay (RIPA) lysis buffer (1 mM phenylmethanesulfonyl fluoride, PMSF) according to the manufacturer's instruction (Beyotime Biotechnology Co. Ltd, China). Protein concentration were measured by the BCA assay kit (ThermoFisher Scientific, Allentown PA). Forty μ g of each lysate sample was boiled for 10 min, separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, USA). Nonspecific reactivity was blocked in 5% nonfat dry milk in Tris-buffered saline (0.05% Tween-20) (TBST) for 1h at room temperature. The membranes were incubated overnight with primary antibodies including

anti-SULT2A1 (ab194113, Abcam, Cambridge, MA), anti-UGT1A1 (ab194697, Abcam, Cambridge, MA) and β -actin (Jinqiao Technology Co. Ltd, China) followed by reaction with a secondary horseradish peroxidase-conjugated (HRP) anti-rabbit IgG antibody (Jinqiao Technology Co. Ltd, China). Specific bands were detected by use of the ECL Chemiluminescence System (Merck Millipore, USA).

2.8. Statistical analysis

Data was analyzed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA). All data were expressed as mean $\pm$ S.E.M. Unpaired Student's *t* test or nonparametric Mann-Whitney U-test was used for statistical analysis between two groups. For multiple group comparison, one-way ANOVA as well as Tukey test was performed. Difference was considered as significant when the *P* values were less than 0.05.

3. Results

3.1. ANIT disrupts the bile acid cycle

To determine how ANIT affects whole-body BA metabolism, the changes of BAs in liver, serum, bile, ileum and cecum that result from ANIT treatment were analyzed using UPLC-ESI-QTOFMS-based BA metabolomics. PLS-DA modelling of the UPLC-ESI-QTOFMS-negative mode data from serum and tissues revealed distinct clustering of the control and ANIT-treated groups (Fig. 1). PLS-DA of the liver metabolome revealed a clear separation between the control group and ANIT-treated group (Fig. 1A). Serum, bile, ileum and cecum metabolomic analysis revealed a similar trend as the liver extracts, including PLS-DA separation trends between the control and ANIT-treated groups (Fig. 1B–E). To determine the contributors to these separations, 12 BA markers were quantified using specific ion monitoring and purified BA metabolite standards (Supplementary Fig 1 and 2). Levels of several BA metabolites were altered in liver, serum, bile, ileum and cecum from ANIT-treated mice (Table 1). In the liver, ω -MCA, β -MCA, CA, T- α/β -MCA and TCA were significantly higher than that of the control group, and the relative abundances of TUDCA, THDCA, TCDCA and TDCA were lower than that of the control group. In the case of serum, the alterations of the 12 BAs were the same as those in liver, but there were no significant differences in T- α/β -MCA and TCA between the control group and ANIT-treated group. In the bile, the levels of most metabolites were decreased, with notable significant reductions of T- α/β -MCA, TCA, TUDCA, THDCA, TCDCA and TDCA compared with the control group. Twelve BA metabolites could be detected in ileum and cecum from the ANIT-treated group that were lower than that of control group, except for CA and DCA in cecum.

3.2. GL and GA prevented ANIT-induced liver injury

To evaluate the protective effect of GL and GA on ANIT-induced hepatic injury, serum ALT, AST, and ALP activities were assessed. A single dose of ANIT to mice resulted in elevated serum ALT (14-fold), AST (6.5-fold), and ALP (3.5-fold) compared with the control group (Fig. 2A). Liver histology revealed the presence of cholangitis and patchy hepatocyte necrosis in ANIT-treated mice (Fig. 3.). Treatment with GL or GA for four days before ANIT injection resulted in marked reductions in serum ALT, AST, and ALP activities and less hepatocyte degeneration (Fig. 2B and C; Fig. 3). Serum cholesterol concentrations

increase 2-fold after ANIT treatment, but the increases dropped near to basal levels found in control mice by pre-treatment with GL or GA prior to ANIT (Fig. 2D). These data suggest that GL and GA prevented ANIT-induced liver damage.

3.3. Effects of GL and GA on ANIT-induced BA disruption

Compared with the ANIT-treated group, GL pre-treatment significantly decreased the levels of T- α / β -MCA ($P < 0.01$) and TCA ($P < 0.01$), and increased the levels of TCDCA ($P < 0.05$) in liver (Fig. 4A). In serum, treatment with GL reduced the levels of ω -MCA ($P < 0.05$), β -MCA ($P < 0.05$), T- α / β -MCA ($P < 0.01$), TCA ($P < 0.01$), TUDCA ($P < 0.05$), TCDCA ($P < 0.05$), and TDCA ($P < 0.01$) elevated by the ANIT treatment (Fig. 4B). Reversal of ANIT-induced BAs by GL was also observed in bile (Fig. 4C). Compared with the ANIT-treated group, the levels of TCA, TUDCA, TCDCA, and TDCA in GL/ANIT group were significantly higher in ileum (Fig. 4D). For the cecum samples, the levels of CA and TCA were lower in the GL/ANIT group than that in the ANIT group (Fig. 4E). In contrast, TUDCA, THDCA, TDCA were higher in the GL/ANIT group than that in the ANIT group (Fig. 4J). A similar trend was noted under GA treatment (Fig. 5A–E). The levels of glycine-conjugated BAs in mice liver were also determined and only glycocholic acid (GCA) could be detected at very low levels. These effects were diminished by treatment with GL and GA (Supplementary Fig. 3).

3.4. Expression of genes responsible for regulation of BA/cholesterol homeostasis

ANIT treatment significantly inhibited hepatic expression of mRNAs involved in BA transport, *Osta* ($P < 0.05$), *Abcc1* ($P < 0.05$), and cholesterol metabolism-related mRNAs *Cyp7a1* ($P < 0.05$) and *Cyp7b1* ($P < 0.05$) (Fig. 6A). The expression of the *Ostb*, *Bsep*, *Abcc3*, *Abcb4*, *Slco1a4*, and *Atp8b1* mRNAs in liver was significantly induced by ANIT treatment (Fig. 6A). Compared with the control group, the expression of *Ostb* and *Abcc3* mRNAs was induced ($P < 0.01$) and suppressed ($P < 0.05$), respectively, in ileum. The cholesterol homeostasis regulation related genes *Acat2*, *Mttp*, and *Npc1l1* were increased in liver from the ANIT-treated group ($P < 0.01$, $P < 0.05$ for *Mttp*). In contrast, the expression of the *Abcg5* and *Acat2* mRNAs were markedly decreased in the ANIT-treated ileum samples (Fig. 6B). Treatment with GL reversed the expression of *Ostb* ($P < 0.01$), *Abcc3* ($P < 0.05$), *Slco1a4* ($P < 0.05$), *Cyp7a1* ($P < 0.05$), *Acat2* ($P < 0.05$), *Mttp* ($P < 0.01$), and *Npc1l1* ($P < 0.01$) mRNAs in liver (Fig. 6A), and *Npc1l1* ($P < 0.05$) mRNA in ileum (Fig. 6B). Treatment with GA significantly reversed the elevation of *Ostb* ($P < 0.001$), *Bsep* ($P < 0.01$), *Abcc1* ($P < 0.01$), *Abcc3* ($P < 0.05$), *Abcb4* ($P < 0.0001$), *Slco1a4* ($P < 0.05$), *Atp8b1* ($P < 0.05$), *Cyp7a1* ($P < 0.001$), *Acat2* ($P < 0.05$), *Mttp* ($P < 0.05$), *Npc1l1* ($P < 0.01$) mRNAs in liver (Fig. 6C), and *Abcg5* ($P < 0.01$) mRNA in ileum (Fig. 6D).

3.5 Western blot

Measurement of protein expression of UGT1A1 and SULT2A1 in mouse liver was carried out to determine if they could influence bile acid levels (Supplementary Fig. 4). Although significant differences could be observed between the ANIT group and the control group for UGT1A1 and SULT2A1 levels, there was no significant difference between the ANIT group and ANIT+GL/GA group, which suggests only a minor effect of UGT1A and SULT2A1

contributing to the influence of GL and GA on ANIT-induced liver injury and bile acid cycle disruption.

4. Discussion

ANIT treatment caused a disruption of the BA cycle and altered distribution of individual BAs in different compartments resulting in cholestasis. TUDCA and UDCA were demonstrated as two major hepatoprotective BAs in various liver toxicity models, including the cholestasis model (Baiocchi et al. 2008; Milkiewicz et al. 1999) and the ethanol-induced liver damage model (Vendemiale et al. 1998). The present results showed a significant decrease in TUDCA in liver, bile, ileum, cecum and serum after ANIT-induced liver damage. A similar trend was also observed for THDCA that was demonstrated to be a hepatoprotective BA (Roda et al. 1998). On the other hand, TCDCA and TDCA are regarded as inducers of cholestatic liver injury (Fukumoto et al. 2002). ANIT treatment also reduced these two BAs possibly as an adaptive response towards ANIT-induced cholestasis. However there was an opposite trend for MCA, CA, TCA and TMCA levels in liver, bile, ileum, cecum and ileum. The elevation of ω -MCA, β -MCA, and T- α/β -MCA in serum and liver, and reduction of these BAs in ileum and cecum indicated that hepatic secretion of these BAs may be inhibited or increase the intestinal absorption of these BA components. The levels of CA and TCA also increased in liver and serum, and decreased in ileum. However, CA and TCA increased in cecum. While the mechanism was unclear, the influence of ANIT towards intestinal bacteria might have resulted in a decrease in the levels of these conjugated BAs in the gut. These alterations likely affect the expression of genes involved in the metabolism and transport of BAs. For example, inhibition of the BA synthesis gene *Cyp7a1* further resulted in the accumulation of cholesterol. The increased expression of *Npc1l1* in liver also may decrease secretion of cholesterol from liver. The increased expression of *Mttp* could affect the assembly and secretion of cholesterol into the blood, and decreased expression of *Abcg5* in intestine could increase blood cholesterol levels. Altered expression of *Acat2* in liver and intestine, respectively, after ANIT could result in increased secretion and decreased absorption of cholesterol that might be a response to the elevation of cholesterol. These findings aid in understanding the mechanism of disruption of BA/cholesterol metabolism in the liver-gut axis following ANIT administration.

The hepatoprotective mechanism of GL and GA was evaluated in various liver toxicity models. For example, GL was reported to exhibit hepatoprotection towards lithocholic acid-induced injury, and the potential mechanism was suggested as induction of *Cyp3a11* through pregnane X receptor activation (Wang et al. 2012). Others demonstrated that GL protected against acetaminophen-induced liver damage through reversing fatty acid metabolism (Yu et al. 2014). GA protected carbon tetrachloride-induced liver injury via the inhibition of the expression of *Cyp2e1* (Jeong et al. 2002). The protection of GA towards free fatty acid-induced hepatic lipotoxicity was attributed to stabilizing the integrity of lysosomes and mitochondria and inhibiting cathepsin B expression and enzyme activity (Wu et al. 2008). In the current study, GL and GA were examined for their protective role of towards ANIT-induced liver damage and cholestasis. A reversing effect of GL and GA towards ANIT-induced BA cycle disruption and cholesterol alteration was observed, along with normalized expression of genes involved in the metabolism and transport of BAs and cholesterol. The

phase II conjugation of BA is mediated by UGT1A1 and SULT2A1. But UGT1A1 and SULT2A1 all exhibited minor effect on the rescuing of GL/GA to ANIT-induced bile acid cycle disruption. Previous studies found that GL and GA can significantly affect the metabolism by intestinal bacteria (Akao 2000), and intestinal bacteria have a key role in the modulation of BA homeostasis (Ridlon et al. 2014). Earlier studies showed that the deficiency of BA-activated transcription factor farnesoid X receptor (FXR) could enhance the susceptibility to ANIT-induced liver injury in mice (Cui et al. 2009). The FXR agonist GW4064 protected against ANIT-induced cholestasis (Liu et al. 2003). Transgenic mice with specific expression of FXR in intestine were used to demonstrate that selective activation of intestinal FXR could protect liver from ANIT-induced cholestasis through regulating the BA pool size (Modica et al. 2012). Additionally, GL/GA were reported to affect the activity of some signaling molecules including nuclear factor kappa B (Wang et al. 2012; Yin et al. 2010), exerting hepatoprotective actions. Thus, the mechanisms of preventive effect of GA and GL against ANIT-induced liver injury are multiple and complex.

Others reported ANIT-induced alterations in BA profiles using serum, liver, urine, and feces (Tang et al. 2016). However, the current study used serum, liver, bile, ileum, and cecum obtained from ANIT-treated mice. Direct determination of BA profiles using bile and intestine tissues, and hepatic/ileal expression of the genes involved in BA/cholesterol metabolism further clarified the comprehensive changes in BA/cholesterol homeostasis and helped determine the role of the liver-gut BA axis in the mechanism of action of cholestatic hepatotoxins. Additionally, serum BA profiles revealed significant increases in ω -MCA, β -MCA, and CA and similar trends in T- α / β -MCA and TCA in ANIT-treated mice, which were reversed by pretreatment with GL and GA. These findings are partially in agreement with the results of a previous study that pre-treatment of gentiopicoside ameliorated increases in serum ALT and ALP activities, as well as serum concentrations of β -MCA, CA, TCA, T- α -MCA, and T- ω -MCA (Tang et al. 2016). Therefore, circulating MCA/CA and their taurine-conjugated derivatives may reflect the severity of ANIT-induced hepatotoxicity.

Drug-induced liver injury may lead to prolonged and refractory cholestasis and can become a serious condition necessitating cessation of medication. Therefore, preventive interventions may be of value for certain medications or patients. Murine ANIT-treated liver injury mimics the condition of drug-induced liver injury in humans and GA/GL are commonly administered to humans having chronic viral hepatitis, but the usefulness of these agents for drug-induced liver injury has not been established. One reason is that the dose of GA to humans is smaller than that administered to mice in experimental models (approximately 3 mg/kg body weight/day equivalent for humans vs. 50 mg/kg body weight mg/day in the present study). This study yields the possibility that higher doses of GA/GL may be a promising preventive agent for drug-induced liver injury in humans.

In conclusion, ANIT-induced BA cycle disruption was determined using BA metabolomic methods and this result was successfully used for the mechanism explanation of the protective role of GL and GA towards ANIT-induced cholestasis (Fig. 7).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

UPLC-ESI-QTOFMS	ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry
CA	cholic acid
ω-MCA	ω -muricholic acid
β-MCA	β -muricholic acid
MDCA	murideoxycholic acid
UDCA	ursodeoxycholic acid
DCA	deoxycholic acid
T-α-MCA	tauro- α -muricholic acid
T-β-MCA	tauro- β -muricholic acid
TCA	taurocholic acid
TUDCA	tauroursodeoxycholic acid
THDCA	taurohydrodeoxycholic acid
TCDCa	taurochenodeoxycholate
TDCA	taurodeoxycholic acid
PLS-DA	partial least squares-discriminant analysis
Osta	organic solute transporter alpha
Ostβ	organic solute transporter beta

Bsep	bile salt export pump
Cyp7a1	cholesterol 7 α -hydroxylase
I-Babp	intestinal bile acid binding protein
Mttp	microsomal triglyceride transfer protein

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Fig. 1.

The effect of GL or GA pretreatment on ANIT-induced liver injury. Significant comparison was based on two-tailed *Student's t*-test or Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.0001$ compared with control in same group.

Fig. 2. Effect of ANIT on the BA metabolites in mice ($n = 4$ or 5 in each group). Based on the score plots from PLS-DA analysis, distinct BA profiles of control and ANIT-treated groups were shown for liver (A), serum (B), bile (C), ileum (D) and cecum samples (E).

Fig. 3. Liver histology of ANIT-treated mice (H&E staining). Representative photomicrographs taken in $\times 40$ magnification are shown. Bars represent 500 μm . Arrows indicate hepatocyte degeneration area.

Fig. 4.

Rescuing effect of GL on the BA composition. Comparison of 9 BA metabolites in liver (A), serum (B), bile (C), ileum (D) and cecum samples (E) from the ANIT-treated and ANIT +GL-treated groups are shown. Significant comparison was based on two-tailed *Student's t*-test or Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$ compared with ANIT-treated group.

Fig. 5.

Rescuing effect of GA on the BA metabolite composition. Comparison of 9 BA compositions in liver (A), serum (B), bile (C), ileum (D) and cecum samples (E) from the ANIT- treated and ANIT+GA-treated groups are shown. Significant comparison was based on two-tailed *Student's t*-test or Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$ compared with ANIT-treated group.

Fig. 6.

Effect of GL/GA on the gene expression profiles involved in BA homeostasis. (A) Hepatic mRNA expression of genes related with BA metabolism, transportation and cholesterol absorption and transpotation in control, ANIT-treated and ANIT+GL-treated mice. (B) Ileum mRNA expression of genes involved in BA metabolism, transportation and cholesterol absorption and transpotation in control, ANIT-treated and ANIT+GL-treated mice. (C) Hepatic mRNA expression of genes related with BA metabolism, transportation and cholesterol absorption and transpotation in control, ANIT-treated and ANIT+GA-treated mice. (D) Ileum mRNA expression of genes involved in BA metabolism, transportation and cholesterol absorption and transpotation in control, ANIT-treated and ANIT+GL-treated mice. Significant comparison was based on Tukey test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$, versus control group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.005$, #### $P < 0.0001$, versus ANIT group

Fig. 7.
Mechanisms of rescuing effects of GL and GA on BA cycle disruption caused by ANIT.

Table 1
Change in BAs levels in serum and tissues from ANIT-treated mice compared with control vehicle-treated mice.

	Liver	Serum	Gallbladder	Ileum	Cecum
w-MCA	↑ ^{**}	↑ [*]	↑	↓	↓ [*]
β-MCA	↑ ^{***}	↑ [*]	↑	↓	↓
CA	↑ ^{***}	↑ [*]	↓	↓	↓
MDCA	--	--	--	--	↓ [*]
UDCA	--	--	--	--	↓ [*]
DCA	--	--	--	--	↓ [*]
T-α,β-MCA	↓ ^{***}	↓	↓ ^{**}	↓ [*]	↓
TCA	↓ ^{***}	↑	↓ ^{**}	↓ [*]	↓ [*]
TUDCA	↓	↓	↓ ^{****}	↓	↓
THDCA	↓	↓	↓ [*]	↓	↓
TCDCa	↓	↓	↓ [*]	↓	↓
TDCA	↓ ^{**}	↓	↓ ^{****}	↓	↓

* $P < 0.05$;

** $P < 0.01$;

*** $P < 0.005$;

**** $P < 0.0001$ compared with the control group.