Effects of diammonium glycyrrhizinate on hepatic and intestinal UDP-Glucuronosyltransferases in rats: Implication in herb-drug interactions

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[ABSTRACT] Glycyrrhizin is a major bioactive component of liquorice, which exerts multiple biochemical and pharmacological activities and is frequently used in combination with other drugs in the clinic. Mycophenolate mofetil (MMF), an immunosuppressant widely used in transplant patients, is metabolized by UDP-glucuronosyltransferases (UGTs). Although significant evidence supports that glycyrrhizin could interact with the cytochrome P450s (CYPs), few studies have addressed its effects on UGTs. The present study aimed at investigating the regulatory effects of diammonium glycyrrhizinate (GLN) on UGTs in vitro and in vivo. We found that long-term administration of GLN in rats induced overall metabolism of MMF, which might be due to the induction of UGT1A protein expression. Hepatic UGT1A activity and UGT1A mRNA and protein expression were significantly increased in GLN-treated rats. UGT1A expression levels were also increased in the intestine, contradicting with the observed decrease in intestinal UGT1A activities. This phenomenon may be attributed to different concentrations of glycyrrhetinic acid (GA) in liver and intestine and the inhibitory effects of GA on UGT1A activity. In conclusion, our study revealed that GLN had multiple effects on the expression and activities of UGT1A isoforms, providing a basis for a better understanding of interactions between GLN and other drugs.

[KEY WORDS] Diammonium glycyrrhizinate; UDP-glucuronosyltransferase; Sprague-Dawley rat; Drug-drug interaction

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Introduction

Liquorice, one of the most ancient medicinal plants, has been used in traditional Chinese medicine to complement other drugs to reduce toxicity and increase efficacy [1]. Glycyrrhizin is a major water-soluble bioactive triterpene glycoside of licorice [2]. After oral intake, glycyrrhizin is hydrolyzed by intestinal bacteria and then absorbed in the form of glycyrrhetinic acid (GA), aglycone of glycyrrhizin [3]. GA is responsible for the main pharmacological effects of glycyrrhizin, such as anti-inflammatory [4], anti-viral [5], and anti-carcinogenic activities [6]. It is commonly used for the treatment of hepatic steatosis [7], acute and chronic liver injury [8], and cancer [9].

It is widely known that cytochrome P450 (CYPs) and UDP-glucuronosyltransferases (UGTs) are the two main enzymatic families affecting the pharmacokinetic and pharmacodynamic properties of xenobiotics, especially with regard to drug-drug interactions (DDIs). Prolonged administration of glycyrrhizin has been reported to induce the...
that may affect therapeutic outcome and safety. Conversely, GA is shown to have a modest inhibitory effect on CYP3A4 and CYP2D6 both \textit{in vitro} and \textit{in vivo} \cite{12}. Glycyrrhiza radix is also found to inhibit the UGT1A1, UGT1A3, and UGT2B7 activities when incubated with human liver microsomes or recombinant human UGTs \textit{in vitro} \cite{13-15}. However, these UGTs-mediated GA-related DDI studies have not been explored in depth and \textit{in vivo} effects are not mentioned.

Mycophenolate mofetil (MMF) is an immunosuppressant prodrug indicated to prevent solid organ transplant rejection \cite{16}, which is rapidly absorbed and de-esterfied into mycophenolic acid (MPA) \textit{in vivo}. MPA is further metabolized in the liver by UGTs to form the pharmacologically inactive compound MPA 7-O-glucuronide (MPAG) \cite{17}. As MPA pharmacokinetics is closely related with its drug-related toxicity and patients’ clinical outcomes \cite{18-20}, based on the aforementioned regulation of glycyrrhizin on UGTs, glycyrrhizin was suspected to alter the pharmacokinetics and pharmacodynamics of MMF when the two agents are combined in the clinic. Therefore, the concomitant use of glycyrrhizin and MPA in the treatment of diseases such as atopic dermatitis \cite{21-22} bears possible herb-drug interactions that may affect therapeutic outcome and safety.

The present study aimed at investigating the effects of diacmonium glycyrrhizinate (GLN) on hepatic and intestinal UGTs activities in rats. The effect of GLN on the activities of UGTs \textit{in vivo} was evaluated by measuring the pharmacokinetic parameters of MMF. Meanwhile, \textit{in vitro} experiments were conducted in isolated microsomal fractions using 17beta-estradiol (E2), 4-methylumbelliferone (4-MU), and MPA, which are typical substrates for UGT1A1, UGT1A6 and UGT1A7, respectively \cite{23-25}. Real-time PCR and Western blotting analyses were used to determine the expression levels of UGT isoforms in the liver and intestine. To explain the differential effects of GA on intestinal and hepatic UGT activities, concentrations of GA in the liver and intestine were detected. Our findings from the present study may be useful for the safe and effective combination therapy of GLN and other prescription drugs in the clinic.

Materials and Methods

Chemicals and reagents

GLN and the corresponding standard substance were provided by Chia-Tai Tianqing Pharma-pharma Co. Ltd. (Jiangsu, China, purity 98%). Ursolic acid was purchased from Zelang Ltd. Co. (Nanjing, China). Urudine 5'-diphospho-sphate-glucuronic acid (UDPGA), d-saccharic acid 1, 4-lactone, alamethicin, 4-MU, MPA, E2, and estradiol -3-glucuronide were purchased from Sigma-Aldrich (Shanghai, China). Goat anti-rat polyclonal antibodies against UGT 1A, UGT 1A1, and UGT 1A6 were obtained from Santa Cruz, Inc. (Santa Cruz, CA,USA). Secondary antibodies were purchased from Bioworld technology Company (Nanjing, China). BCA Protein Assay Kit was purchased from Beyotime Institute of Biotechnology (Haimen, China). Prime Script Real time reagent kit for RT-PCR was purchased from Biotechnology Co. Ltd. (Dalian, China). HPLC-grade acetonitrile and methanol were from Merck (Darmstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore Corporation, Billerica, MA, USA).

Animals

Adult male Sprague-Dawley rats (weighing 180–220 g) were purchased from Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China)and housed in an air-conditioned room at a temperature of 25 ± 2 °C with a 12 h/12 h light/dark cycle, with free access to food (standard rodent chow diet) and tap water. The rats were allowed to acclimatize for one week and fasted for 12 h prior to the experiments. The study protocol was approved by the Animal Care and Use Committee of the College of Pharmacy, China Pharmaceutical University, Nanjing, China. Animals were randomly allocated to two groups (6/group). For 15 consecutive days, GLN dissolved in physiological saline was administered by oral gavage at a dose of 40.5 mg·kg⁻¹ body weight. Control animals received physiological saline only. On the 16th day, the animals were sacrificed and the liver and intestine were removed, weighed, and frozen in liquid nitrogen for further analysis.

Determination of the effects of GLN on pharmacokinetics of MMF in rats

In this experiment, the rats were randomly allocated into two groups (n = 6). For 15 consecutive days, control group received saline and GLN group received 40.5 mg·kg⁻¹ of GLN. The drug doses were based on data available in literature \cite{26-27}. On the 16th day, both groups were intragastrically administered MMF at 20 mg·kg⁻¹ 2 hours after GLN pretreatment. The dosing time interval was designed based on the previous pharmacokinetic study of GLN, so as to ensure maximal exposure of MMF to GLN. Blood samples were collected in heparinized tubes at 0, 0.083, 0.167, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, and 24 h after intragastric administration of MMF to determine plasma MPAG (glucuronidation metabolite of MPA) concentrations. Blood samples were immediately centrifuged and stored at −20 °C until analysis.

Enzyme assay

Differential centrifugation was used to prepare rat hepatic and intestinal microsomes as described by Hao \textit{et al.} \cite{28}. Protein concentrations were determined by BCA Protein Assay kit according to the manufacturer’s instructions. The prepared microsomes were frozen and stored at −80 °C until analysis. UGT activities were determined using the typical substrate E2 for UGT1A1, 4-MU for UGT1A6, MPA for UGT1A7 \cite{25, 29}. For the UGT1A1 test, incubations were performed in 1.5-ml polypropylene tubes. First, microsomes were pretreated with alamethicin (2 mg·mL⁻¹ of protein) for 20 min to diminish the latency of UGTs activity. After incubating with 10 mmol·L⁻¹ of MgCl₂, 20 mmol·L⁻¹ of d-saccharic acid 1, 4-lactone, 500 µmol·L⁻¹ of E2 and 50
mmol·L⁻¹ of Tris-HCl buffer (pH 7.4) in a total volume of 200 µL for 30 min at 37 °C, 2 µL of 20 mmol·L⁻¹ of UDPGA was added to initiate the reaction. At the end of incubation, 400 µL of ice-cold acetonitrile was added to terminate the reaction. After centrifuging at 12,000 g for 10 min, 100-µL aliquots were injected onto the LC-MS system (Shi-madzu, Kyoto, Japan) as described previously with minor modifications [24]. For determination of UGT1A6 and UGT1A7 activities, the alamethicin-permeabilized microsomes were incubated with 4-MU (1,000 µmol·L⁻¹) for 10 min or MPA (1,000 µmol·L⁻¹) for 30 min at 37 °C. The reactions were initiated by the addition of 20 mmol·L⁻¹ of UDPGA and stopped by the addition of 40 µL of 10% ice-cold perchloric acid. After centrifuging at 12,000 g for 10 min, 100-µL aliquots were injected into the HPLC system (Shi-madzu, Kyoto, Japan) for measurement of glucuronides of 4-MU and MPA. The detection method was carried out as previously described with minor modifications [30].

**Inhibition of UGTs enzymatic activity assay**

The concentrations of GA ranging from 0 to 100 µmol·L⁻¹ and those of substrates were as follows: E₂ (20, 40, 60, 80 µmol·L⁻¹), 4-MU (50, 125, 200, 500 µmol·L⁻¹), and MPA (20, 50, 100, 200 µmol·L⁻¹). The mixture of different concentrations of GA and substrates were incubated in the rat intestinal microsomal incubation systems. Other reacting procedures were the same as described in the aforementioned enzyme assay.

**Quantitative real-time PCR**

Total RNA was extracted using Trizol reagents (Takara Biotechnology Co., Ltd., Dalian, China) according to the protocol. The cDNA samples were kept at −80 °C until use. The sequences of primers for different isoforms used in the present study were as follows: UGT1A1, 5'-ACC TGT CTCTGCTGCTG TGT-3', 5'-ATGCGATCTGTGTTCGAG GAC-3'; UGT1A6, 5'-AATCCTATGGGTAAC ATC TTG-3', 5'-GTAGGGACACATCCT TCT-3'; UGT1A7, 5'-TGAGGG AGTTTGTGTAAAGG-3', 5'-TAC TTGGCACTAAAG-3'; and GAPDH 5'-CGGGAGCTTGTCATCAATGG-3', 5'-GGCTTACGATGGCTG-3'. The PCR mixture containing 4.5 µL of DEPC water, 7.5 µL of 2 × Premix Ex Taq buffer, 1 µL of each primer (10 µmol·L⁻¹) and 1 µL of cDNA template was initially denatured at 95 °C for 90 s, followed by 40 cycles of amplification. Each cycle was performed as the following steps: 10 s at 95 °C (denaturation); 30 s at 60 °C (annealing); and 30 s at 70 °C (extension). The final extension was performed at 60 °C for UGT1A1, UGT1A6 and UGT1A7, or 55 °C for GAPDH for 10 min.

**Western blotting assay**

Liver and intestinal homogenates were prepared as described previously [28, 31], were separated on 10% SDS polyacrylamide gels at 115 V for 1.5 h. Then proteins on the gels were transferred to PVDF membranes for approximately 2.5 h using wet transfer method. The membranes were then blocked with 5% nonfat milk in TBST (0.05% Tween 20 in TBS) at 37 °C for 1 h and then were separately incubated with UGT1A UGT1A1 and UGT1A6 primary antibodies (diluted 1 : 500, 1 : 200 and 1 : 400 respectively with 5% nonfat milk in TBST) overnight at 4 °C. The membranes were washed for 3 times with TBST (15 min/time), followed by incubation with goat anti-rabbit (UGT1A1, 1 : 10,000) or mouse anti-goat (UGT1A1 and UGT1A6, 1 : 10,000) secondary antibodies for 1 h at 37 °C. For reference, GAPDH was used as reference and detected with a polycolonal antibody (diluted 1 : 400) for 1 h and then incubated with goat anti-rabbit IgG secondary antibody (diluted 1 : 10,000) for 1 h at 37 °C. Protein/antibody complexes were visualized by chemiluminescence detection method with reagents from BioWorld Technology Company (Nanjing, China). The chemiluminescent signals were captured by a ChemiDoc XR System (Bio-Rad Laboratories, Shanghai, China).

**Determination of GA concentrations in liver and intestine**

Concentrations of GA in liver and intestine were determined by LC-MS method described previously with minor modifications [32]. The separation was performed on a VP-ODS (2.0 mm × 150 mm) column (Shimadzu Co., Kyoto, Japan). The column temperature was maintained at 40 °C. The mobile phase was consisted of 200 mg L⁻¹ of ammonium acetate (A) and acetonitrile (B) with a flow rate of 0.2 mL·min⁻¹. The gradient program was as follows: 65% B from 0 to 4.5 min, 65% B to 95% B from 4.5 to 4.55 min, holding 95% B for 3.55 min, 95% B to 65% B from 9.0 to 9.05 min, and maintaining for another 5 min. LC-ESI-MS was performed in negative mode using selected ion monitoring (SIM) mode with target ions at m/z 469.5 for GA and m/z 455.2 for the internal standard ursolic acid (IS). The samples were prepared by adding 10 µL of IS (ursolic acid in methanol, 100 ng·mL⁻¹) to the homogenized tissue sample (100 µL) and vortexing, followed by addition of 1 ml of ethyl acetate. After vortex and centrifugation (8000 r·min⁻¹, 10 min), the upper organic layer (800 µL) was collected and evaporated using nitrogen gas at less than 40 °C. The residue was reconstituted with 120 µL of mobile phase and 100 µL of supernatant was injected onto the LC-MS system for analysis after centrifugation.

**Statistical analysis**

The results were expressed as mean ± standard deviation (SD). Pharmacokinetic parameters such as Tₘax, Cₘax; t₁/₂, AUCₐ₀₋ₚ, and V/F were calculated using WinNonlin 6.3 software (Pharsight Corporation, Princeton, USA), and statistical analyses were performed with SPSS ver. 13.0 software (SPSS Inc., Chicago, IL). P < 0.05 was considered statistically significant.

**Results**

**Pharmacokinetics of mycophenolate mofetil co-administrated with GLN**

MMF is an immunosuppressive agent that has been shown to undergo UGT-catalyzed metabolism in vivo [17]. Pharmacokinetic parameters of different administered groups were calculated to determine the effects of GLN on MMF metabolism.
As shown in Table 1 and Fig. 1, after long-term GLN administration, the GLN group showed lower $T_{\text{max}}$ (77%) and $V/F$ values (34%) for mPAG, compared with the control group. Besides, a significant increase in $C_{\text{max}}$ and $AUC_{0-\infty}$ values (2.7-fold) was observed in the GLN group, indicating an accelerated metabolism of MMF after GLN pretreatment.

### Table 1  The main pharmacokinetic parameters of MPAG in different groups ($n = 6$)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>$T_{\text{max}}$ (h)</th>
<th>$C_{\text{max}}$ (mg·mL$^{-1}$)</th>
<th>$t_{1/2}$ (h)</th>
<th>$AUC_{0-\infty}$ (h·mg·mL$^{-1}$)</th>
<th>$V/F$ (ml·kg$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.85 ± 0.14</td>
<td>6.55 ± 2.90</td>
<td>8.82 ± 3.10</td>
<td>65.6 ± 7.86</td>
<td>5.51 ± 1.30</td>
</tr>
<tr>
<td>GLN</td>
<td>0.65 ± 0.14*</td>
<td>21.5 ± 7.2*</td>
<td>9.71 ± 2.3</td>
<td>174.9 ± 45.3**</td>
<td>1.86 ± 0.57**</td>
</tr>
</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$ vs control group

**Effects of GLN on hepatic and intestinal UGT activities**

To examine the effects of GLN on the activities of UGTs in the liver and intestine, we measured UGT activities after GLN treatment. The rats were treated with either with GLN or saline, and the activities of UGT1A1, UGT1A6, and UGT1A7 were determined using specific substrates. As shown in Fig. 2, the hepatic activities of UGT1A1, UGT1A6, and UGT1A7 were significantly increased upon GLN administration to 122.6%, 116.3% and 189.4% of control animals. In contrast, intestinal UGT activities of UGT1A1, UGT1A6 and UGT1A7 were significantly decreased to 66.1%, 78.2% and 73.8% upon GLN treatment as compared to the control group.

**Effects of GLN on the mRNA expression of UGTs in rat liver and intestine**

To further explain the changes of pharmacokinetic parameters, UGT1A1, UGT1A6, and UGT1A7 mRNA expression were determined by real-time PCR. As evidenced from Fig. 3, in GLN group, the hepatic mRNA expression levels...
of all three UGT isoforms were significantly elevated, which may well explain the up-regulation of hepatic UGTs activities. However, intestinal mRNA levels showed no statistical significance compared with control group, discrepant with the decrease of intestinal UGTs’ activities.

**Influence of GLN on the protein levels of UGTs in rat liver and intestine.**

To validate the above findings, Western blot analysis was performed. We measured the protein levels of UGT1A, UGT1A1, and UGT 1A6 (Fig. 4 A, B). Protein levels in the liver were up-regulated to a 1.2–1.3 fold increase of the protein levels of all three UGT isoforms compared with the normal control, which were in accordance with the increased mRNA expression and enzymatic activities. Surprisingly, we observed an evident increase of intestinal UGT1A1 (227% of control), UGT1A6 (199% of control), and UGT1A (192% of control), in GLN-treated group, which were in contrast with the down-regulated UGT activities.

![Fig. 4  Effects of GLN on intestinal and hepatic UGT protein expression. A. Representative Western blot stains of UGTs in rat liver and intestine samples (n = 4); B. Relative stain intensity for respective UGT levels were presented in comparison with those of control rats. Rats were gavaged once daily with vehicle (control group) or 40.5 mg·kg⁻¹·d⁻¹ GLN for 15 consecutive days. Intestinal and hepatic tissues were removed on the 16th day. Bars represent mean ± SD. *P < 0.05, **P < 0.01 vs the control group](image)

**Inhibitory effects of GA on UGTs activities**

The contradiction between intestinal expression and enzyme activities prompted us to hypothesize that GA might directly interact with UGTs and inactivate their activities. To verify this hypothesis, E₂ (20, 40, 60, and 80 µmol·L⁻¹), 4-MU (50, 125, 200, and 500 µmol·L⁻¹) or MPA (20, 50, 100, and 200 µmol·L⁻¹) were added to the reaction mixture containing different concentrations of GA (0, 10, 50, and 100 µmol·L⁻¹) in the rat intestinal microsomal incubation systems. As shown in Fig. 5, incubation of different concentrations of substrates with microsomes moderately inactivated the activity of UGTs.

**Hepatic and intestinal distributions of GA**

For determinations of hepatic and intestinal GA exposure the hepatic and intestinal samples were promptly removed, diluted in 4 fold of volumes (V/W) of saline and then homogenized. The samples were frozen at –20 °C until analysis. GA concentrations were 0.232 ± 0.062 µmol·L⁻¹ in livers and 1.819 ± 0.282 µmol·L⁻¹ in intestines. Intestinal GA concentration was appropriately 8-fold higher than that of liver. High exposure of GA resulted in the exacerbation of UGTs activities inhibition, which might explain the different alterations in hepatic and intestinal UGTs activities.

**Discussion**

GLN is frequently co-administered with synthetic drugs as well as other traditional Chinese medicine. Thus, prediction of interactions between GLN and other drugs is important for dosage modification in order to avoid adverse reactions. To date, several reports have described the potential of GLN to modulate CYPs [13, 33-34], but little is known about interactions of GLN with the glucuronidation metabolism mediated by UGTs. The present study aimed to explore the potential of GLN to interact with UGTs.

MMF, a powerful immunosuppressive drug, is hydrolyzed in the gastrointestinal tract to form its active metabolite MPA. Increasing evidence indicates that therapeutic MPA monitoring may be helpful to improve the efficacy of MMF.
Fig. 5 Lineweaver-Burk Plots Of GA Inhibition Of UGT-catalyzed 4-MU (A, substrate of UGT1A6), E2 (B, substrate of UGT1A1) Aand MPA (C, substrate of UGT1A7) In Rat Intestinal Microsomes. E2 (20, 40, 60, 80 µmol L⁻¹), 4-MU (50, 125, 200, and 500 µmol L⁻¹) or MPA (20, 50, 100, and 200 µmol L⁻¹) were added to the reaction mixture systems containing GA at 0 to 100 µmol L⁻¹. The data represent the mean of triplicate determinations. Double reciprocal plots for the kinetics of inhibition of UGT1A by GA in normal rat intestinal microsomes and optimize the outcome of MMF treatments [20, 35]. As liquorice and GLN are both used in the treatment of oral lichen planus or atopic dermatitis [17, 36-37], possible interactions of these compounds with MMF would pose a risk of allograft rejection for transplant patients. Therefore, in the present study, we chose MMF as the probe to evaluate effects of GLN on UGTs in vivo. Our results indicated that GLN significantly accelerated the metabolism of MPA, indicating an inductive effect on UGTs activities, which was in accordance with previous studies [38]. After long-term oral administration, GLN presented an overall up-regulated effect on UGTs activities in vivo, but changes of hepatic and intestinal UGT1A1, UGT1A6 and UGT1A7 activities were opposite. Long-term pretreatment of GLN significantly increased hepatic UGT1A activities, and meanwhile decreased intestinal UGT1A activities to a large extent in microsome incubation study. Additional experiments on intraperitoneal injection of magnesium isoglycyrrhizinate, the metabolite of which is also GA, exhibited the same results (data not shown). To investigate whether the expression of UGT1A mRNA and protein contributed to the paradoxical phenomenon, real-time PCR and Western blotting experiments were subsequently conducted. Up-regulation of mRNA and protein levels of UGT1A1, UGT1A6, and UGT1A7 in liver corresponded with the increased hepatic UGTs activities. The intestinal UGTs mRNA and protein expressions were also up-regulated, contradictory with the decreased UGTs activities.

GLN is hydrolyzed to yield GA in vivo, which subsequently undergoes a two-step pathway of phase II metabolism, including glucuronidation and sulfation reactions [39]. Previous researches have shown that this biotransformation process strongly promotes the inhibitory potential of GA on UGT1A1, UGT1A3, and UGT2B7 [14-16]. Thereafter, we carried out a series of experiments to confirm the inhibitory capability of GA on UGT1A1, UGT1A6, and UGT1A7 isoforms, and then determined exposure of GA in hepatic and intestinal tissues. We confirmed an inhibitory effect of GA on the three UGT isoforms. Accordingly, we hypothesized that the exposure of different GA concentrations in liver and intestine led to the opposite results. Intestinal GA concentration was proven to be much higher than that in liver, which may help explain the decrease in UGTs enzymatic activities.

In conclusion, long-term GLN treatment--induced glucuronidation may be due to the induction of UGTs protein expression. However, GA, the active metabolite of GLN, showed inhibitory activities towards UGTs in microsome incubation experiment. Our study illustrated that GLN significantly increased the oral bioavailability of MMF, which would be applicable for assessing the possible interaction between GLN and other UGTs-metabolized drugs. Opposite effects on hepatic and intestinal UGTs activities were attributed to the major metabolite GA, which exhibited different exposures in different tissues and direct inhibition on UGTs activities.

References


