

DMD #21089

Down-regulation of a hepatic transporter Mrp2 is involved in alteration of pharmacokinetics of glycyrrhizin and its metabolites in a rat model of chronic liver injury

Toshiaki Makino, Nobuhiro Ohtake, Akito Watanabe, Naoko Tsuchiya, Sachiko Imamura, Seiichi Iizuka, Makoto Inoue, Hajime Mizukami

Department of Pharmacognosy, Graduate School of Pharmaceutical Science, Nagoya City University, Nagoya (T.M., A.W., H.M.); Tsumura Research Laboratories, Tsumura Co. Ltd. (N.O., N.T., S.I., S.I.); and Laboratory of Medicinal Resources, School of Pharmacy, Aichi Gakuin University, Nagoya (M.I.)

DMD #21089

Running title: Mrp2 and pharmacokinetics of glycyrrhizin

Corresponding Author:

Toshiaki Makino, Ph. D., Department of Pharmacognosy, Graduate School of Pharmaceutical Science, Nagoya City University, 3-1 Mizuho-ku, Nagoya 467-8603, Japan.

E-mail: makino@phar.nagoya-cu.ac.jp

Tel. & Fax: +81 52 836 3416

Text pages: 17

Tables: 4

Figures: 5

References: 30

Abstract: 244 words

Introduction: 660 words

Discussion: 649 words

Abbreviations: ALT, alkaline aminotransferase; ALP, alkaline phosphatase; AUC, area under the plasma concentration-time curve; cMOAT, canalicular multispecific organic anion transporter; CDAA, choline-deficient L-amino acid-defined; CSAA, choline-supplemented L-amino-defined; EHBR, Eisai hyperbilirubinemic rat; GA, glycyrrhetic acid; GL, glycyrrhizin; HE, hematoxylin and eosin; 3MGA, 3-monoglucuronyl-glycyrrhetic acids; Mrp, multi-drug resistance-associated protein; PL, phospholipid; 11 β -HSD2, type 2 11 β -hydroxysteroid dehydrogenase

DMD #21089

ABSTRACT

Glycyrrhizin (GL) has been used to treat chronic hepatitis in Japan and Europe. It is thought to induce pseudoaldosteronism via inhibition of type 2 11 β -hydroxysteroid dehydrogenase (11 β -HSD2) by glycyrrhetic acid (GA), a major metabolite of GL. A previous clinical study suggested that 3-monoglucuronyl-glycyrrhetic acid (3MGA), another metabolite of GL, might play a more important role in the pathogenesis of pseudoaldosteronism. The present study evaluates the pharmacokinetics of GL and its metabolites in rats with chronic liver injury induced by a choline-deficient L-amino acid-defined (CDAA) diet to clarify the relationship between 3MGA and pseudoaldosteronism. In rats fed a CDAA-diet, plasma concentrations and urinary eliminations of GL and 3MGA were markedly higher than in the rats fed control diet; the plasma concentration of GA was unaffected, when GL was orally administered. Immunohistochemical analysis revealed the suppression of levels of multi-drug resistance-associated protein 2 (Mrp2) and its localization in the hepatic tissue of rats fed a CDAA-diet. When 3MGA was intravenously injected in rats fed a CDAA-diet or injected in Mrp2-dysfunctional Eisai hyperbilirubinemic rats (EHBRs), plasma concentrations of 3MGA were higher and biliary excretion of 3MGA was lower than in each control group. The results suggested that 3MGA would be excreted to bile via hepatic Mrp2, and that its dysfunction would reduce 3MGA clearance. 3MGA accumulated by liver fibrosis resulted in the increased excretion through renal tubule and might be strongly related to the pathogenesis of pseudoaldosteronism because 11 β -HSD2 is expressed in renal

DMD #21089

tubular epithelial cells.

DMD #21089

Glycyrrhizin (GL) is a glycoside of glycyrrhetic acid (GA) with two molecules of glucuronic acid (Fig. 1). GL is one of active ingredients of licorice, roots of *Glycyrrhiza glabra* and *G. ularensis*. This was frequently prescribed in the herbal formula of Japanese traditional kampo medicine to treat various diseases. GL has also been used as a chemical drug to treat chronic hepatitis and allergic diseases for more than 20 years in Japan and Europe (Arase et al., 1997; van Rossum et al., 1998; Kumada, 2002). GL treatment has led to a significant decrease in serum alkaline aminotransferase (ALT) levels in patients with chronic hepatitis C (van Rossum et al., 2001).

An excessive quantity of licorice extract or GL preparation induces peripheral edema, hypokalemia, myopathy, and hypertension, which are the symptoms of pseudoaldosteronism; it can be potentially lethal (Morimoto and Nakajima, 1991). The frequency of pseudoaldosteronism caused by kampo medicines depend on the dosage and the duration of licorice-treatment (Homma et al., 2006). It was reported that administration of licorice extract corresponding to a daily dosage of 814 mg of GL (equivalent to 466 mg of GA) induced pseudoaldosteronism in healthy volunteers (Bernardi et al., 1994). Long-term (4 weeks) ingestion of licorice extract also induced a rise in blood pressure, even at low dosage (50 g/day; corresponding to 75 mg/day of GA) in healthy volunteers (Sigurjonsdottir et al., 2001). Repeated intravenous infusion of GL (200 mg/day) for the treatment of liver disease for five weeks induced hypotension with hypokalemia (Kanaoka et al., 1986). The pseudoaldosteronism caused by GL is thought to be due to the inhibition of type 2 11β -hydroxysteroid dehydrogenase (11β -HSD2) in renal tubular epithelial cells by GA, a major metabolite of GL (Tanahashi et al., 2002). The inhibition leads to an elevated level of renal cortisol, a potent agonist of

DMD #21089

mineralocorticoid receptors, leading to sodium retention, hypokalemia, hypertension and, eventually, to suppression of renin and aldosterone (Conn et al., 1968).

Orally administered GL was enzymatically hydrolyzed to GA by intestinal bacterial flora before absorption into the blood stream (Akao, 1998). The circulated GA was further metabolized to 3-monoglucuronyl glycyrrhetic acid (3MGA) in the liver by UDP-glucuronyltransferase, and then excreted with bile into the intestine (Ploeger et al., 2001). When GL was injected intravenously, it was partially metabolized to 3MGA in the liver by lysosomal β -D-glucuronidase; and GL and 3MGA could be excreted with bile. The biliary excreted GL and 3MGA were hydrolyzed by intestinal bacteria into GA, which was then re-absorbed into the blood stream (Akao et al., 1991; Akao, 1998).

It was reported that the plasma concentration of 3MGA was significantly higher in the hypokalemia group than in the normal group in patients with chronic hepatitis who had been treated with GL for more than four weeks; the plasma concentration of GA did not differ between the two groups (Kato et al., 1995). It was also shown that the 3MGA level was higher in patients treated with GL via the oral route than that in patients treated via the intravenous route (Kato et al., 1995). Plasma clearance of GL in cirrhosis patients was markedly delayed compared to that in healthy subjects when GL was given intravenously (Yamamura et al., 1995). These results suggest that 3MGA plays a more important role in inducing pseudoaldosteronism than GL and GA, and that the altered pharmacokinetic profile of GL caused by hepatic diseases is related to the mechanisms leading to pseudoaldosteronism.

In the present study, we investigated the pharmacokinetics of GL orally administered to rats with

DMD #21089

chronic liver injury and fibrosis induced by ingestion of a choline-deficient L-amino acid-defined (CDAA-) diet. We also examined the relationship between levels of multidrug resistance-associated protein 2 (Mrp2) in the liver and bile excretion of 3MGA using rats fed a CDAA-diet and Eisai hyperbilirubinemic rats (EHBRS) expressing the loss of function without a canalicular multispecific organic anion transporter (cMOAT/Mrp2) (Ito et al., 1997; Gotoh et al., 2000; Akita et al., 2001).

Materials and Methods

Animals. Male Wistar rats weighing 120–140 g, male Sprague-Dawley (SD) rats weighing 160–180 g, or male EHBRS weighing 160–250 g were purchased from Japan SLC (Hamamatsu, Japan). SD strain rats were used as the control of EHBRS, since EHBRS have the backgrounds of SD strain (Ito et al., 1997). Animals were housed three per cage, and received food and water *ad libitum* under controlled temperature (25°C), humidity, and lighting (12 h light, 12 h dark) in accordance with the guidelines for animal care at our institution.

Compounds and Diets. GL (purity > 99 %), GA (97%), and 3MGA (99%) were purchased from Calbiochem (San Diego, CA, USA), Tokyo Kasei Kogyo (Tokyo, Japan), and Nacalai Tesque (Kyoto, Japan), respectively. Carbenoxolone sodium was bought from LKT Laboratories, Inc. (St. Paul, MN, USA). The CDAA-diet and the control, choline-supplemented L-amino acid-defined (CSAA-) diet, were obtained from Dyets, Inc. (Bethlehem, PA, USA). The compositions of these diets were previously described (Nakae et al., 1992; Endoh et al., 1996; Sakaida et al., 1996).

Biochemical analysis. ALT, alkaline phosphatase (ALP), total bile acids (TBA), and

DMD #21089

phospholipids (PL) in plasma were measured using Biochemical Analyzer TBA-40FR (Toshiba Medical Systems Co., Tokyo, Japan). Plasma hyaluronic acid was estimated using a human ELISA kit (Seikagaku Co., Tokyo, Japan).

Liver Histology. Liver tissue was fixed in 10% formalin for 48 h and embedded in paraffin. Sections of 3 μm -thickness were examined under light microscopy after staining with hematoxylin and eosin (HE), oil red O, or Sirius red.

Immunoblot Analysis. Liver total homogenates and crude liver membranes were prepared as described by Okada et al. (2007). Transporter protein levels in rat liver membranes were determined using a monoclonal antibody against Mrp2 (Alexis Biochemicals Co., San Diego, CA, USA) according to the method described by Shoda et al. (2004). The immunoblot membranes were reprobed with an antibody against β -actin (Santa Cruz Biotechnology, Inc., CA, USA). The signals were detected with ECL plus western blotting detection system (GE Healthcare Bioscience, Buckinghamshire, UK), and densitometrically quantified by an image analyzer, Typhoon 9410 (GE Healthcare Bioscience). Signals of Mrp2 were normalized to those of β -actin in each specimen; the percentage of values in rats fed the CDAA-diet to that in rats fed a CSAA-diet for 4 and 12 weeks were then calculated ($n = 6$).

Immunohistochemical detection of Mrp2. Liver tissue specimens (each 50 mg) were frozen in Freon/liquid nitrogen, embedded in OCT Compound (Sakura Finetechnical Co., Ltd., Tokyo), cut into 6 μm -thick sections, and mounted on slides. The tissue sections were immunostained with monoclonal antibody against Mrp2 as previously described (Okada et al., 2007). Mrp2-staining area

DMD #21089

in 20 hyperfields of the specimen was measured by NIH image (National Institutes of Health, Bethesda, MD), and the average was determined as the value of Mrp2 level in each specimen. Data were expressed as the ratio of Mrp2 levels in the specimens of CDAA-rats to those in CSAA-rats ($n = 6$).

Bile Secretion. Rats were anesthetized with urethane (1 g/kg body weight, intraperitoneally injected) and the common bile duct cannulated with a polyethylene tube (outer diameter, 0.6 mm; SP10, Natsume, Tokyo, Japan). Starting from 10 min after cannulation, bile was collected into preweighed tubes at 15–30 min intervals over 120 min. Cumulative amounts of bilirubin excretion in bile were calculated based on the weight and total concentration of bilirubin of each specimen.

Pharmacokinetic analysis. GL (100 mg/kg body weight) was dissolved in distilled water containing 0.1% (v/v) NH_4OH and orally administered to rats fed the CDAA- or CSAA-diet. Blood (0.3 ml) was withdrawn via the right jugular vein 1, 2, 4, 8, 12, 24, and 48 h after treatment under light anesthesia with ether. Urine was collected over a period of 24 h using a metabolic cage after GL treatment. For studying the biliary excretion of 3MGA in rats fed the CSAA- or CDAA-diet, and in SD rats and EHBRs, the animals were anesthetized with urethane, and the common bile duct was cannulated with a polyethylene tube as mentioned above; the right jugular vein and carotid artery were cannulated with a polyethylene tube (SP10). 3MGA (5 mg/kg body weight) was dissolved in a mixture of polyethyleneglycol (PEG) 400/ethanol 4:1 and infused to the catheter of the jugular vein 10 min after cannulation. Blood (0.3 ml) was withdrawn via the carotid catheter at 5, 20, 40, 90 and 120 min, and was centrifuged (1,200g, 20 min, 4°C) to obtain plasma. Samples of plasma, bile, and urine

DMD #21089

were stored at -80°C until analysis. The area under the plasma concentration–time curve (AUC) was calculated using the trapezoidal rule from the first measurement point to the last measurement point.

Preparation of Sample for Liquid chromatography Analysis. Fifty microliters of each sample and standard was spiked with internal standard, carbenoxolone sodium (IS). The sample was loaded onto the Oasis HLB μ Elution plate (Waters, Milford, MASS, USA), which was pre-conditioned with methanol followed by 0.1 M HCl solution. The plate was washed with 0.1 M HCl solution and the analytes eluted with 200 μl of methanol. The eluate was evaporated to dryness under nitrogen gas and redissolved in 100 μl of mixture of 2% acetic acid in water and acetonitrile (1:1). Aliquots of the sample solutions were analysed by LC/MS/MS (10 μl) and HPLC/UV (30 μl).

Analysis of GL and its Metabolites. The concentrations of GL, 3MGA, and GA in plasma and urine were measured by LC/MS/MS analysis when GL was orally administered. The concentrations of 3MGA in plasma and bile were measured using HPLC/UV analysis when 3MGA was intravenously infused because these concentrations were too high for LC/MS/MS analysis.

LC/MS/MS analysis was done using the API 2000 system (Applied Biosystems Japan, Tokyo, Japan). The mass spectrometer used an electrospray ionization (ESI-) source in the negative ion mode with multiple reaction monitoring (MRM). The analytical column was an Inertsil ODS-3, 50×2.1 mm, 5 μm (GL Sciences Inc., Tokyo, Japan) and column temperature was kept at 40°C . The mobile phase was delivered with a linear gradient elution system, acetonitrile (A): 2% (v/v) acetic acid (B), at a flow rate of 200 $\mu\text{l}/\text{min}$. The gradient profile was as follows: 50% A increasing to 70% over 6 min, 70% A increasing to 90% over 4 min, and A:B (90:10) maintained for 5 min. The optimal

DMD #21089

operating parameters of MRM were found by infusing a mixture of standard solutions of GL, 3MGA, and GA with IS in the mobile phase at 1 $\mu\text{g/ml}$ each. The optimum conditions of the interface were as follows: curtain gas flow-rate (CUR), 20 l/min; nebulizing gas pressure (GS1), 50 psi; tube gas pressure (GS2), 50 psi; ion spray voltage, -4.5 kV; heater gas temperature (TEM), 400°C ; collision-activated dissociation gas (CAD), 6×10^{-3} Torr. Ions of the standards, GL, 3MGA, GA, and IS, were activated at -110 , -70 , -40 , and -32 V of collision energies (CE), respectively. To assay all analytes, both quadrupoles were maintained at unit resolution and the transitions (precursor to daughter) monitored were m/z 821.4 to 70.9 for GL, m/z 645.4 to 74.9 for 3MGA, m/z 469.3 to 425.1 for GA, and m/z 569.3 to 469.4 for IS. The dwell time for each transition was 150 ms. MRM data was acquired and the chromatograms integrated using MDS Sciex Analyst 1.4 software. A weighted $1/\text{concentration}^2$ quadratic regression was used to generate a standard calibration curve and calculate sample concentrations.

HPLC/UV analysis was done using the Shimadzu LC-10AD system (Shimadzu, Kyoto, Japan) with a photodiode array detector. The analytical column was a Symmetry ShieldTM RP-18, 75×4.6 mm, $3.5 \mu\text{m}$ (Waters), and column temperature was kept at 40°C . The mobile phase was delivered with a linear gradient elution system acetonitrile (A): 0.5% (v/v) acetic acid (B) at a flow rate of 1.0 ml/min. The gradient profile was as follows: 45% A increasing to 60% over 20 min, and then A:B (60:40) maintained for 5 min. The UV detector was set at 254 nm.

Statistical Analysis. Statistical analysis was carried out using the Student *t*-test. Statistical significance was considered at a *p* value of less than 0.05. Data were expressed as mean \pm standard

DMD #21089

deviation.

Results

Characteristics of the rats fed CDAA-diets. Chronic liver injury model was made using Wistar strain rats fed CDAA-diet (CDAA-rats) for 4 or 12 weeks. For the control, rats fed CSAA-diet (CSAA-rats) were prepared. Plasma levels of ALT, ALP, total bilirubin, and TBA were significantly higher in CDAA-rats than in CSAA-rats, while PL concentration was significantly decreased (Table 1). Liver weights in CDAA-rats were markedly greater than those in CSAA-rats (Table 1). Hepatic steatosis was observed in the liver tissues of rats fed the CDAA-diet for 4 and 12 weeks (Fig. 2). Plasma hyaluronic acid concentration, a plasma parameter of tissue fibrosis, was significantly higher only in rats fed the CDAA-diet for 12 weeks compared with CSAA-rats. The change in the plasma hyaluronic acid level was consistent with the histological observation, indicating severe hepatic fibrosis only in rats fed the CDAA-diet for 12 weeks (Fig. 2). Bilirubin excretion into bile was lower in CDAA-rats than in CSAA-rats, though bile flow did not change. Urinary bilirubin excretion was higher in the rats fed the CDAA-diet for 12 weeks than CSAA-rats (Table 2).

Pharmacokinetics of GL and its metabolites in CDAA-rats. GL was orally administered to rats fed the CDAA- or CSAA-diet for 4 or 12 weeks, and samples of blood and urine were collected. Time course of changes in the plasma concentration of GL, 3MGA, and GA in rats fed the CDAA-diet for 12 weeks are shown in Fig. 3. The plasma concentrations of GL and 3MGA in CDAA-rats were higher than those in CSAA-rats until 12 h for GL and 4 h for 3MGA after GL administration. The

DMD #21089

plasma concentration of GA for all the measurement periods after GL treatment was almost identical in CDAA- and CSAA-rats. AUC from 1 to 24 h (AUC_{1-24h}) of GL and its metabolites were compared between CDAA- and CSAA-rats (Table 3). The AUC_{1-24h} of GL and 3MGA in CDAA-rats were five- to ten-fold higher than those in CSAA-rats, respectively. In contrast, the AUC_{1-24h} of GA in CDAA-rats was almost identical as that in CSAA-rats. The volume of urine over 24 h showed no difference between CDAA- and CSAA-rats treated for 12 weeks; urinary excretions of GL and 3MGA in CDAA-rats were 5.4 times and 4 times, respectively, greater than those in CSAA-rats (Table 4). Urinary excretion of GA was below the detection limit (1 ng/100 g body weight) in CSAA-rats, and at trace levels in CDAA-rats.

Immunoblot and immunohistochemical analyses of liver Mrp2. Levels of Mrp2 in the livers of CDAA-rats were evaluated by western blotting and immunohistochemical analysis. Mrp2 levels in the plasma membrane fraction from liver homogenate of the rats fed the CDAA-diet for 12 weeks were lower than those of the rats fed the CSAA-diet (Fig. 4A). Densitometric analysis revealed that Mrp2 levels in the CDAA-diet-fed-rats for 4 and 12 weeks were $40.8 \pm 6.5\%$ ($p < 0.01$) and $37.0 \pm 5.5\%$ ($p < 0.001$), respectively, compared to those of CSAA-rats. Mrp3 levels were down-regulated only in the rats treated with the CDAA-diet for 12 weeks, and Mrp4 levels were unaffected by CDAA-diets (data not shown). Immunohistochemical analysis of Mrp2 in liver tissue sections confirmed remarkable differences between the rats fed the CSAA-diet and those fed the CDAA-diet for 12 weeks (Fig. 4B). The Mrp2-derived signals were much lower in rats fed the CDAA-diet than those fed the CSAA-diet for 4 weeks ($27.7 \pm 6.3\%$, $p < 0.001$) and 12 weeks ($23.6 \pm 5.3\%$, $p < 0.001$).

DMD #21089

Pharmacokinetics of intravenously administered 3MGA in rats fed the CDAA-diet, and in EHBRs. 3MGA was intravenously injected into rats fed the CSAA- or CDAA-diet for 13 weeks, and the plasma and bile concentrations of 3MGA traced (Fig. 5A and 5B). In CSAA-rats, 3MGA disappeared quickly from the plasma and about 90% of 3MGA was excreted in the bile within 120 min; the rate of disappearance of 3MGA from plasma was slower in CDAA-rats than in CSAA-rats. The plasma concentration of 3MGA in CDAA-rats was significantly augmented compared to that in CSAA-rats. The excretion of 3MGA into bile of CDAA-rats was markedly suppressed by liver injury. The pharmacokinetics of intravenously injected 3MGA were also evaluated using EHBRs which are expressing dysfunctional Mrp2 protein (Fig. 5C and 5D). The plasma concentration of 3MGA was immediately decreased, and approximately 85% of 3MGA was excreted into the bile 40 min post-injection. In contrast, biliary excretion of 3MGA was significantly slower in EHBR than in SD rats, and plasma concentrations of 3MGA were significantly higher by 120 min post-injection in EHBRs than in SD rats (Fig. 5C and D).

Discussion

Feeding CDAA-diets to rats is known to induce hepatic fibrosis and cirrhosis, and has been used to establish an experimental model of hepatitis in animals (Jin et al., 2007; Ustundag et al., 2007). Biochemical analysis and histological observation in this study revealed that severe liver injury was induced by feeding the CDAA-diet to Wistar rats for as little as 4 weeks. For CDAA-rats given GL orally, the plasma concentration, AUC, and urinary excretion of GL and 3MGA were significantly

DMD #21089

increased, while those of GA were not affected. In contrast, excretion of 3MGA into bile was significantly reduced in CDAA-rats when 3MGA was intravenously injected. These results clearly indicate that the pharmacokinetics of GL and its metabolites are altered when severe liver injury is induced. They are consistent with a clinical report stating that the elimination of GL from plasma was significantly slower when GL was injected in patients with chronic hepatitis than when it was injected in healthy individuals (Yamamura et al., 1995).

The increased plasma concentrations of GL and 3MGA in the hepatic fibrosis model rats may be related to the decreased level of Mrp2 in their livers. Mrp2 is an organic anion transporter located on the bile canalicular membrane, and is responsible for the excretion of various organic anions, including glutathione-conjugates and glucuronide-conjugates of xenobiotics into bile (Keppler and König, 1997; Gotoh et al., 2000). The Mrp2 level in the livers of CDAA-rats was drastically down-regulated, which might lead to a decrease in excretion of 3MGA in bile, and with a subsequent increase in tubular secretion (as shown in the increased urinary concentration of 3MGA in CDAA-rats). EHBRs was originally found as mutant rats with chronic conjugated hyperbilirubinemia (Hosokawa et al., 1992). They were subsequently confirmed to express the dysfunctional Mrp2 protein by a point mutation in the open reading frame (Ito et al., 1997). Excretion of 3MGA into bile was shown to be suppressed in EHBRs, which also supports the expectation that Mrp2 plays a role in the biliary excretion of 3MGA, and suggests that the reduction and/or dysfunction of Mrp2 by liver injury or mutation led to a decrease in bile excretion of 3MGA. Suppression of biliary excretion of GL and 3MGA resulted in increased plasma concentrations and

DMD #21089

eventually increased urinary excretion of GL and 3MGA through renal tubular epithelial cells.

The inhibitory effect of GL on 11 β -HSD2 activity was reported to be 200- to 1,000-fold less potent than GA (Buhler et al., 1994). In contrast, 3MGA exhibited potent inhibition of 11 β -HSD2 activity in kidney microsomes at a similar extent similar to GA (Kato et al., 1995). It was reported that patients with GL-induced hypokalemia exhibited higher plasma concentration of 3MGA than GL-treated patients without hypokalemia, although the plasma concentrations of GA between these two groups did not differ (Kato et al., 1995). GA would not be transported into renal tubular epithelial cells from the circulation because GA is scarcely eliminated into urine in rats as well as in humans (Ploeger et al., 2001). Therefore, it is predicted that the inhibitory effects of 3MGA on 11 β -HSD2 in renal tubular epithelial cells might be more potent than the effect of GA *in vivo*. Indeed, intravenous injection of 3MGA into guinea pigs was shown to decrease the plasma concentration of potassium (Ohtake et al., 2007). These studies suggest that 3MGA, not GA, is likely to play a central part in inducing pseudoaldosteronism by inhibiting 11 β -HSD2. Therefore, the accumulation of 3MGA in renal tissues by reduced biliary excretion due to liver dysfunction might be involved in the pathogenesis of pseudoaldosteronism caused by ingestion of licorice and GL. Further investigations of Mrp2 function for the biliary transportation of 3MGA and its transport mechanisms through tubular epithelium are required, but the present study provides basic but important information for the clinical use of GL and GL-containing herbal medicines because they are often prescribed to patients with liver dysfunction.

DMD #21089

References

- Akao T (1998) Distribution of enzymes involved in the metabolism of glycyrrhizin in various organs of rat. *Biol Pharm Bull* **21**:1036-1044.
- Akita H, Suzuki H, Ito K, Kinoshita S, Sato N, Takikawa H and Sugiyama Y (2001) Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. *Biochim Biophys Acta* **1511**:7-16.
- Arase Y, Ikeda K, Murashima N, Chayama K, Tsubota A, Koida I, Suzuki Y, Saitoh S, Kobayashi M and Kumada H (1997) The long term efficacy of glycyrrhizin in chronic hepatitis C patients. *Cancer* **79**:1494-1500.
- Bernardi M, D'Intino PE, Trevisani F, Cantelli-Forti G, Raggi MA, Turchetto E and Gasbarrini G (1994) Effects of prolonged ingestion of graded doses of licorice by healthy volunteers. *Life Sci* **55**:863-872.
- Buhler H, Perschel FH, Fitzner R and Hierholzer K (1994) Endogenous inhibitors of 11 β -OHSD: existence and possible significance. *Steroids* **59**:131-135.
- Conn JW, Rovner DR and Cohen EL (1968) Licorice-induced pseudoaldosteronism. Hypertension, hypokalemia, aldosteronopenia, and suppressed plasma renin activity. *JAMA* **205**:492-496.
- Endoh T, Tang Q, Denda A, Noguchi O, Kobayashi E, Tamura K, Horiguchi K, Ogasawara H, Tsujuchi T, Nakae D, Sugimura M and Konishi Y (1996) Inhibition by acetylsalicylic acid, a cyclo-oxygenase inhibitor, and p-bromophenacylbromide, a phospholipase A2 inhibitor, of both cirrhosis and enzyme-altered nodules caused by a choline-deficient, L-amino acid-defined diet in rats. *Carcinogenesis* **17**:467-475.
- Gotoh Y, Suzuki H, Kinoshita S, Hirohashi T, Kato Y and Sugiyama Y (2000) Involvement of an organic anion transporter (canalicular multispecific organic anion transporter/multidrug resistance-associated protein 2) in gastrointestinal secretion of glutathione conjugates in rats. *J Pharmacol Exp Ther* **292**:433-439.
- Homma M, Ishihara M, Qian W and Kohda Y (2006) Effects of long term administration of

DMD #21089

- Shakuyaku-kanzo-To and Shosaiko-To on serum potassium levels. *Yakugaku Zasshi* **126**:973-978.
- Hosokawa S, Tagaya O, Mikami T, Nozaki Y, Kawaguchi A, Yamatsu K and Shamoto M (1992) A new rat mutant with chronic conjugated hyperbilirubinemia and renal glomerular lesions. *Lab Anim Sci* **42**:27-34.
- Ito K, Suzuki H, Hirohashi T, Kume K, Shimizu T and Sugiyama Y (1997) Molecular cloning of canalicular multispecific organic anion transporter defective in EHBR. *Am J Physiol* **272**:G16-22.
- Jin H, Yamamoto N, Uchida K, Terai S and Sakaida I (2007) Telmisartan prevents hepatic fibrosis and enzyme-altered lesions in liver cirrhosis rat induced by a choline-deficient L-amino acid-defined diet. *Biochem Biophys Res Commun* **364**: 801-807.
- Kanaoka M, Yano S, Kato H and Nakada T (1986) Synthesis and separation of 18 β -glycyrrhetyl monoglucuronide from serum of a patient with glycyrrhizin-induced pseudo-aldosteronism. *Chem Pharm Bull* **34**:4978-4983.
- Kato H, Kanaoka M, Yano S and Kobayashi M (1995) 3-Monoglucuronyl-glycyrrhetic acid is a major metabolite that causes licorice-induced pseudoaldosteronism. *J Clin Endocrinol Metab* **80**:1929-1933.
- Kepler D and Konig J (1997) Hepatic canalicular membrane 5: Expression and localization of the conjugate export pump encoded by the MRP2 (cMRP/cMOAT) gene in liver. *FASEB J* **11**:509-516.
- Kumada H (2002) Long-term treatment of chronic hepatitis C with glycyrrhizin [stronger neo-minophagen C (SNMC)] for preventing liver cirrhosis and hepatocellular carcinoma. *Oncology* **62 Suppl 1**:94-100.
- Morimoto Y and Nakajima C (1991) Pseudoaldosteronism induced by licorice derivatives in Japan. *J. Trad. Med.* **8**:1-22.
- Nakae D, Yoshiji H, Mizumoto Y, Horiguchi K, Shiraiwa K, Tamura K, Denda A and Konishi Y

DMD #21089

- (1992) High incidence of hepatocellular carcinomas induced by a choline deficient L-amino acid defined diet in rats. *Cancer Res* **52**:5042-5045.
- Ogawa K, Suzuki H, Hirohashi T, Ishikawa T, Meier PJ, Hirose K, Akizawa T, Yoshioka M and Sugiyama Y (2000) Characterization of inducible nature of MRP3 in rat liver. *Am J Physiol Gastrointest Liver Physiol* **278**:G438-446.
- Ohtake N, Kido A, Kubota K, Tsuchiya N, Morita T, Kase Y and Takeda S (2007) A possible involvement of 3-monoglucuronyl-glycyrrhetic acid, a metabolite of glycyrrhizin (GL), in GL-induced pseudoaldosteronism. *Life Sci* **80**:1545-1552.
- Okada K, Shoda J, Kano M, Suzuki S, Ohtake N, Yamamoto M, Takahashi H, Utsunomiya H, Oda K, Sato K, Watanabe A, Ishii T, Itoh K, Yokoi T, Yoshizato K, Sugiyama Y and Suzuki H (2007) Inchinkoto, a herbal medicine, and its ingredients dually exert Mrp2/MRP2-mediated choleresis and Nrf2-mediated antioxidative action in rat livers. *Am J Physiol Gastrointest Liver Physiol* **292**:G1450-1463.
- Ploeger B, Mensinga T, Sips A, Seinen W, Meulenbelt J and DeJongh J (2001) The pharmacokinetics of glycyrrhizic acid evaluated by physiologically based pharmacokinetic modeling. *Drug Metab Rev* **33**:125-147.
- Sakaida I, Matsumura Y, Kubota M, Kayano K, Takenaka K and Okita K (1996) The prolyl 4-hydroxylase inhibitor HOE 077 prevents activation of Ito cells, reducing procollagen gene expression in rat liver fibrosis induced by choline-deficient L-amino acid-defined diet. *Hepatology* **23**:755-763.
- Shoda J, Miura T, Utsunomiya H, Oda K, Yamamoto M, Kano M, Ikegami T, Tanaka N, Akita H, Ito K, Suzuki H and Sugiyama Y (2004) Genipin enhances Mrp2 (Abcc2)-mediated bile formation and organic anion transport in rat liver. *Hepatology* **39**:167-178.
- Sigurjonsdottir HA, Franzson L, Manhem K, Ragnarsson J, Sigurdsson G and Wallerstedt S (2001) Licorice-induced rise in blood pressure: a linear dose-response relationship. *J Hum Hypertens* **15**:549-552.

DMD #21089

- Tanahashi T, Mune T, Morita H, Tanahashi H, Isomura Y, Suwa T, Daido H, Gomez-Sanchez CE and Yasuda K (2002) Glycyrrhizic acid suppresses type 2 11 β -hydroxysteroid dehydrogenase expression *in vivo*. *J Steroid Biochem Mol Biol* **80**:441-447.
- Ustundag B, Bahcecioglu IH, Sahin K, Duzgun S, Koca S, Gulcu F and Ozercan IH (2007) Protective effect of soy isoflavones and activity levels of plasma paraoxonase and arylesterase in the experimental nonalcoholic steatohepatitis model. *Dig Dis Sci* **52**:2006-2014.
- van Rossum TG, Vulto AG, de Man RA, Brouwer JT and Schalm SW (1998) Review article: glycyrrhizin as a potential treatment for chronic hepatitis C. *Aliment Pharmacol Ther* **12**:199-205.
- van Rossum TG, Vulto AG, Hop WC and Schalm SW (2001) Glycyrrhizin-induced reduction of ALT in European patients with chronic hepatitis C. *Am J Gastroenterol* **96**:2432-2437.
- Yamamura Y, Tanaka N, Santa T, Kotaki H, Aikawa T, Uchino K, Osuga T, Sawada Y and Iga T (1995) The relationship between pharmacokinetic behaviour of glycyrrhizin and hepatic function in patients with acute hepatitis and liver cirrhosis. *Biopharm Drug Dispos* **16**:13-21.

DMD #21089

Legends for Figures

Fig. 1 Chemical Structures of glycyrrhizin (GL), 3-monoglucuronyl-glycyrrhetic acid (3MGA), and glycyrrhetic acid (GA). GlucUA, glucuronic acid.

Fig. 2 Histology of the liver tissues of Wistar rats fed CSAA- or CDAA-diet for 4 and 12 weeks. Histological analysis was conducted using light microscopy at 40×magnification. HE, hematoxylin and eosin staining.

Fig. 3 Plasma concentration profiles of GL (A) and its metabolites (B, C) when GL was orally administered to Wistar rats with CSAA- or CDAA-diet for 12 weeks. GL (100 mg/kg) was orally administered to the rat and blood samples were collected 1, 2, 4, 8, 12, 24 and 48 h after treatment. Plasma concentrations of GL and its metabolites, 3MGA and GA were measured as described in Materials and Methods. Open square, CSAA-rats; closed circle, CDAA-rats ($n = 5-6$).

Fig. 4 Western blot and immunohistological analysis of Mrp2 in the rats fed CSAA- or CDAA-diet. A: Immunoblot analysis of Mrp2 in crude plasma membrane fraction isolated from liver of Wistar rats treated with CSAA- or CDAA- diet for 12 weeks. B: Immunohistological localizations of Mrp2 in the livers were analyzed using light microscopy at 100×magnification. Brownish area shows Mrp2-positive staining.

DMD #21089

Fig. 5 Plasma concentration profiles and biliary excretion of 3MGA when 3MGA was intravenously infused to Wistar rats fed CSAA- or CDAA-diet for 13 weeks (A, B) and SD rats or EHBRs (C, D). 3MGA (5 mg/kg) was intravenously infused to the rats and blood samples were collected at 5, 20, 40, 60, 90, and 120 min (A, C), and bile samples were collected over a period of 120 min (B, D). Plasma and bile concentrations of 3MGA were measured as described in Materials and Methods. Open square, CSAA-rats; closed circle, CDAA-rats (A, B); open circle, SD rats; closed square, EHBRs (C, D); ($n = 6$).

Table 1 Plasma characteristics of rats fed the CSAA- or CDAA-diet.

	4 weeks				12 weeks			
	CSAA		CDAA		CSAA		CDAA	
Body weight (g)	274	± 13.9	248	± 14.2 **	361	± 25	293	± 18 **
Liver weight (g)	7.3	± 0.7	16.3	± 1.6 ***	8.1	± 0.8	12.7	± 1.4 ***
ALT (U/l)	37.8	± 6.5	568	± 81 ***	34.3	± 6.1	422	± 85 ***
ALP (U/l)	617	± 41	965	± 97 ***	369	± 22	723	± 88 ***
Total bilirubin (µg/ml)	0.18	± 0.06	0.94	± 0.13 ***	0.23	± 0.04	0.89	± 0.19 ***
Bile acids (µmol/l)	5.33	± 0.44	46.0	± 5.19 ***	4.40	± 1.04	49.8	± 11.4 ***
Phospholipids (mg/dl)	143	± 15	88.7	± 8.5 ***	125	± 7	77.3	± 11.6 ***
Hyaluronic acid (ng/ml)	79.7	± 9.0	112	± 39	70.0	± 6.1	92.1	± 7.2 **

Wistar rats were fed CSAA- or CDAA-diet for 4 or 12 weeks, and then sacrificed. Plasma markers were measured as described in Materials and Methods. ** $p < 0.01$, *** $p < 0.001$; significantly different from the CSAA-rats ($n = 6$).

Table 2 Bilirubin excretions in bile and urine in rats fed the CSAA- or CDAA-diet.

	CSAA	CDAA
Bile flow ($\mu\text{l}/\text{min}/\text{g}$ liver)	0.8 ± 0.1	0.8 ± 0.1
Total bilirubin concentration ($\mu\text{g}/\text{ml}$)	85.4 ± 18.9	$51.2 \pm 6.0^{**}$
Bilirubin excretion ($\text{ng}/\text{min}/\text{g}$ liver)	69.0 ± 13.7	$42.0 \pm 7.2^{**}$
Urine excretion ($\text{ml}/100\text{g}$ body weight)	0.9 ± 0.8	1.4 ± 0.8
Total bilirubin concentration ($\mu\text{g}/\text{ml}$)	0.1 ± 0.1	$0.5 \pm 0.1^{***}$
Bilirubin excretion ($\mu\text{g}/100\text{g}$ body weight)	0.1 ± 0.1	$0.7 \pm 0.6^*$

Wistar rats were fed CSAA- or CDAA-diet for 12 weeks. Bile was collected from rats under anesthesia with urethane over a 60 min period to estimate the choleric activity and urine was collected over a 24 h period using a metabolic cage. The total bilirubin levels of bile and urine were determined as described in Materials and Methods. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; significantly different from CSAA-rats ($n = 5-6$).

Table 3 Area under plasma concentration-time curve from 1 to 24 h (AUC_{1-24h}) of GL and its metabolites when GL was orally administered to rats fed the CSAA- or CDAA-diet.

Compound	4 weeks		12 weeks	
	CSAA	CDAA	CSAA	CDAA
GL ($\mu\text{g}\cdot\text{h}/\text{ml}$)	2.58 \pm 2.11	18.6 \pm 8.8 ***	2.67 \pm 0.66	14.3 \pm 2.0 **
3MGA ($\text{ng}\cdot\text{h}/\text{ml}$)	102 \pm 30	404 \pm 34 ***	48.8 \pm 34.6	487 \pm 109 ***
GA ($\mu\text{g}\cdot\text{h}/\text{ml}$)	30.9 \pm 9.8	34.4 \pm 18.4	30.0 \pm 12.8	38.9 \pm 16.9

Wistar rats were fed CSAA- or CDAA-diet for 4 or 12 weeks. GL (100 mg/kg) was orally administered to the rat and the blood samples were collected 1, 2, 4, 8, 12, and 24 h after the treatment. Plasma concentrations of GL and its metabolites, 3MGA and GA, were measured as described in Materials and Methods. AUC_{1-24h} was calculated from the plasma measurements using the trapezoidal rule from first point to the last point. ** $p < 0.01$, *** $p < 0.001$; significantly different from the CSAA-rats ($n = 5-6$).

Table 4 Urinary excretion of GL and its metabolites when GL was orally administered to the rats fed the CSAA- or CDAA-diet.

	CSAA	CDAA
Urinary volume (ml/100 g body weight)	1.2 ± 0.5	1.4 ± 0.4
GL (ng/100 g body weight) [□]	75.2 ± 55.3	408 ± 170 ^{**}
3MGA (ng/100 g body weight) [□]	35.8 ± 14.5	145 ± 87.5 [*]
GA (ng/100 g body weight) [□]	1.1 (1/6) ^a	3.3 ± 3.9 (4/5) ^a

Wistar rats were fed with CSAA- or CDAA-diet for 13 weeks, and then, 100 mg/kg of GL was orally administered. Urine samples were collected over 24 h using metabolic cages. The collected urinary volume and the concentrations of GL and its metabolites, 3MGA and GA were measured as described in Materials and Methods. Excretive amounts of GL, 3MGA, and GA were calculated from the urinary volume and these concentrations. ^aThe incidence rate of detection (limit, 1 ng/100 g body weight). * $p < 0.05$, ** $p < 0.001$; significantly different from the CSAA-rats ($n = 5-6$).

Figure 2

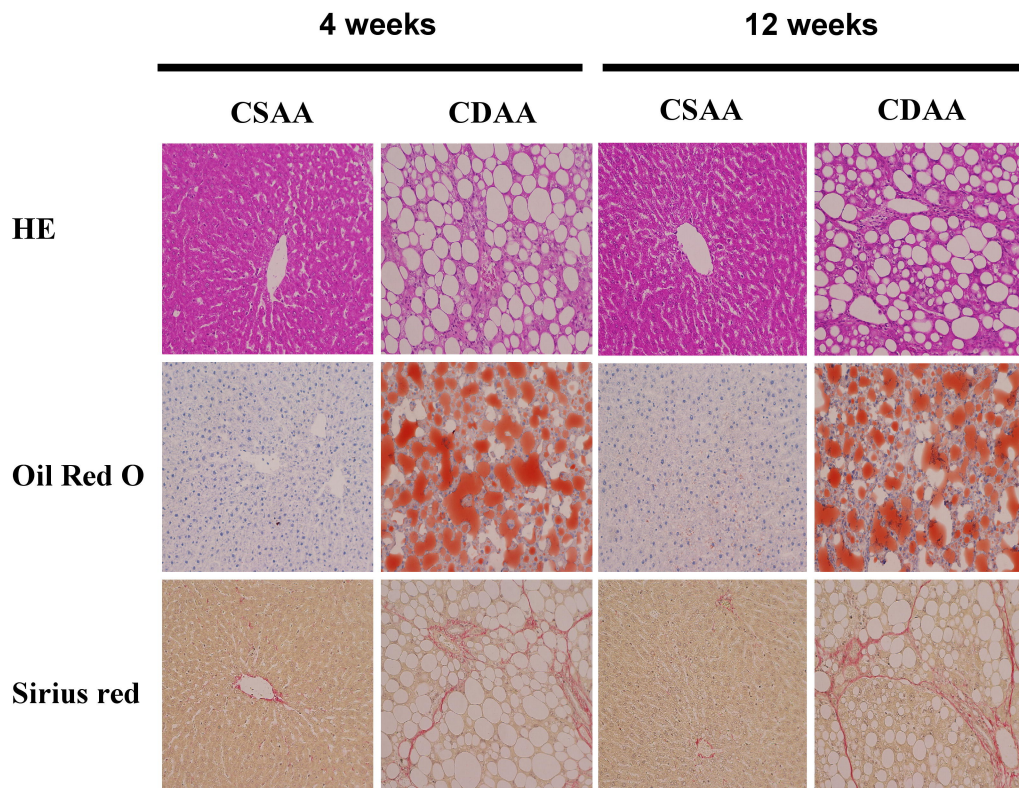


Figure 3

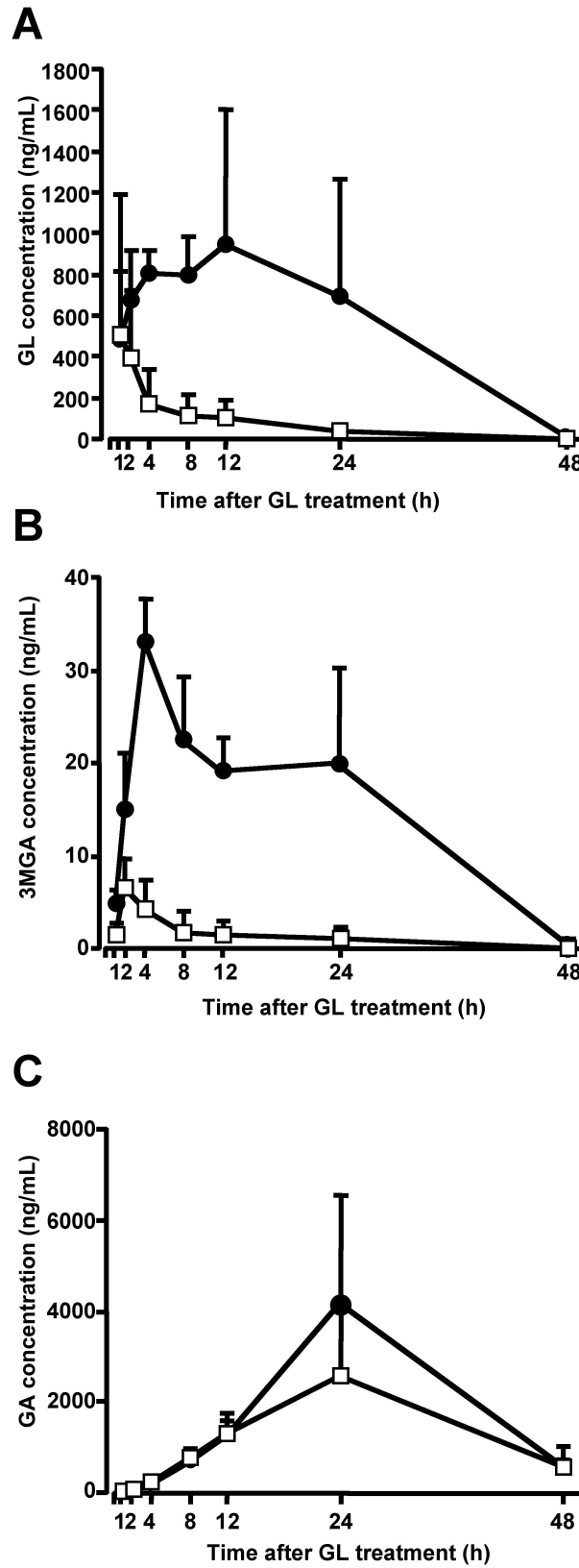


Figure 4

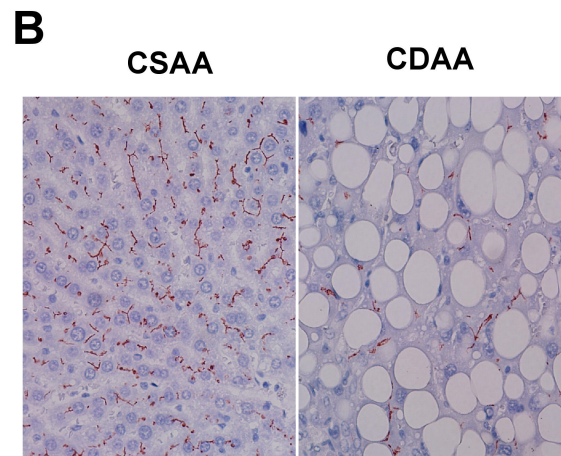
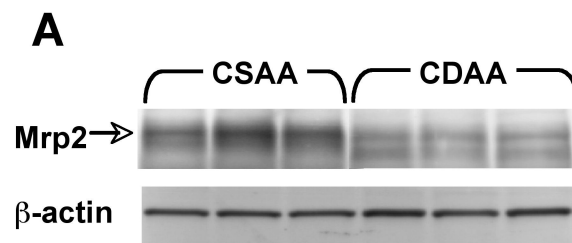


Figure 5

