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Inhibitory effects of endogenous linoleic acid and glutaric acid on the renal glucuronidation of berberrubine in mice and on recombinant human UGT1A7, 1A8, and 1A9

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Running title: Endogenous molecules inhibit UGTs

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Abbreviations: Acox, acyl-Coenzyme A oxidase; BRB, berberrubine; BRBG, berberrubine-9-O-β-D-glucuronide; CAR, constitutive androstane receptor; DDIs, drug–drug interactions; DMEs, drug metabolizing enzymes; Fabp, fatty acid-binding protein; HFD, high fat diet; MPA, mycophenolic acid; MPAG, mycophenolic acid glucuronide; Nrf2, NF-E2-related factor 2; PPARα, peroxisome proliferator-activated receptor α; PXR, pregnane X receptor; UDP-GLcA, uridine diphosphate glucuronic acid; UGDH, UDP-glucose 6-dehydrogenase; UGP, UDP–glucose pyrophosphorylase; UGTs, UDP-glucuronosyltransferases; ρNP, ρ-nitrophenol; ρNPG, ρ-nitrophenyl

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glucuronide.

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Abstract

Berberrubine (BRB) has a strong lipid-lowering effect and can be extensively metabolized into berberrubine-9-O-β-D-glucuronide (BRBG) *in vivo*. Recently, pharmacokinetics studies showed that the production of BRBG was significantly decreased in the urine of mice fed with a high fat diet (HFD), indicating a decreased glucuronidation capacity. Based on the UGT isoform identification, hepatic and renal microsomal incubation, glucuronidation was examined to suggest the metabolism of BRB in liver and kidneys. The results showed that the renal UGT activity for metabolizing BRB markedly decreased, which may be highly related to the decreased expression and activity of renal Ugt1a7c. Surprisingly, *in vitro* studies revealed neither BRB nor BRBG inhibited the renal UGT activity. By employing an integrated strategy of metabolomics and pharmacokinetics, we identified and confirmed for the first time the inhibitory effect of some potential endogenous molecules on the renal glucuronidation of C57BL/6J mice, such as glutaric acid and linoleic acid. By employing recombinant human UGTs, we found that glutaric acid and linoleic acid efficiently affect the activity of recombinant human UGT1A7, 1A9 and 1A8 at their normal or abnormal physiological levels *in vivo*. Glutaric acid (2 mM) markedly inhibited the activity of UGT1A7 by 89.4% and UGT1A9 by 32.8%. The inhibition rates reached 99.3% for UGT1A9, 48.3% for UGT1A7, and 46.8% for UGT1A8 with linoleic acid at 200 μM. It has been suggested that the endogenous molecules have the potential to affect the efficiency of glucuronidation, which might be a key factor contributing to individual differences in drug metabolism.

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Introduction

Drugs or other xenobiotics usually undergo two phases of biotransformation which determine their pharmacokinetic profiles, therapeutic effects and toxic side effects (Guengerich, 2006; Oda et al., 2015). As one of the most important phase II reactions, glucuronidation is responsible for ~35% of all drug metabolism by phase II enzymes (Kiang et al., 2005). The toxicity of many toxic xenobiotics could be attenuated during the metabolic process *in vivo*, and many of them are dependent on the glucuronidation process for detoxification (Tephly and Burchell, 1990). For instance, the metabolite of irinotecan, SN-38, is correlated to severe toxicities including diarrhea and leucopenia, and it could be detoxified after conjugation with glucuronic acid (van der Bol et al., 2011). Benzo-[a]pyrene, a dangerous inducer related to lung cancer, could also be detoxified through glucuronidation (Kua et al., 2012). Hence, the disturbance of the glucuronidation process is closely related with drug-induced toxicity or the risk of some diseases.

In most cases, the capacity of drug glucuronidation is dependent on the activity of UDP-glucuronosyltransferases (UGTs). The mammalian UGT superfamily can be divided into UGT1 (1A), and UGT2 (2A, 2B) families. Previous studies have reported that the expression or activity of UGTs can be affected by many factors involving nuclear receptors, diverse diseases, inflammation, oxidative stress and other pathological or abnormal physiological effects (Gradinaru et al., 2012; Xu et al., 2012; Gruber et al., 2013; Xie et al., 2013). For instance, the relative mRNA levels of partial UGT isoforms were influenced by steatosis induced by obesity (Xu et al., 2012). The

glucuronidation of β-estradiol and 4-methylumbelliferone in the kidneys was significantly reduced in chronic renal insufficiency rats (Yu et al., 2006). It is well known that disease states or other abnormal physiological states are generally accompanied with complicated changes in internal homeostasis involving endogenous molecules, enzymes, metabolic pathways and signaling pathways. However, the direct influence of endogenous molecules with altered levels in those processes are rarely reported, and some potential endogenous molecules may be highly related with altered activity of UGTs; e.g., the activities of UGT1A1, 1A6 and 1A7 are altered in rats with diabetes, which is accompanied by disorders of the metabolism of carbohydrates, protein and fatty acids (Xie et al., 2013). As a result of their remarkable significance in clinical drug safety, drug–drug interactions (DDIs) have drew an extensive attention. Many drugs have been reported to exert inhibitory or inducing effects on UGTs that can affect glucuronidation and cause DDIs. However, little is known about the effect of endogenous molecules on UGTs.

In addition to being located in the liver, UGTs are also distributed in the kidneys, gastrointestinal tract, brain, and some other tissues (Court et al., 2012; Rowland et al., 2013). Some previous studies have indicated that UGTs are highly expressed in the kidneys, which is second only to the expression in the liver (Lohr et al., 1998; Kerdpin et al., 2008; Mutsaers et al., 2013). Since the kidneys possess a high blood flow (~25% cardiac output) and participate in the formation of urine (Atherton, 2012), the glucuronidation of drugs in the kidneys is extremely important for drug metabolism and elimination. Meanwhile, the kidney is exposed to many endogenous

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or exogenous molecules, which may influence the activity of renal UGTs.

Berberrubine (BRB), an active lipid-lowing metabolite of berberine (Li et al., 2010; Zhou et al., 2014), showed a high proportion of glucuronidation metabolism *in vivo* in our previous study (Yang et al., 2017a). In this study, for the first time, based on the specific metabolic pattern of BRB, our data suggested that some important endogenous molecules are potentially effective endogenous inhibitors on mouse renal glucuronidation via the employment of an integrated strategy of metabolomics and pharmacokinetics. Considering the species differences in UGT isoforms and to provide more evidence for clinical research, recombinant human UGTs were used for the evaluation of these potential endogenous inhibitors.

This study provides important evidence that some important endogenous molecules could disturb the renal glucuronidation process and influence the activity of several UGTs, which could be a constructive example demonstrating the interaction of endogenous molecules on drug metabolizing enzymes (DMEs).

Materials and methods

Reagents

BRB (purity>95%) was synthetized by ChemzamPharmtech Co (Nanjing, China), BRBG was prepared and identified by Key Lab of Drug Metabolism and Pharmacokinetics in China Pharmaceutical University (Yang et al., 2017a). Control diet (AIN-93M) and high fat diet (HFD, 60% calories from fat and 1% cholesterol) were purchased from Trophic Animal Feed High-Tech Co., Ltd (Nantong, China).

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ρ-nitrophenol (ρNP), ρ-nitrophenyl glucuronide (ρNPG), UDP-glucuronic acid (UDP-GLcA), alamethicin, D-Saccharic acid 1,4-lactone, 4-methylumbelliferone (4-MU), β-estradiol, naloxone, mycophenolic acid (MPA), glutaric acid, linoleic acid, hydroxyglutaric acid, urea, aminoisobutyric acid, alanine, 3-hydroxybutyric acid, palmitic acid, stearic acid, taurine and glyceric acid were all purchased from Sigma-Aldrich (St Louis, MO, USA). The stable-isotope-labeled internal standard compound (IS) myristic-1,2–$^{13}C_2$ acid (99 atom%^{13}C), methoxyamine hydrochloride (purity 98%), pyridine (≥99.8% GC), N-Methyl-N-trimethylsilyltrifluoroacetamide and 1% trimethylchlorosilane were also provided by Sigma-Aldrich (St Louis, MO, USA). Recombinant human UGTs were purchased from BD Biosciences (San Jose, CA).

Pharmacokinetic studies

C57BL/6J mice (male; six weeks old; weighing 18–22 g) were purchased from the College of Animal Science and Technology (Yangzhou University, China) and were housed under a 12-hour light-dark cycle with free access to food and water (temperature, 22± 3°C； humidity, 55%± 5%). All animal studies were performed with the approval of the Animal Ethics committee of China Pharmaceutical University. The mice were randomly divided into CSB (control diet with a single dose of BRB), CMB (control diet with multiple doses of BRB), and HMB (high fat diet with multiple dose of BRB) groups. Over consecutive six weeks, mice in the CMB and HMB groups were intragastrically administered BRB (50 mg/kg/d), and mice in the CSB group were gavaged with vehicle CMC-Na (5%) as a control. All mice were fasted overnight

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and given free access to water (12 h) before the experiments. Blood samples of all mice were collected into heparinized tubes at 0.083, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8 h after the last BRB administration (50 mg/kg). Similarly, urine samples were collected in the metabolic cages for 12 h after the last administration. All samples were prepared in the manner previously reported (Yang et al., 2016).

Measurement of UGT activity and UDP-GLcA levels in the liver and kidneys of C57BL/6J mice

C57BL/6J mice (male; six weeks old; weighing 18–22 g) were randomly divided into four groups: C (control diet), CB (control diet with BRB administration), H (high fat diet), HB (high fat diet with BRB administration). Mice were housed under a 12-hour light-dark cycle with free access to food and water (temperature, 22± 3°C； humidity, 55%± 5%). All animal studies were performed with the approval of the Animal Ethics committee of China Pharmaceutical University. The mice in CB and HB were intragastrically administered 50 mg/kg BRB for six consecutive weeks and the mice in C and H were gavaged with vehicle CMC-Na (5%) as control. The body weights of the mice were recorded every week. UGT enzyme assays were performed as the previous method (Liu et al., 2013; Wang et al., 2015). Hepatic and renal microsomes were prepared from C57BL/6J mice according to the method described previously (Feere et al., 2015). Hepatic and renal microsomes of C57BL/6J mice from the four groups were pre-incubated with alamethicin for 30 min at 4°C. An incubation contained microsomes (0.2 mg/mL protein), 50 mM Tris-HCl buffer (pH 7.4), 20 μg/mL alamethicin, 2 mM UDP-GLcA, 10 mM $MgCl_2$, 5 mM D-Saccharic acid

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1,4-lactone, and 50 μM BRB at 37°C for 1 h. Similarly, UDP-GLcA levels were determined in the incubation system according to the previous methods (Bánhegyi et al., 1996; Yamamura et al., 2001; Kang et al., 2010) based on the formation of ρNPG. All reactions were terminated by ice-cold acetonitrile (containing 10 ng/mL IS) at a ratio of 1:3 (V/V) and prepared for LC-MS/MS analysis.

Identification of UGT isoenzymes

The total RNA of the liver and kidneys was isolated and reverse-transcribed into cDNA based on previous method (Mannhalter et al., 2000). Quantitative real-time PCR was used to determine the relative mRNA levels of the UGT isoenzymes. Based on previous methods (Livak and Schmittgen, 2001; Margaillan et al., 2015), quantitative PCR data for each UGT (Ct_{UGT}) were first normalized with an internal standard (ΔCt_{UGT}), and then normalized with ΔCt values (ΔCt_{Low}) of the sample expressing the lowest levels of UGTs to determine $\Delta\Delta Ct_{UGT.}$ The equation $2^{-\Delta\Delta Ct\ UGT}$ was then used to determine the relative quantification of UGTs.

Competitive UGT inhibitory effects were determined *in vitro* to confirm the subtype of UGTs in glucuronidation of BRB using previously described methods (Liu et al., 2013; Wang et al., 2015) with a slight modification. Then, 50 μM BRB and different concentrations of MPA (40, 200, or 1000 μM, n=3) were simultaneously incubated in blank renal microsomes. The incubation system and processing method were the same as the UGT activity assay mentioned above. The amount of BRBG was measured.

The renal microsomes of the four groups (C, CB, H and HB) were prepared and individually incubated with 1 mM MPA for 30 min; the other constituents of this

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incubation system were the same as previously mentioned. The amount of MPAG formation was quantified by LC-MS/MS. The direct inhibition effects of BRB or BRBG on MPA metabolism were also evaluated by co-incubating MPA with BRB or BRBG (50 μM, respectively, n=3) in blank renal microsomes.

Metabolomic study

The urine metabolomic study was performed according to the well-developed metabolic platform based on the GC/MS technique, as described previously (J et al., 2005; Gu et al., 2015). Briefly, 30 μL of urine samples were pre-incubated with 30 μL of urease (10 mg/mL) at 37°C for 1 h. The incubation was terminated with methanol (containing 5 μg/mL internal standard [$^{13}C_2$]-myristic acid) at a ratio of 1:4 (V/V) and vortexed for protein precipitation. An aliquot of 100 μL supernatant was dried and derivatized using the same method as was used for the plasma samples (Guo et al., 2016). The raw data were processed, and the endogenous compounds were identified based on previous methods (J et al., 2005).

Evaluation of inhibitory capability of the typical endogenous molecules on UGT isoforms

The concentrations of the compounds administered are designed and calculated according to the physiological level reported in the Human Metabolome Database (HMDB). Linoleic acid: low dose, 2 μM, middle dose, 20 μM, high dose, 200 μM; glyceric acid, palmitic acid, stearic acid, alanine, glutaric acid, hydroglutaric acid, taurine, and 2-aminoisobutyric acid: low dose, 20 μM, middle dose, 200 μM, high dose, 2 mM; 3-hydrobutyric acid: low dose, 30 μM, middle dose, 300 μM, high dose,

3 mM; urea: low dose, 2 mM, middle dose, 20 mM, high dose, 200 mM. *In vitro* microsomal and recombinant human UGT incubation systems were performed as previously described (Maul et al., 2015; Song et al., 2015). MPA (1mM) was used as the probe substrate of UGT1A7 to evaluate the activity of microsomes and recombinant UGT1A7 enzyme (Mohamed et al., 2008). Since BRB could also be metabolized well by recombinant human UGT1A9, 1A1, 1A8, or 1A3 (data not shown), BRB (50 μM) was selected as the probe substrate of those four isoforms. Valproic acid (VPA,1 mM) was selected for UGT1A4 and 1A10 (Argikar and Remmel, 2009), and 4-MU (1 mM) was selected for UGT1A6, 2B4 and 2B7(Udomuksorn et al., 2007; Zhu et al., 2016). The microsomal incubation system was performed in the same way as mentioned above. The concentrations of different recombinant human UGTs and the incubation time were selected following the instruction recommendations.

Effects of linoleic acid and glutaric acid on the glucuronidation of BRB in human renal proximal tubular cells (HK-2) and mice

HK-2 cells were purchased from China Center for Type Culture Collection (CCTCC, Shanghai, China) and cultured in DMEM/F-12 medium with 10% fetal serum. Cells were then cultured in 12-well plates and exposed to 50 μM BRB with or without the administration of linoleic acid (200μM) and glutaric acid (2mM) for 12h at 37 °C.

C57BL/6J mice were randomly divided into three groups: BRB, BRB+LA, and BRB+GA. Mice in BRB group were orally administered with a single dose of BRB (50mg/kg), while mice in BRB+LA and BRB+GA group were co-administered with a

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single dose of linoleic acid (4mg/kg, i.p.) or glutaric acid (20mg/kg, i.p.) Urine samples were collected in the metabolic cages for 24 h.

LC-MS/MS conditions

The sample analysis work was performed based on liquid chromatography-tandem mass spectrometry which contains a Shimadzu Ultra Performance LC-20A system (Shimazu Corpo-ration, Kyoto, Japan), coupled with API 4000 Triple Quadrupole Mass Spectrometer (AB SCIEX, Forster City, CA, USA). A Turbo Ion-spray source (ESI) was used. An Agilent Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 3.5 μm particle size) was used for chromatographic separation. Mass spectrometry detection was conducted in the positive mode and the source parameters were set as follows: spray voltage, 5500 V; curtain gas, 30 Arb; ion source gas 1, 70 Arb; ion source gas 2, 70 Arb. Compounds were detected in MRM monitoring conditions. The transitions of m/z 322.4→307.1 (DP: 80 V; CE: 34 eV), m/z 498.3→322.1 (DP: 80 V, CE: 30 eV), m/z 321.0→207.0 (DP: 80 V, CE: 30 eV), m/z 514.3→321.0 (DP: 50 V, CE: 25 eV), m/z 321.2→321.2 (DP: 80 V, CE: 5 eV), m/z 353.0→177.0 (DP: 50, CE: 25 eV) and 339.800 → 176.2 (DP: 80, CE: 35 eV),were monitored for BRB, BRBG, MPA, MPAG, VPAG, 4-MUG, andtetrahydroberberine (internal standard, IS), respectively.

Statistical analysis

Data were analyzed with Graph Pad Prism5.01 (La Jolla, CA). Multiple groups of one-factor experiments were assessed using one-way ANOVA, followed by Tukey *post-hoc* multiple comparison test. Two-tailed unpaired Student's test was used in the

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comparison of two groups. The differences were considered to be significant when $p < 0.05$. Data were expressed as mean ± SD, and each group had at least three experiments performed in triplicate.

Partial least squares discriminant analysis (PLS-DA), a method by using partial least squares regression in the discriminant analysis in SIMCA-P+13.0 (Version 13.0, Umetrics, Umeå, Sweden). Briefly, the data matrix was constructed using compound index as variable names, sample ID as observations, and normalized peak areas as variables. All data was mean-centered and UV scaled. The goodness of fit for a model was evaluated using three quantitative parameters based on cross-validation: R^2X, the explained variation in X; R^2Y, the explained variation in Y; Q^2Y, the predicated variation in Y. The number of principal components was determined once the Q2Y value decreased continuously. The range of these parameters is between 0 and 1, the closer they approach 1, the better they could predict or explain.

Results

HFD decreased the ratio of BRBG to BRB in urinary excretion

A simultaneous assaying of BRB and BRBG was developed using an LC-MS/MS analytical approach based on our previous studies (Yang et al., 2017a). The plasma concentration-time curves and urinary excretion of both BRB and BRBG in the three groups (CSB, CMB and HMB) were investigated. As shown in Fig.1 (A-D), HFD slightly increased the mean plasma AUC_{0-t} of both BRB and BRBG in HMB compared to CMB, and did not show an obvious influence on the ratio of BRBG to

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BRB. BRBG in CSB showed shoulder peaks which indicates the possibility of enterohepatic circulation and metabolic interconversion, since BRBG could be hydrolyzed via the gut flora. The enterohepatic circulation may decelerate the excretion of BRB and BRBG in CSB. As a primary metabolite of berberine, BRB may have a similar antibacterial effect. Therefore, the shoulder peaks were not observed in multiple dose groups.

Interestingly, although the excretion of BRBG in HMB and CMB into the urine did not show any significant difference, the ratio of BRBG to BRB significantly decreased in HMB compared with CMB, since the excretion of BRB was significantly higher in HMB, Fig. 1E, 1F and 1G. The ratio in CMB group is lower than that in CSB. However, there is only a marginal significant between these two groups (p=0.051). These results indicated the fact that HFD could significantly increase the urinary excretion of BRB and decrease the ability of glucuronidation. Moreover, pharmacokinetic studies showed BRBG is at an extremely low level in the bile of mice, which suggests a much lower level of glucuronidation metabolism of BRB in the liver (data not shown). In addition to the liver, the glucuronidation process in the kidneys is also at an extremely high level. Some studies have reported that the activity of UGTs in the kidneys is second only to that in the liver (Lohr et al., 1998; Kerdpin et al., 2008; Mutsaers et al., 2013). The inconsistency of glucuronidation proportion of BRB in the plasma and urine indicated that the kidneys may play an important role in BRB glucuronidation.

HFD slightly increased hepatic glucuronidation, but markedly decreased renal

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glucuronidation of BRB

In the process of glucuronidation, compounds (contain polar groups, i.e., hydroxyl, amine and carboxyl, etc.) are conjugated with uridine-5'-diphospho-α-D-glucuronic acid (UDP-GLcA) under the catalysis of UGTs. UGP and UGDH are two essential enzymes that catalyze glucose-1-phosphate to form UDP-GLcA. As our previous study indicated, the overall ability of glucuronidation was decreased since the ratio of BRBG to BRB decreased significantly after HFD combined with BRB administration. We investigated the correlative factor involved in the glucuronidation process to uncover the characteristics of glucuronidation after treatment. No significant difference was observed in the levels of renal and hepatic UDP-GLcA in HB compared with H or CB in spite of the inconsistent changes of UGP and UGDH in liver and kidney after treatment (Fig. 2A, 2B and 2C).

To further confirm the activities of UGTs after BRB treatment on HFD, hepatic and renal microsomes of different groups were isolated, prepared, and then incubated with BRB *in vitro*. Based on the rate of BRBG formation in the liver, UGTs in the HB group showed higher activity than in the CB and H groups (HB vs CB, $p<0.05$; HB vs H, $p<0.05$), Fig. 2D. These results demonstrated the elevated hepatic glucuronidation ability after HFD combined with BRB treatment. To interpret the paradox between decreased overall glucuronidation ability and increased hepatic glucuronidation ability, the glucuronidation processing in the kidney was also determined. The rate of BRBG formation in renal microsomes is similar to that in hepatic microsomes which indicated the important role of kidney in the glucuronidation of berberrubine.

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Interestingly, in comparison to the liver, the renal glucuronidation ability in the HB group significantly decreased on the contrary (HB vs CB, $p<0.01$; HB vs H, $p<0.05$). In conclusion, the amount of renal UDP-GLcA showed no significant difference despite the decreased relative levels of UGP and UGDH, but there was a distinctly decreased renal UGT activity in the renal microsomes of HB which may contribute to the decreased overall glucuronidation ability.

As an important nuclear receptor, peroxisome proliferator-activated receptor α (PPARα) recently has been reported to regulate the expression and activity of UGT isoenzymes (Xu et al., 2012; Zhou et al., 2013). To further investigate the potential mechanism of renal and hepatic UGT metabolism differences, the relative mRNA levels of *Pparα* and its targeting genes, including *Fabp* and *Acox*, were also determined (Supplemental Figure 1). There was no significant difference in hepatic *Pparα* and the relative amount of its targeting genes after treatment (HB vs H). Differently, there was a significant decrease in the renal *Pparα* signaling pathway after BRB treatment on HFD, which may be correlative with the decreased activity of UGTs measured before. These results suggested that there was a specific down-regulation of the renal UGT signaling pathway after BRB treatment combined with HFD. Interestingly, *in vitro* studies indicated that neither BRB nor BRBG exerted significant inhibitory effect on the *Pparα* signaling pathway in renal cells (HK-2) which suggests the existence of some endogenous factors (Supplemental Figure 2).

UGT isoenzymes related to the glucuronidation of BRB in C57BL/6J mice

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Since HFD combined with BRB greatly inhibited renal UGT activity, we examined the differential effects on hepatic and renal UGTs in C57BL/6J mice, and profiled the relative mRNA levels of different UGT isoenzymes in the liver and kidneys considering the lack of commercial antibodies for mouse UGTs. As shown in Fig. 3A and 3C, there was an obvious tissue specificity of the main UGT isoenzymes. In mouse kidneys, *Ugt1a7c* showed the absolutely highest mRNA level, which was almost 700-fold higher than that of *Ugt2b35*, and the mRNA levels of other UGTs （*1a6*, *1a2*, *1a1*, *1a9* and *2b1*） were also at a relatively low level except for *Ugt2b5*. The UGT distribution in the kidneys is in accordance with previous studies(Buckley and Klaassen, 2007). In the liver, however, the relative mRNA level of *Ugt1a7c* was at the lowest level and was nearly 40-fold lower than that of *Ugt2b5*, which showed the highest hepatic UGT expression. Moreover, the relative amounts of hepatic *Ugt1a1*, *2b1* and *1a6* were also relatively high, but *Ugt1a2*, *1a9* and *2b35* were at very low levels. After the high fat diet combined with BRB treatment, there was a distinct decrease in renal *Ugt1a7c*, which was the highest expressed UGT in the kidneys, Fig. 3D. While in the liver, the UGTs which expressed at relatively high levels had no significant difference in HB (vs CB or H, Fig.3B).

Although the lack of commercial antibodies and recombinant enzymes for mouse UGTs makes the investigation on mouse UGT isoenzymes only at mRNA levels, these results were in accordance with the renal and hepatic UGT activities determined before, as shown in Fig. 2D.

There is a high degree of similarity and homology in terms of the genes and metabolic

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specificity of UGTs between human and rodents (Hanioka et al., 2001; Williams et al., 2002; Mackenzie et al., 2005; Antonilli et al., 2008; Xie et al., 2013). MPA was used as the potential probe substrate of Ugt1a7c to determine the competitive inhibitory effects on the renal glucuronidation of BRB (Inoue et al., 2007; Zhang et al., 2013). Different concentrations of MPA were incubated with 50 μM BRB in the normal mouse renal microsomal system. The formation of BRBG significantly decreased after incubation with high concentrations of MPA, which indicated that the decreased renal glucuronidation of BRB may be highly related to Ugt1a7c, as shown in Fig. 3E. Moreover, the glucuronidation of BRB under Ugt2b was also investigated using different concentrations of naloxone as a probe substrate in mouse renal microsomes (Supplemental Figure 3) (Zhou et al., 2013; Wang et al., 2015). It was suggested that Ugt2b was not the important enzyme for the glucuronidation of BRB. Hence, the slight increase in Ugt2b5 in HB should not influence the glucuronidation of BRB, which is consistent with decreased glucuronidation ability.

In addition, BRB could also be metabolized by recombinant human UGT1A7 enzyme, as shown in Fig. 3F. Since UGT genes share high homology between humans and rodents (Mackenzie et al., 2005), the decreased *Ugt1a7c* may be highly responsible for the decreased glucuronidation of BRB, since the absolutely highest level in the kidneys.

Accordingly, the glucuronidation of MPA in the renal microsomes of the different groups (C, CB, H and HB) were also investigated, as shown in Fig. 3G. The formation of MPAG in the renal microsomes of the HB group also decreased significantly (HB

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vs CB, $p<0.01$; HB vs H, $p<0.001$), which confirmed the fact that the UGT isoform for MPA is similar to that for BRB and Ugt1a7c would be the most relevant isoform. Besides, neither BRB nor its primary metabolite, BRBG, exerted inhibitory effect on MPAG formation, Fig. 3H, which indicated the existence of other endogenous factors that influenced the activity of UGTs.

Metabolomic approach to identifying end ogenous molecules potentially inhibiting glucuronidation of BRB in mice

Previous studies have suggested that some uremic toxins could inhibit renal metabolic capacity through interference with glucuronidation (Mutsaers et al., 2013). To explore the potential endogenous factors that influence the activity of renal UGTs and to provide a new strategy for clinical detection of drug interactions, the urinary metabolic patterns in C57BL/6J mice were evaluated with GC/MS. A PLS-DA model was created with the samples classified into C, CB, H, and HB groups, Fig. 4A. The scores plot revealed obvious differences between those four groups. Intragroup samples were prone to cluster closely, while intergroup samples scattered to different extents. Remarkably, the samples in the HB showed a distinct shift from that in the H group, which is consistent with the inhibition rate of UGT activity in the HB compared to the H group (Fig.2D). Volcano plot showed a statistical difference in the metabolic profiles between HB and H, Fig. 4B. Based on the discriminant metabolites identified, we found that urine samples in the HB group showed an obviously increased level of purine and pyrimidine metabolites compared to that in the H group, including allantoin, urea, aminoisobutyric acid, dihydrouracil, and alanine. Moreover,

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the levels of three fatty acids (linoleic acid, palmitic acid, and stearic acid), one ketone body (3-hydroxybutyric acid), and several other organic acids (glyceric acid, taurine, glutaric acid and hydroxyglutaric acid) were also significantly elevated, Fig. 4C, 4D and 4E.

Evaluation of the inhibitory effect of the typical endogenous molecules on UGT activity in renal microsomes of mice

In this study, to further explore the possibility of endogenous factors influencing the activity of renal UGTs, eleven elevated compounds were selected based on previous statistical analyses of urinary metabolic profiles, which contained three end products of purine or pyrimidine metabolism (urea, aminoisobutyric acid and alanine), four fatty acid metabolism related compounds (linoleic acid, palmitic acid, stearic acid and 3-hydroxybutyric acid), and four other organic acids (glyceric acid, taurine, glutaric acid and hydroxyglutaric acid).

The inhibitory effects of those selected compounds on UGT activity were evaluated using renal microsomes of C57BL/6J mice. As shown in Fig. 5, urea, glutaric acid, linoleic acid, 3-hydroxybutyric acid and palmitic acid could significantly inhibit the formation of MPAG at the high dose or in a dose-dependent way. The inhibitory rates of these compounds at high doses could reach 73.1%, 99.0%, 44.0%, 43.7% and 61.6%, respectively, compared with the control group. Besides, no significant effects were observed of these compounds on *Pparα* signaling pathway (data not shown). These evidence demonstrate the possibility that the elevated endogenous metabolites in urine may contribute to the decreased glucuronidation ability of BRB in C57BL/6J

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mice.

Evaluation of the inhibitory effect of the typical endogenous molecules on recombinant human UGT1A7

Based on our previous evidence, those selected compounds could influence the glucuronidation of MPA in renal microsomes of C57BL/6J mice, which may be highly attributed to the inhibitory effect on Ugt1a7c. UGTs show high homology between humans and rodents (Mackenzie et al., 2005). For instance, estradiol is used as the typical substrate for human UGT1A1, rat UGT1A1 and mouse Ugt1a1 (Williams et al., 2002; Antonilli et al., 2008; Wang et al., 2015). Although the existence of species difference, we firstly investigated the inhibitory effect of those endogenous compounds on human UGT1A7, as shown in Fig. 6. Remarkably, glutaric acid and linoleic acid could dose-dependently inhibit the activity of UGT1A7, and the inhibition rates could reach 89.4% and 48.3% at high doses, respectively, which are highly consistent with the result from mouse renal microsomes. In contrast, urea, aminoisobutyric acid, alanine, 3-hydroxybutyric acid, palmitic acid, stearic acid, taurine and glyceric acid did not affect the activity of UGT1A7, and hydroxyglutaric acid only exerted a slight inhibitory effect at low doses. Besides, some of the selected molecules could also influence the expression of UGT1A7 in HK-2 cells (Supplemental Figure 4). It was suggested that the endogenous molecules are extremely important for influencing both the expression and activity of UGTs.

Linoleic acid and glutaric acid are potent candidates for inhibiting the glucuronidation of berberrubine and the activities of recombinant human UGTs

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Since linoleic acid and glutaric acid could exert direct inhibition on the activity of mouse microsomes and recombinant human UGT1A7 *in vitro*, the influence of them on the glucuronidation of BRB in HK-2 cells and mice were also evaluated, as shown in Fig.7. The inhibition rates of linoleic acid (200 μM) and glutaric acid (2mM) on the BRBG formation in HK-2 cells could reach 83.4 % and 55.4%, respectively. The excretion of BRBG in the urine samples decreased significantly after intraperitoneal administration with linoleic acid (4 mg/kg) or glutaric acid (20 mg/kg) in the mice orally administered with a single dose of BRB (50mg/kg).

Glutaric acid and linoleic acid were selected as two inhibitory candidates for UGTs. Considering the species difference of UGTs in human and mouse, we systematically evaluate their influence on other human UGT isoforms, which may help to provide some valuable suggestions for clinical studies (Fig.8 and Fig.9). As the results indicated, glutaric acid could also significantly inhibit the activity of UGT1A9 (32.8%) and 1A8 (12.4%) at high doses, although the inhibition rates were much lower than that of UGT1A7. Meanwhile, glutaric acid could slightly induce UGT1A3 at high dose. No significant influences were observed in the groups of UGT1A1, 1A4, 1A6, 1A10, 2B4 and 2B7. Linoleic acid showed powerful inhibition on UGT1A9 and 1A8. The inhibition rates could even reach 99.3% for UGT1A9 and 46.8% for UGT1A8 at 200μM. Moreover, linoleic acid at the middle dose also significantly inhibited UGT1A9 by 36%. Meanwhile, linoleic acid could dose-dependently inhibit the activity of UGT1A1, an important UGT isoform for metabolizing bilirubin and irinotecan.

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Importantly, according to the reports from HMDB, the adopted doses of glutaric acid and linoleic acid could be achieved in the urine or blood samples of some individuals (HMDB00673, HMDB00661). It is suggested that the levels of endogenous compounds, such as linoleic acid and glutaric acid, would be closely related with some clinical diseases and drug toxicity resulted from metabolic disturbances.

Discussion

The glucuronidation process *in vivo* could be influenced by the alteration of UGT expression (gene or protein) and their enzyme activity. Exogenous or endogenous molecules could directly affect the enzyme activity through bonding with the protein domains. According to our data, the selected endogenous molecules could directly inhibit the UGT activity in an *in vitro* microsomal or recombinant UGT system. Among these selected molecules, urea, 3-hydroxybutyric acid and palmitic acid could significantly inhibit the glucuronidation of renal microsomes rather than recombinant human UGT1A7. This may be due to the species difference between the protein domains of UGTs and the contributions of other isoenzymes existed in the microsomes. Besides, some of the selected endogenous molecules (i.e., urea, glyceric acid, and taurine, etc) could also exert significant inhibition on the gene expression of UGT1A7 in HK-2 cells. Generally, inducers or inhibitors could alter the gene expression through the responsive transcription factors, such as PPARα. In this study, the decreased expression of Ugt1a7c in kidney was accompanied by the down-regulated renal *Pparα* signaling pathway (Supplemental Figure 1). However, no

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significant effects on *Pparα* signaling pathway were observed in the HK-2 cells treated with these selected molecules. The regulation of these molecules on the gene expression of UGTs may through other nuclear receptors, such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), etc(Buckley and Klaassen, 2009), and the down-regulated renal *Pparα* signaling pathway *in vivo* should be caused by some other unknown reasons.

Ugt1a7c is a predominantly abundant isoform in mouse kidneys and UGT1A7 is also highly expressed in human kidneys (Harbourt et al., 2012). In this study, the recombinant human UGT1A7 was employed and related with mouse Ugt1a7c since they possess high similarity and homology. However, due to the existence of species difference, Ugt1a7c and UGT1A7 may possess different metabolic properties. Moreover, UGT1A7 is involved in the metabolism of some clinical drugs and xenobiotics, e.g., acetaminophen and benzo(a)pyrene (Maruo et al., 2005). In Fig. 6, glutaric acid and linoleic acid were found to exert significant inhibitory effect on UGT1A7. Hence, the altered levels of them *in vivo* would possibly contribute to the perturbation on glucuronidation through UGT1A7, which may result in the alteration of glucuronidation capacity in the kidneys. Previous evidence indicated that UGT family share a high degree of similarity in terms of gene and protein structure (Wildt et al., 1999; Tukey and Strassburg, 2000; Yang et al., 2017b). In this study, glutaric acid and linoleic acid are also proven to significantly inhibit the activity of UGT1A8 and 1A9, as shown in Fig.8 and Fig.9. UGT1A9 is also an important UGT isoform that is highly expressed in both the liver and kidneys(Ohno and Nakajin, 2009), and it

is responsible for the metabolism of numerous clinical drugs.

To date, the influence of endogenous molecules on drug metabolism has been paid less attention as compared to DDIs. Very few evidence confirmed the effect of endogenous regulators and those studies were carried out merely by employing an *in vitro* microsomal or recombinant enzymes (Tsoutsikos et al., 2004; Fang et al., 2013). No confirmed *in vivo* results have been obtained in terms of the effects of endogenous molecules on glucuronidation. In this study, for the first time, we demonstrated that an elevation of endogenous molecules including glutaric acid, linoleic acid, 3-hydroxybutyric acid and palmitic acid could significantly contribute to the inhibition on mouse renal glucuronidation. Among those compounds, glutaric acid and linoleic acid are the two strongest potential inhibitors for human recombinant UGT1A7, 1A8 and 1A9. Because there is no intact signal pathway available in the *in vitro* incubation system, the inhibition of the endogenous molecules on the activity of mouse microsomes and human UGTs suggests the direct effect of the molecules on UGTs. Besides, for the first time, we confirm the inhibitory effects of endogenous linoleic acid and glutaric acid on the glucuronidation metabolism in human renal proximal tubular cells (HK-2) and mice, in addition to microsomes and recombinant UGTs.

This study was performed based on the specific inhibitory effect on renal glucuronidation of BRB under a high fat diet. In fact, there may be more endogenous compounds that have changed during this process. Although the selected molecules are proved to exert the inhibitory effect on UGTs, some other endogenous molecules

or potential mechanisms may also be involved in the inhibited glucuronidation of BRB in this study. Most importantly, this study provided a valuable strategy by employing metabolomics methodology to identify endogenous molecules on DMEs. Furthermore, the effective concentrations of glutaric acid and linoleic acid administered could be achieved in normal or abnormal human urine or blood samples according to the reports from HMDB, i.e., the actual concentrations of linoleic acid in the blood samples of some normal and abnormal individuals are higher than 200 μM (HMDB00673), and the urinary levels of glutaric acid in some individuals with glutaric aciduria are high than 2mM (HMDB00661). The data suggested that linoleic acid and glutaric acid would possibly contribute to the individual variation of glucuronidation and some endogenous molecules might be key factors contributing to individual differences in drug metabolism. Furthermore, previous study has confirmed the inhibitory potential of endogenous bile acids towards cytochrome P450 activity(Chen and Farrell, 1996) and indicated the possibility that the increased levels bile acids during the hepatic diseases may interfere with the glucuronidation of xenobiotics, such as drugs (Fang et al., 2013). Therefore, the metabolic perturbation in some metabolic diseases which involving an altered level of these endogenous molecules has the potential to induce the variation of glucuronidation of xenobiotics. Importantly, individual variances can be evaluated, and a personalized medication can be suggested for the substrates of UGT based on the *in vivo* levels of the endogenous molecules.

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Authorship Contributions

Participated in research design: Jiye Aa, Guangji Wang, Yuan Xie and Na Yang.

Conducted experiments: Na Yang, Sijia Li, Caixia Yan and Ying Peng.

Performed data analysis: Na Yang, Runbin Sun and Jun He.

Wrote or contributed to the writing of the manuscript: Na Yang, Jiye Aa and Guangji Wang.

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Footnotes:

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Conflicts of Interest

None

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Figure Legends

Figure 1. Pharmacokinetics studies of BRB and BRBG after oral administration of BRB in C57BL/6J mice.

CSB, control diet with single dose of BRB; CMB, control diet with multiple doses of BRB; HMB, high fat diet with multiple doses of BRB. The mice in CMB and HMB were p.o. administered with 50 mg/kg/d BRB for six consecutive weeks. (A) The plasma concentration-time curves of BRB. (B)The plasma concentration-time curves of BRBG. (C) Mean plasma AUC0-t of BRB and BRBG. (D) The ratio of BRBG to BRB in the plasma. (E) Excretion of BRB in the urine. (F) Excretion of BRBG in the urine. (G)The ratio of BRB to BRBG in the urine. The plasma samples were collected at 0.083, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8 h after administration. Error bars show SD of replicates at each time point in each group (0-6h: n=6; 8h: n=4). The urine samples were collected in metabolic cages for 12 h after the last administration. Values are means ± SD (n=5). The data were assessed using two-tailed Student's t test. *p< 0.05, **p<0.01, compared with CSB; # p< 0.05, ## p<0.01, compared with CMB.

Figure 2. The alteration of hepatic and renal glucuronidation pathway after treatment.

C, normal standard diet with vehicle CMC-Na; CB, normal standard diet with BRB (50 mg/kg/d, i.g.); H, high-fat diet with vehicle CMC-Na; HB, high-fat diet combined with BRB (50 mg/kg/d, i.g.). Mice were treated for six consecutive weeks. (A) Relative mRNA levels of UGP and UGDH in the liver (n=5). (B) Relative mRNA levels of UGP and UGDH in the kidneys (n=5). (C) Relative amount of UDP-GLcA in the kidneys and liver (n=4). (D)Activities of UGTs in the renal and hepatic

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microsomes prepared from tissues of different groups, the rate was determined according to the formation of BRBG after incubation with BRB *in vitro* (n=6). Values are means ± SD. Two-tailed unpaired Student's test was used in the comparison of two independent groups. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, N.S., not significant.

Figure 3. Identification of UGT isoenzymes related to the glucuronidation of BRB. (A) The relative amount of different UGT isoforms in the liver. (B)The relative mRNA levels of hepatic UGT isoenzymes in C57BL/6J mice after treatment. (C) The relative amount of different UGT isoforms in the kidneys. (D) The relative mRNA levels of renal UGT isoenzymes in C57BL/6J mice after treatment (n=5). (E) Competitive inhibitory effects of MPA on the formation of BRBG in normal renal microsomes of C57BL/6J mice (n=3). (F) The metabolism of BRB by recombinant human UGT1A7 enzyme (n=3). (G) The activity alteration of renal microsomes on the metabolism of MPA in C57BL/6J mice after treatment (n=6). (H) The influence of BRB and BRBG on the metabolism of MPA in normal renal microsomes of C57BL/6J mice (n=3). Values are means ± SD. Two-tailed unpaired Student's test was used in the comparison of two independent groups. ** $p< 0.01$, *** $p< 0.001$, compared with CB; ### $p<0.001$, compared with H.

Figure 4. Metabolomic study of mouse urine after treatment. (A) PLS-DA scores plots. R2X：0.574；R2Y：0.445；Q2Y:0.213. (B) Volcano plot of urinary metabolites in HB compared with H. The P values were assessed using two-tailed Student's t test. (C-E) Phase diagrams showed the deviations of the key molecules with significantly elevated levels. The digital labels represent the fold change of the selected molecules

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in HB group compared to H group (C) Metabolites involved in purine/pyrimidine metabolism. (D) Metabolites involved in fatty acid metabolism. (E) Other organic acids.

Figure 5. Evaluation of th e inhibitory capability of the typical endogenous molecules on UGT activity in mouse r enal microsomes. Eleven elevated compounds were selected and administered in the incubation system of mouse renal microsomes at three different doses for 30 min at 37°C.The relative amount of MPAG formation represents the activity of Ugt1a7c in mouse renal microsomes after treatment. C, blank control group; L, low dose; M, middle dose; H, high dose. The selected compounds contain urea (A), glutaric acid (B), linoleic acid (C), 3-hydrobutyric acid (D), palmitic acid (E), aminoisobutyric acid (F), alanine (G), stearic acid (H), taurine (I), glyceric acid (J), and hydroxyglutaric acid (K). Values are means ± SD (n=3). The data were assessed by one-way ANOVA, followed by Tukey *post-hoc* multiple comparison test. * $p< 0.05$, *** $p< 0.001$, compared with control.

Figure 6. Evaluation of th e inhibitory capability of the typical endogenous molecules on recombinant human UGT1A7. Recombinant human UGT1A7 was incubated with eleven selected compounds at three different doses for 30 min (37°C). The relative amount of MPAG formation represents the activity of UGT1A7. C, blank control group; L, low dose; M, middle dose; H, high dose. The selected compounds contain glutaric acid (A), linoleic acid (B), hydroxyglutaric acid (C), urea (D), aminoisobutyric acid (E), alanine (F), 3-hydrobutyric acid (G), palmitic acid (H), stearic acid (I), taurine (J), and glyceric acid (K). Values are means ± SD (n=3). The

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data were assessed by one-way ANOVA, followed by Tukey *post-hoc* multiple comparison test. **p*< 0.05, ***p*< 0.01, ****p*< 0.001, compared with control.

Figure 7. Effects of linoleic acid and glutaric acid on the glucuronidation of BRB in HK-2 cells and mice

(A) Effects of linoleic acid and glutaric acid on BRBG formation in HK-2 cells. Cells were exposed to 50 μM BRB with or without the administration of linoleic acid (200μM) and glutaric acid (2mM) for 12h at 37 °C (n=4). (B) Effects of linoleic acid and glutaric acid on BRBG excretion in urine. Mice in BRB group were orally administered with a single dose of BRB (50mg/kg), and mice in BRB+LA and BRB+GA group were co-administered with a single dose of linoleic acid (4mg/kg, i.p.) or glutaric acid (20mg/kg, i.p.). Urine samples were collected in the metabolic cages for 24 h (n=5). The data were assessed by one-way ANOVA, followed by Tukey *post-hoc* multiple comparison test. **p*< 0.05, ***p*< 0.01, ****p*< 0.001, compared with BRB group.

Figure 8. Evaluation of the inhibitory capability of glutaric acid on recombinant human UGTs. Recombinant human UGT1A9 (A), 1A8 (B), 1A3 (C), 1A1 (D), 1A4 (E), 1A6 (F), 1A10 (G), 2B4 (H), 2B7 (I) were incubated with three different doses of glutaric acid (L, 20μM**;** M, 200μM; H, 2mM) at 37°C **.** BRB (50μM) was selected as the probe substrate of UGT1A9, 1A8, 1A3 and 1A1. Valproic acid (VPA, 1mM) was selected for UGT1A4 and 1A10. 4-MU (1mM) was selected for UGT1A6, 2B4 and 2B7. The concentrations of different recombinant human UGTs and the incubation time were selected following the recommendation of instructions. The data were

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assessed by one-way ANOVA, followed by Tukey *post-hoc* multiple comparison test. $^{**}p< 0.01$, $^{***}p< 0.001$, compared with control.

Figure 9. Evaluation of the inhibitory capability of linoleic acid on recombinant human UGTs. Recombinant human UGT1A9 (A), 1A8 (B), 1A1 (C), 1A6 (D), 1A3 (E), 1A4 (F), 1A10 (G), 2B4 (H), 2B7 (I) were incubated with three different doses of linoleic acid (L, 2 μM**;** M, 20 μM; H, 200 μM) at 37 °C**.** BRB (50μM) was selected as the probe substrate of UGT1A9, 1A8, 1A3 and 1A1. Valproic acid (VPA, 1mM) was selected for UGT1A4 and 1A10. 4-MU (1mM) was selected for UGT1A6, 2B4 and 2B7. The concentrations of different recombinant human UGTs and the incubation time were selected following the recommendation of instructions. The data were assessed by one-way ANOVA, followed by Tukey *post-hoc* multiple comparison test. $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$, compared with control.

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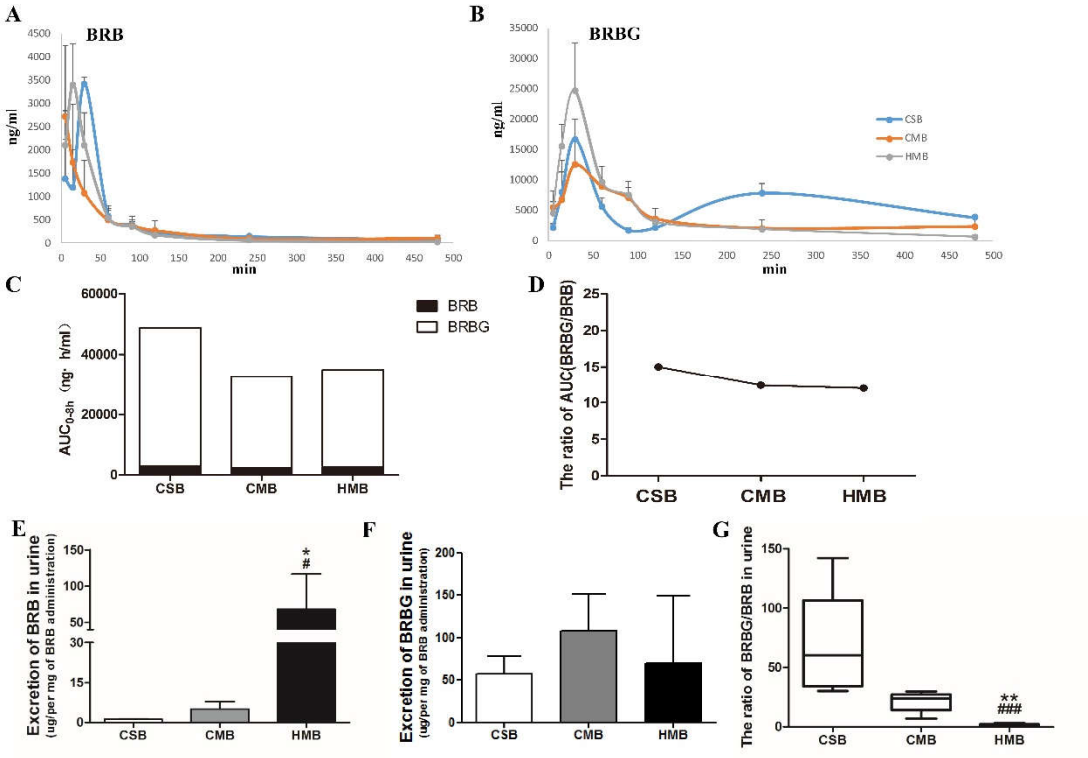

Figure 1

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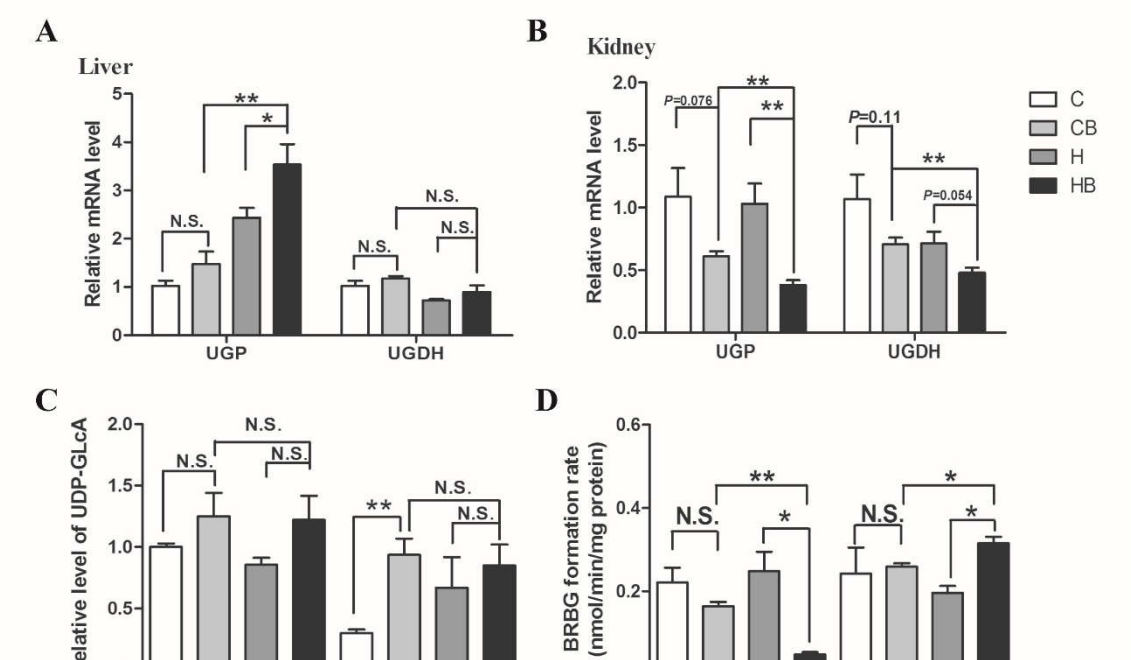

Figure 2

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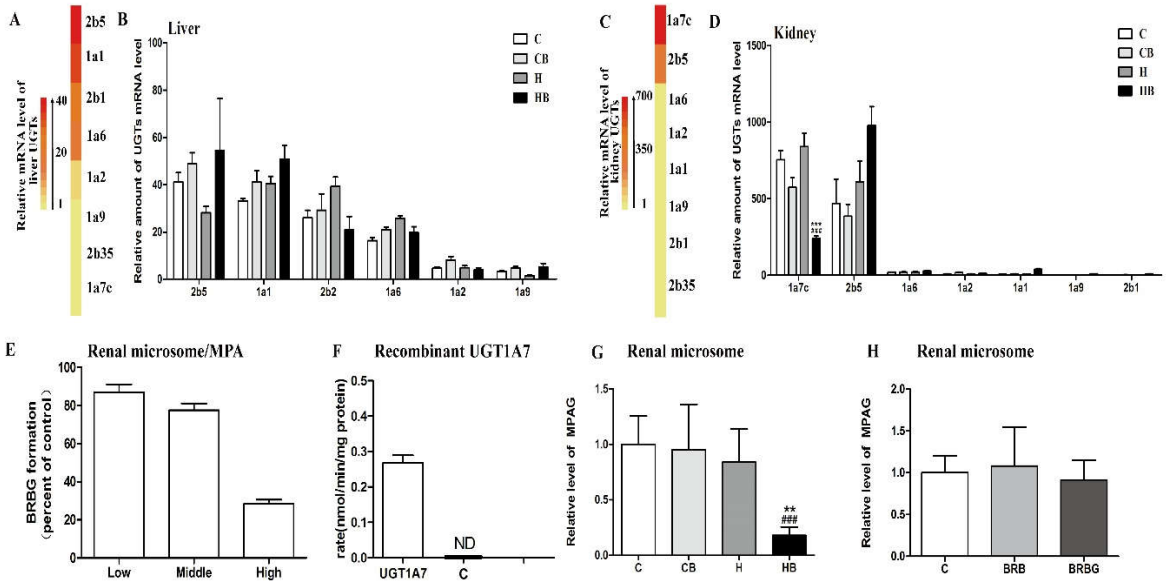

Figure 3

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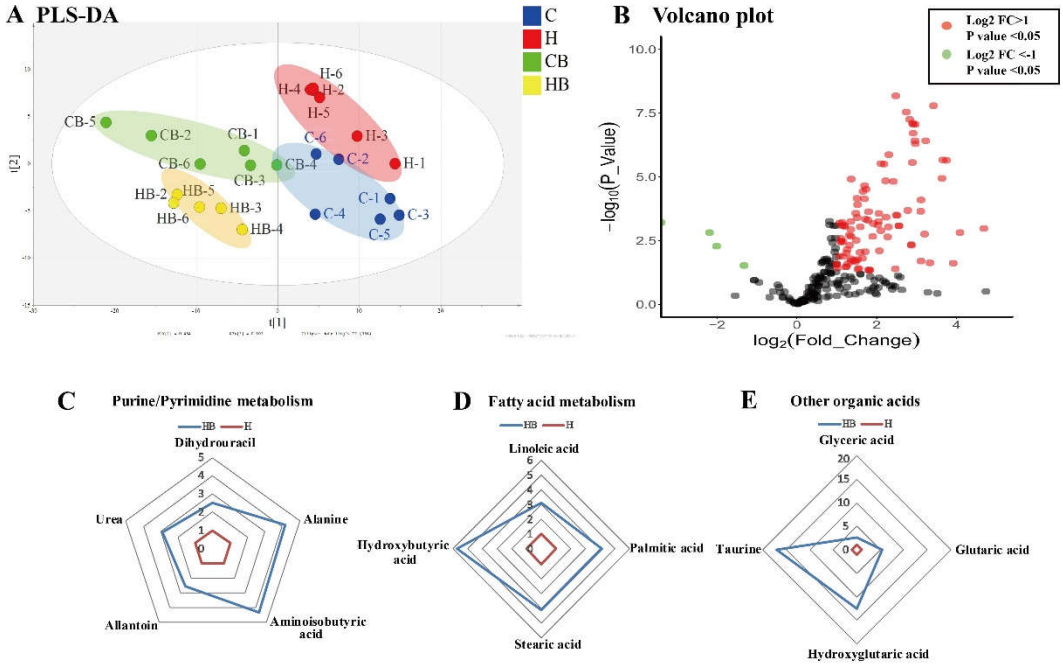

Figure 4

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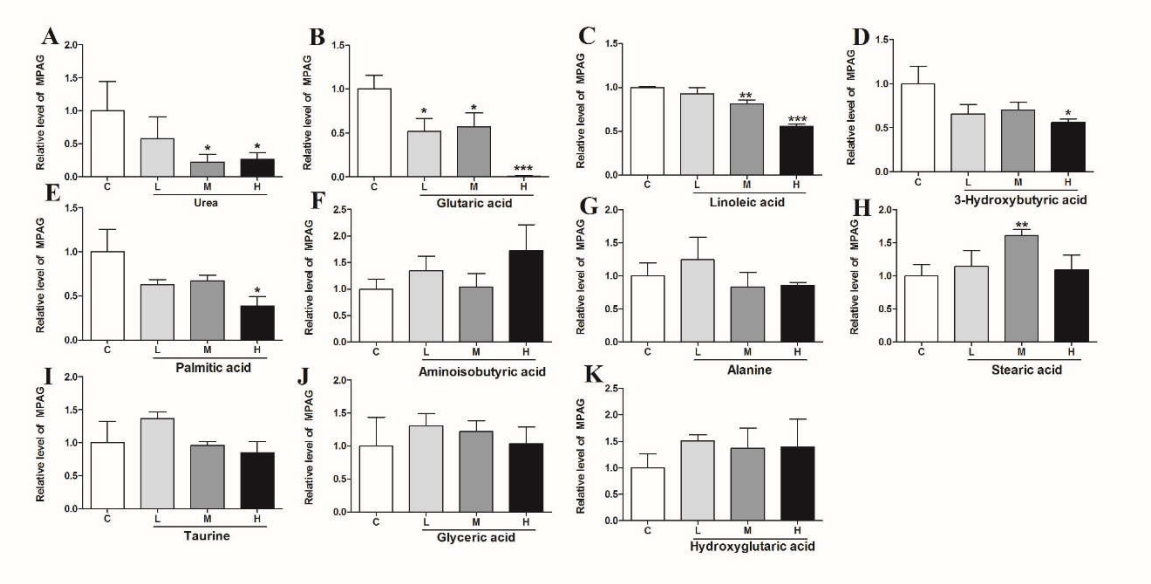

Figure 5

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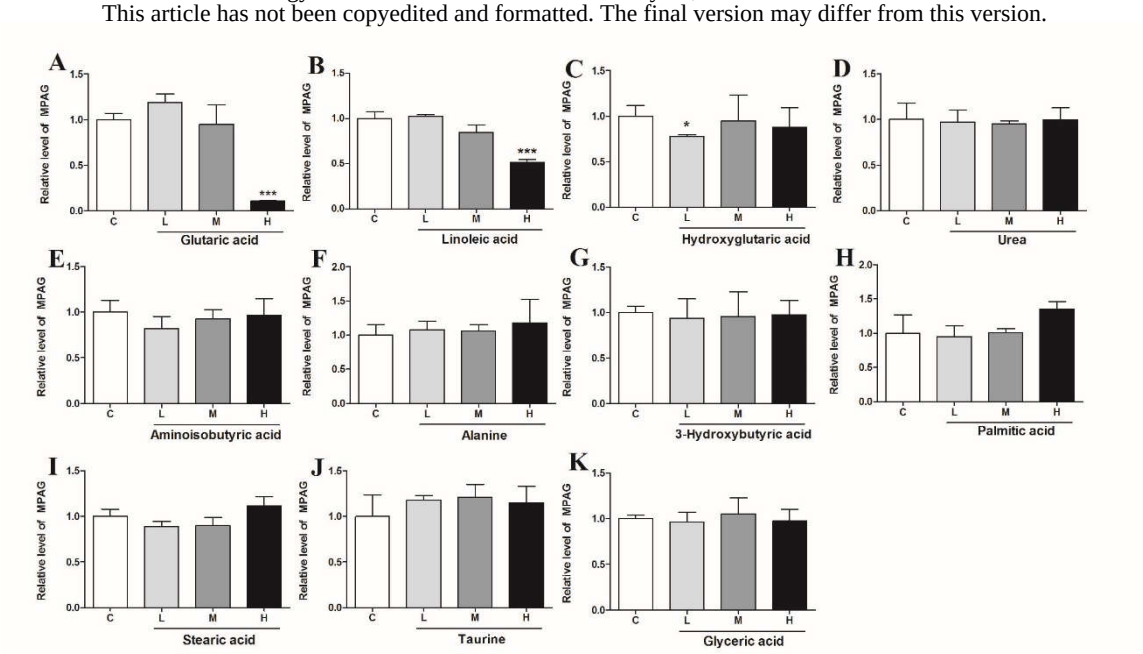

Figure 6

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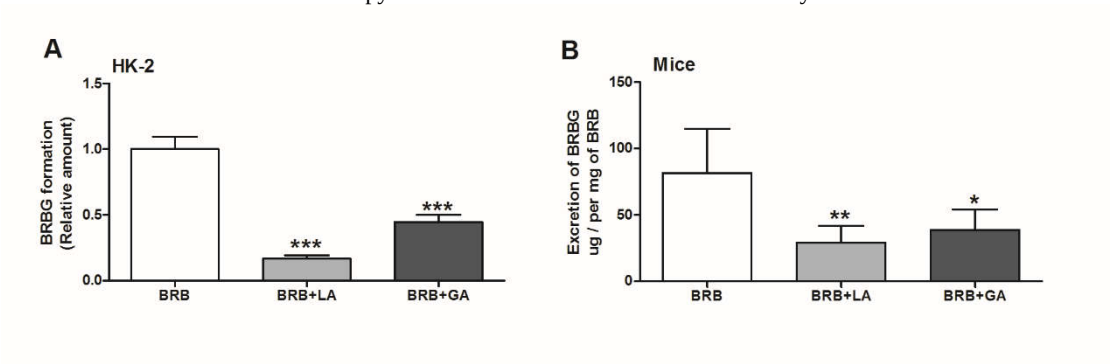

Figure 7

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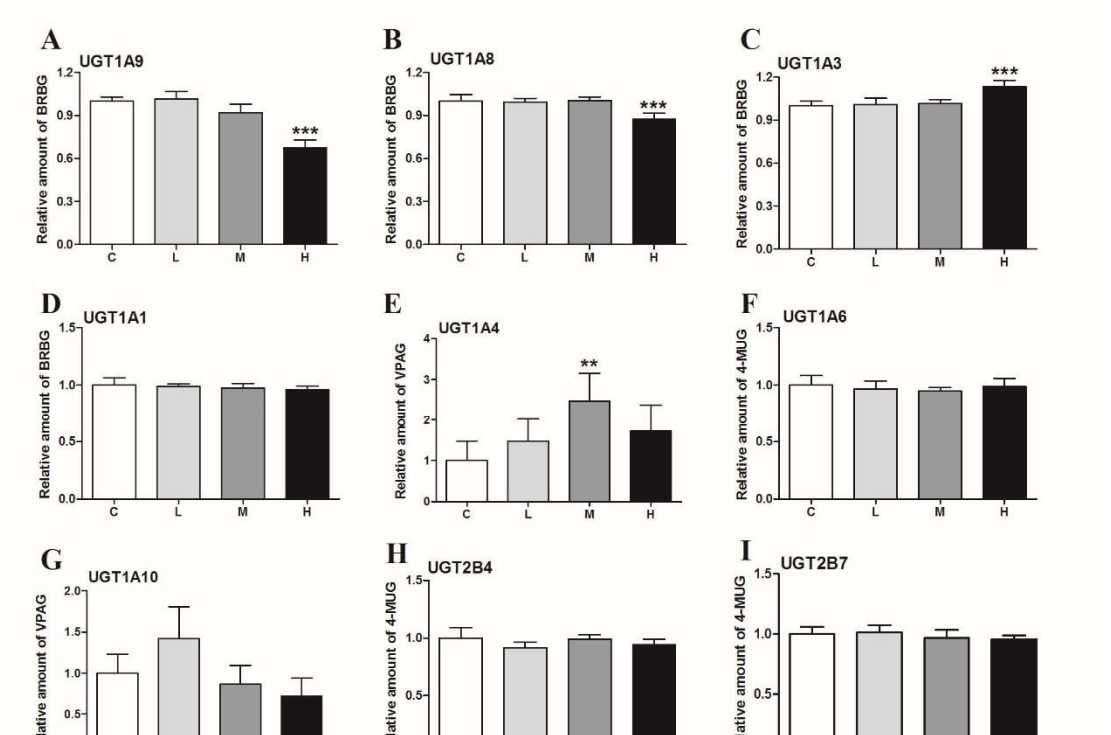

Figure 8

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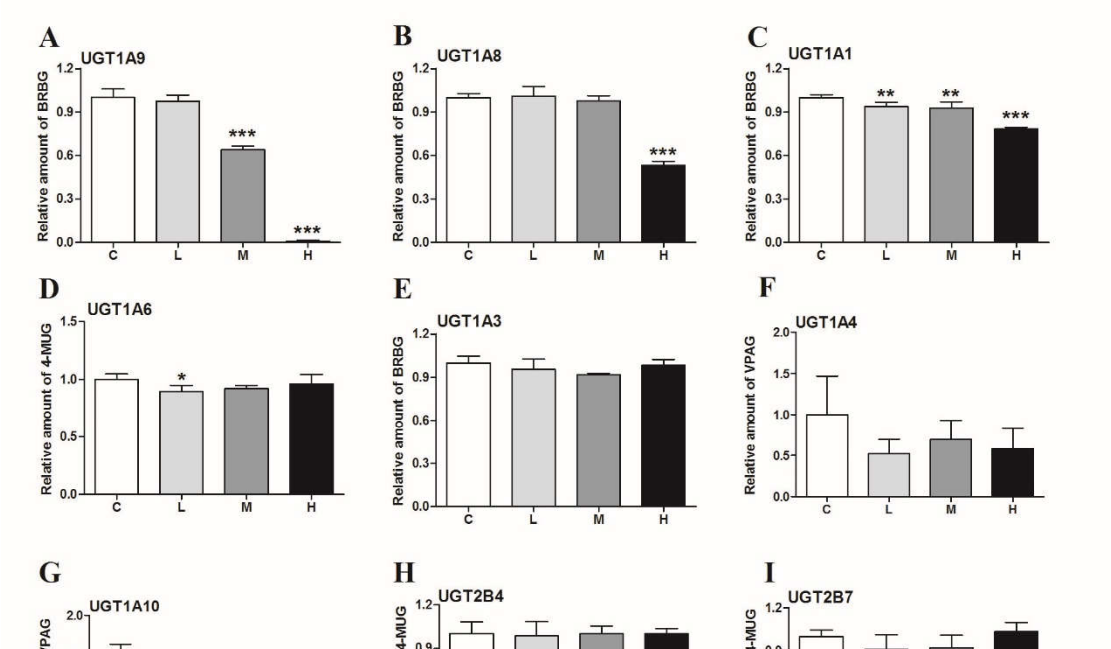

Figure 9