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Thioacetamide Intoxication Triggers Transcriptional Upregulation but Enzyme Inactivation of UDP-glucuronosyltransferases

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Running title: TAA induced dysregulations of UGT

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ABBREVIATIONS: TAA, thioacetamide; UGTs, UDP-glucuronosyltransferases; SLE, *Schisandra* lignans extract; DDB, dimethyl diphenyl bicarboxylate; TES, testosterone; 4-MU, 4-methylumbelliferone

ABSTRACT

Thioacetamide (TAA) is a potent hepatotoxicant and has been widely used to develop experimental liver fibrosis/cirrhosis models. Although the liver toxicity of TAA has been extensively studied, little is known about its potential influence on UDP-glucuronosyltransferases (UGTs) associated with the development of liver fibrosis. The present study aimed to uncover the regulation patterns of UGTs in TAA induced liver fibrosis of rats. Potential counteracting effects of hepatoprotective agents were also determined. TAA treatment for 8 weeks induced a significantly transcriptional upregulation of the major UGT isoforms including UGT1A1, UGT1A6, and UGT2B1, accompanied with the dramatic elevations of most typical serum biomarkers of liver function and fibrosis scores. Upon TAA intoxication, the mRNA and protein levels of the major UGT isoforms were increased to 1.5-2.5-fold and 2.5-3.3-fold of that of the normal control, respectively. The hepatoprotective agents *Schisandra* lignans extract (SLE) and dimethyl diphenyl bicarboxylate (DDB) could largely abolish TAA induced upregulation of all the three UGT isoforms. Enzyme activities of UGTs, however, remained unchanged after TAA treatment. The dissociation of protein expression and enzyme activity could possibly be attributed to the inactivating effects of TAA, upon a NADPH dependent bioactivation, on UGTs. This study suggests the transcriptional upregulation of UGTs may be an alternative mechanism of their preserved activities in liver fibrosis/cirrhosis.

Introduction

UDP-glucuronosyltransferases (UGTs), a group of membrane-bound enzymes that reside in the endoplasmic reticulum, catalyze the conjugation of UDP-glucuronic acid to the hydroxyl, carboxyl, amine, or thiol group of numerous structurally-diverse endogenous substances and xenobiotics (Radominska-Pandya *et al.*, 2005). Since the addition of glucuronic acids generally increases the water solubility of substances and thus, facilitates elimination, UGTs play an important role on detoxifying or inactivating endogenous and xenobiotic substances (Wang *et al.*, 2010). Alternatively, UGTs catalyzed glucuronidation could contribute to the bioactivation and/or toxification of some substances, as in the case of acyl glucuronidation of carboxylic drugs such as non-steroidal anti-inflammatory drugs (Southwood et al., 2007; Koga et al., 2011). Nevertheless, the dysregulations of UGTs caused by various pathological factors and exogenous toxicants could trigger significant changes of pharmacological, toxicological, and/or pathological consequences of various substances.

The dysregulations of cytochrome P450s in hepatic fibrosis/cirrhosis have been extensively studied (Gomez-Lechon et al., 2009; Morgan, 2009). In contrast, the current understanding of UGTs dysregulation in hepatic injury remains still very limited and the previous results are largely controversial. UGTs activities were found largely preserved in liver diseases, possibly because of the activation of latent UGT enzymes (Desmond *et al.*, 1994), the increased expression in remaining viable cells (Debinski *et al.*, 1995), and the increased contribution of extrahepatic UGTs (Omar *et al.*, 1996). However, more recent

studies focusing on the mRNA levels of individual UGT isoforms from both the clinical patients with liver diseases (Congiu *et al.*, 2002) and the mice with inflammation (Richardson *et al.*, 2006) indicated downregulations of most UGT isoforms. The major limitation of previous studies concerning UGT regulation in liver injury is that the expression and enzyme activity of individual isoforms have not been concomitantly evaluated, resulting in inconsistencies across various studies with different measures. In addition, results obtained from clinical patients may be complicated by other confounding factors including gene polymorphisms and concomitant drugs, tobaccos, and dietary components. Therefore, it is important to determine simultaneously the expression and enzyme activity of individual UGT isoforms in the experimental models of liver injury for the better understanding of liver injury *per se* on regulating UGTs.

Thioacetamide (TAA), originally used as a fungicide, is a potent hepatotoxicant and has been widely used in developing experimental liver fibrosis/cirrhosis models mimicking the human liver fibrosis/cirrhosis (Nozu et al., 1992; Kang et al., 2005). As the largest metabolic organ in biological systems, the liver endowed with many types of drug metabolizing enzymes plays a critical role on the metabolic elimination of many endogenous substances and exogenous toxicants. Therefore, extensive understanding of the dysregulation of drug metabolizing enzymes in liver injury should be an essential step for exploring pathological processes and consequences of liver diseases. It had been previously reported that most CYP450 isoforms were down-regulated in a TAA induced rat cirrhosis model (Nakajima *et al.*, 1998). In contrast, the profile of UGTs regulation in TAA induced liver injury models remains largely unknown.

Considering that some recent reports suggested a dysregulation of most individual UGT isoforms in liver injury, we hypothesized herein that TAA induced fibrosis/cirrhosis would also result in a dysregulation of UGT isoforms. The major purpose of this study was to test the hypothesis by concomitant evaluation of the mRNA, protein, and enzyme activity of major rat UGT isoforms including UGT1A1, UGT 1A6, and UGT 2B1 in a TAA induced liver injury model. In addition, it was of great interest to determine whether hepatoprotective agents would be effective in counteracting TAA induced dysregulation of UGTs. For this purpose, *Schisandra* lignan extract (SLE) and dimethyl diphenyl bicarboxylate (DDB), both of which had been well proven to possess powerful hepatoprotective effects against liver injury induced by various pathological factors(Gao et al., 2005; Abdel-Hameid, 2007; Xie et al., 2010), were included in this study to further verify our hypothesis.

Materials and Methods

Chemicals and Reagents

TAA was obtained from Jiahui Medicine Chemical Co. Ltd (Anhui, China). DDB and SLE were obtained from Qingze Science and Technology Co. Ltd (Nanjing, China). BCA protein assay kit, SDS-polyacrylamide gel electrophoresis, and sample loading buffer were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). UGT1A1, UGT1A6, and UGT2B antibodies (Goat anti-rat IgG) were obtained from Santa Cruz Biotechnology Inc (CA, USA). Bilirubin was purchased from Laboratory for the Control of Drugs of Jiangsu Province (Nanjing, China). Testosterone (TES), 4-methylumbelliferone (4-MU), 4-MU-O-glucuronide, glucose-6-phosphate, NADP+, glucose-6-phosphate dehydrogenase, UDPGA, alamethicin, and D-Saccharic acid 1,4-lactone monohydrate were all purchased from Sigma-Aldrich (Shanghai, China). The purity of all the chemicals was proven to exceed 99%. HPLC grade acetonitrile and methanol were obtained from Merck (Darmsadt, Germany). Deionized water was purified using a Milli-Q system (Millipore Corporation, Billerica, MA).

Animals and Treatment

Male Sprague–Dawley rats (200–250g) were caged and fed at 20-25°C, with a 12-hour light-dark cycle and free access to food and water. The animals were acclimatized to the environment for a week, and fasted with free access to water for 12 h prior to each experiment. All animal experimental procedures were approved by the Animal Care and Use Committee of China Pharmaceutical University and were in accordance with the

guiding principles in the use of animals in toxicology adopted by Chinese Society of Toxicology. Animals were randomly divided into 5 groups with 6 individuals in each group. Four groups of rats were intraperitoneally injected with TAA (200 mg/kg, 3 % in physiological saline) twice a week for a consecutive 8 weeks to induce hepatic fibrosis (Barash et al., 2008). Another group of rats serving as normal control was treated with normal saline. After an initial TAA treatment for 4 weeks, the rats were intragastrically administered with 1 % CMC-Na (TAA control), SLE low dose (100mg/kg, suspended in 1% CMC-Na), SLE high dose (400mg/kg, in 1% CMC-Na), or DDB (200 mg/kg, in 1% CMC-Na) once a day, respectively, for another 4 weeks. All the rats were weighed before treatment and at appropriate time points throughout the whole experimental duration, and were observed daily for signs of illness. Rats were sacrificed by cervical dislocation under ether anesthesia at the end of 8th week. Blood samples were collected for biochemical determinations; the liver tissues were quickly removed, weighed, and frozen immediately in liquid nitrogen until analysis.

To determine the acute effects of TAA on regulating UGTs, rats were intraperitoneally injected with a single dose of TAA at 200 mg/kg, while the normal control group of rats was injected with normal saline. Six hours after treatment, the rats were sacrificed under ether anesthesia and the livers were quickly removed and immediately frozen in liquid nitrogen. The liver samples were used for the determination of mRNA levels and enzyme activities of UGTs.

Hepatic Function Biomarkers and Fibrosis Determination

Hepatic function biomarkers including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), albumin (ALB), γ-Glutamyl Transpeptidase (γ-GT), and total bilirubin (T-Bil) were determined by an automatic blood biochemical analyzer (Beckman Counter LX20, USA).

Slices of the same part of each rat liver were cut off and fixed in phosphate buffered 10 % formaldehyde solution and then embedded in paraffin wax. Sections of liver tissues with 5 µm thickness were cut and stained with hematoxylin–eosin (HE) according to standard procedures, and then examined for histopathological changes under the microscope (Olympus BH2, Japan). The extent of fibrosis was graded, according to the semi-quantitative METAVIR scoring system (Huwart et al, 2008), as no fibrosis (score 0), portal fibrosis without septa (score 1), portal fibrosis with few septa (score 2), numerous septa without cirrhosis (score 3), and established cirrhosis (score 4). The assessment of the extent of fibrosis was performed in blind by an experienced pathologist.

RT-PCR Analysis

Frozen liver samples were crushed with a glass mortar in liquid nitrogen. RNA was extracted using a RNA extraction kit (Keygene, Shanghai, China) according to the manufacturer's instructions. The RNA pellet was dried and dissolved in RNase free water and stored at -80°C until use. RNA concentrations were determined with a spectrophotometer at 260 nm. For cDNA synthesis, a reaction media containing 5 µg of total RNA , 1µl of Oligo dT (10 µM), 1µl of dNTPs (10 mM), and RNase free water to a final volume of 15 µl was heated at 65 °C for 5 min and then immediately placed in ice

water bath for 5 min. To each sample, 0.5 μl of RNase inhibitor (40 U), 2 μl of 10×AMV

Reaction Buffer, 1 μl of DTT (1 M), and 1 μl of reverse transcriptase (AMV) was add and

kept at 37°C for 60 min and then at 70°C for 15 min. The cDNA samples were kept at

-80°C until use.

Primers for rat UGT1A1, UGT 1A6, UGT 2B1, and glyceraldehyde phosphate

dehydrogenase (GADPH) were designed using the primer select software (Primer

Premier, 5.0, CA) based on the previous reports (Nozu et al., 1992; Kang et al., 2005).

Sequences of the PCR primers are as follows (sense and antisense for each isoform,

5'-3'): *UGT1A1* (554 bp), ACG AAG TGG TGG TCA TAG CA, CTG TAA GAT TTC

AGT GGC AAG; *UGT1A6* (270 bp), TGG TGC TAG TGC CAG AAG TCA A, GAG

CAT CAA ACT GGT TCT CCC T; *UGT2B1* (439 bp), GTC ACG GTT CTT GTA TCT

TCG G, GAA CAA CAG GCA CAT AGG AAG G; *GAPDH* (352 bp), CGG GAA GCT

TGT CAT CAA TGG, GGC AGT GAT GGC ATG GAC TG. The PCR mixture

containing 5 μl of 10×Taq buffer, 1 μl of dNTPs (10 mM), 2 μl of each primer (10 μM), 2

μl of cDNA template, 0.5 μl of Taq DNA polymerase, and 4 μl of MgCl$_2$ (2.5 mM) was

initially denatuated at 95°C for 90 seconds, followed by 32 cycles amplification for

UGT1A1, 30 cycles for UGT1A6, 28 cycles for UGT2B1, or 30 cycles for GAPDH.

Each cycle was performed as the following steps: 90 seconds at 94°C (denaturation); 45

seconds at 59.5°C for UGT1A1, 59°C for UGT1A6, 56°C for UGT2B1, or 55°C for

GAPDH (annealing); and 45 seconds at 72°C (extension). The final extension was

performed at 72°C for 10 minutes. Then, 8 μL of the PCR-amplified mixture was

subjected to electrophoresis on 1.5% agarose gel and visualized by ethidium bromide

staining. The gel was then photographed by ultraviolet gel image formation (BIO-RAD Gel DocTM XR, USA) and the gray scale was determined by a digitalized software (Quantity one, BIO-RAD, USA).

Western Immunoblotting

Liver tissue proteins (70 μg) were resolved by SDS-10% polyacrylamide gel electrophoresis and transferred to a PVDF membrane at 180 mA for 60 min using a wet transfer method (Bio-Rad, wet blotter). After transferring, the membranes were blocked with 5 % non-fat dry milk in Tris-buffered saline/Tween 20 buffer (TBST) at 37 °C for 90 minutes. Then, the membranes were incubated overnight at 4°C with each diluted antibody (1:200 for UGT1A1, 1:150 for UGT 1A6, and 1:200 for UGT2B) in 5 % non-fat dry milk-TBST and washed 3 times with TBST for 15 minutes each time. For references, β-actin was detected using a polyclonal antibody (1:200). A peroxidase-conjugated rabbit anti-goat IgG (bioworld, USA) (1:5000) was used as second antibody and incubated with the membranes for 60 minutes at 37°C. The membranes were washed extensively again (3 times, 15 min/time) with TBST buffer before detection using enhanced chemiluminescence (ECL) detection reagents (bioworld, USA). After exposure in the darkroom, the film was then photographed and the signal intensity was detected by a digitalized software (Quantity one, BIO-RAD, USA). The protein levels of UGTs were normalized to that of the reference band β-actin.

Preparation of Rat Liver Microsomes

For enzyme activities analysis of UGTs, each group of rats' livers were pooled together

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and the hepatic microsomal suspensions were prepared by differential centrifugation as previously described (Hao *et al.*, 2007) and resuspended in 0.25 M sucrose/1 mM EDTA solution (pH 7.4). Protein concentrations were determined with a BCA protein assay kit according to the manufacturer's instructions. Final protein concentration was adjusted to about 10 mg/ml with the same buffer. The suspensions were frozen and maintained at -80°C until use.

Enzyme Activities Assay of UGTs

Enzyme activities of UGTs in rat liver microsomes were determined using the typical substrate bilirubin for UGT1A1, 4-MU for UGT1A6, and TES for UGT2B1. For UGT1A1 test, the pooled and alamethicin-permeabilized microsomes（2mg/ml） from

each group were incubated for 30 min at 37°C in the presence of 10 mM $MgCl_2$ and 320 μM bilirubin in a final volume of 0.2 ml. The reactions were initiated by the addition of 20 mM UDPGA and terminated by the addition of 200 μl ice cold glycine/HCl (2 M, pH 2.7). The bilirubin glucuronidation was assayed by the electrophotometrical determination of metabolites as described elsewhere with minor modifications (Gordon et al., 1983). Bilirubin glucuronides were determined by measuring the absorbance at 550 nm using a detection kit, based on the conversion of mono- and di-bilirubin glucuronides to dipyrrolic azo derivatives. Since bilirubin is a light sensitive compound, all handling procedures for bilirubin were carried out under dim light.

For UGT1A6 and UGT2B1 test (Hirotada S, et al., 2008; Nobumitsu H, et al., 2001), the pooled and alamethicin-permeabilized microsomes (2mg/ml) of each group were

incubated at 37°C with 4-MU (500 μM) for 5 min, or TES (75 μM) for 18 min in the media containing 10 mM $MgCl_2$ in a final volume of 0.2 ml in PBS (PH 7.4) and. The reactions were initiated by the addition of 20 mM UDPGA and terminated by the addition of 200 μl of ice cold acetonitrile. After centrifugation, 10 μl of supernatant was injected into a HPLC system (Shimadzu, Japan) for the detection of 4-MU, TES, and their glucuronides. The UV detector was set at 220 nm for the detection of 4-MU and its glucuronide, and at 250 nm for TES and its glucuronide, respectively. Separation was achieved on a Diamonsil C18 column (150×4.6 mm, 5 μm, Dikma Technologies, USA).

A mobile phase consisting acetonitrile and 50 mM ammonium phosphate buffer (30:70, v:v) was used for TES and its glucuronide detection. For 4-MU and its glucuronide detection, the mobile phase was composed of acetonitrile and 20 mM ammonium phosphate buffer (10:90, v:v). The flow rate was set at 1.0 ml/min in both cases.

To determine the potential inactivating effects of TAA on UGTs, TAA (5, 50, and 200 μM) was pre-incubated with rat liver microsomes for 0.5 h with or without the addition of NADPH before the addition of UDPGA and probe substrates to initiate the UGTs catalyzed reactions. Effects of hydrogen peroxide (0.2, 1, and 5 mM) pre-incubation were also determined likewise. In another set of incubations, DDB (10, 50, and 200μM) or GSH (1 and 5 mM) was added concomitantly with TAA in the UGT1A6 activity test.

TAA disposition in liver microsomes

To investigate whether the hepatoprotective agents were influential on the TAA disposition, an assay of NADPH dependent metabolic depletion of TAA was performed

in the in vitro liver microsomes incubation system. The incubation mixture contained 1 mg/ml microsome protein, TAA (0.1 mM), NADPH-regenerating system, and the hepatoprotective agent SLE (0-500 μg/mL) or its lignan component schizandrol A (0-200 μM) and deoxyschizandrin (0-100 μM), or DDB (0-500 μM), in a final volume of 200 μl. NADPH-regeneration system was finally added to initiate the reaction. All the incubations were conducted at 37°C for 0.5 h and terminated by adding ice-cold acetonitrile. The concentration of remaining TAA was determined by HPLC-UV (Chilakapati et al., 2005). Five replicates were performed for each of the incubations.

Statistical Analysis

Data are expressed as mean ± SD, and statistically significant differences were assessed with the one-way ANOVA followed by post-hoc analysis (Dunnett's test) in most cases, except for the liver fibrosis scores that were analyzed by a chi-squared test. All statistical analyses were carried out with SPSS software version 16.0 (SPSS, Chicago, USA); the difference was considered statistically significant when the probability value was less than 0.05 ($P<0.05$).

Results

Body and Liver Weight

TAA treatment for 8 weeks caused a significant growth retardation of rats, as evidenced by the significant decrease of final body weight and body weight gains. Although the absolute liver weights were not changed significantly, TAA treatment for 8 weeks induced a significant increase of liver weight index. SLE or DDB treatment exerted a slight ameliorating effect on the body weight loss, but little impact on the increase of liver weight index, induced by TAA (Supplemental Table 1).

Histopathology

Induction of hepatic fibrosis was notable in rats exposed to TAA for 8 weeks, characterized with a fibrosis score of 2.83±0.41. Both SLE and DDB exerted a significant attenuating effect on TAA induced hepatic fibrosis, as evidenced from both histopathological examinations (Supplemental Fig.1) and fibrosis scores (Table 1).

Serum ALT, AST, AP, Albumin, γ-GT, and Total Bilirubin

Typical serum biomarkers of hepatic functions including ALT, AST, AP, ALB, γ-GT, and T-Bil were determined. The results are shown in Fig. 1. Compared with the normal control, the levels of all serum biomarkers except ALB were significantly increased after TAA treatment for 8weeks, indicating a serious hepatic injury caused by TAA. Rats treated with SLE and DDB showed remarkably lower levels of serum biomarkers, compared with those in TAA group.

mRNA levels of UGTs

The mRNA levels for the major rat liver UGTs including UGT1A1, UGT 1A6, and UGT 2B1 were determined by RT-PCR. TAA treatment significantly increased the mRNA levels of all the three UGT isoforms in rat livers, with UGT1A1 increased to 182%, UGT1A6 to 157%, and UGT2B1 to 206% of those in the control group, respectively (Fig.2). It was of interest to find that both hepatoprotective agents SLE and DDB treatment exhibited a significant counteracting effect on the TAA induced adaptive mRNA upregulation of UGTs. The mRNA levels of all the three UGT isoforms in the SLE (400 mg/kg) and DDB treatment group were even restored back to the normal levels (97%-132% of the normal control).

Protein Expression of UGTs

For assessing the protein expression levels of the major rat liver UGTs, a western blotting analysis using the polyclonal antibody of UGT1A1, UGT1A6, and UGT2B was applied in this study. In accordance with the mRNA levels, TAA treatment resulted in a nearly 3-fold increase of the protein levels of all the three UGT isoforms, compared with the normal control (Fig. 3). SLE and DDB treatment significantly counteracted the TAA induced upregulation of UGT protein levels. Both high dose SLE (400 mg/kg) and DDB treatment restored the UGT protein levels back to normal levels (97%-135% of normal control).

To evaluate the regulating effects of SLE and DDB *per se* on UGTs, SLE (400 mg/kg) or DDB (200 mg/kg) was administered intragastrically to healthy rats once a day for 2

weeks and the protein levels of hepatic UGTs were determined by western blotting. As a result, no significant differences were found between the hepatoprotective agents treated groups and the control group, suggesting that SLE and DDB themselves have little direct effect on regulating UGTs (Supplemental Fig.2).

Enzyme Activities of UGTs

Enzyme activities for the major rat UGT isoforms UGT1A1, UGT1A6, and UGT2B1 were determined using the typical substrate bilirubin, 4-MU, and TES, respectively, in pooled liver microsomes incubation systems. The incubation conditions including microsomal protein levels and incubation time were optimized to ensure a linear production of glucuronidation metabolites of each substrate.

In contrast to the UGT mRNA and protein expressions, it was surprising to find that TAA treatment exhibited little effect on the enzyme activities of all the three UGT isoforms tested in the microsomal incubation systems. In addition, neither SLE nor DDB treatment showed significant influence on the enzyme activities of UGTs, when compared with either normal control or TAA group (Fig.4).

The lack of correlation between protein expression and enzyme activity upon TAA intoxication prompted us to hypothesize that TAA may directly inactivate UGTs. To test this hypothesis, TAA (0, 5, 50, and 200 μM) was pre-incubated in liver microsomes with the addition of NADPH for 0.5 h, and then the remaining enzyme activities of UGTs were determined by adding the respective probe substrates as described in the UGT

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activity test. The pre-incubation of TAA without the addition of NADPH showed little effect on UGT activities. In contrast, TAA pre-incubation with NADPH dramatically and concentration-dependently inactivated the enzyme activities of all the three UGT isoforms (Fig.5). Likewise, hydrogen peroxide pretreatment significantly inactivated the enzyme activities of UGTs (Fig.6). Taking the UGT1A6 test as an example, the addition of DDB and GSH partially abolished TAA induced inactivation of enzyme activity (Fig.7).

Acute Effects of TAA on Regulating UGTs in Rats

To observe the acute effects of TAA on regulating UGTs, rats were intraperitoneally injected with a single dose of TAA (200mg/kg). The mRNA levels and enzyme activities of liver microsomal UGTs after six hours TAA treatment were determined. As shown in Fig.8, a single dose of TAA treatment exerted little influence on both the mRNA levels and enzyme activities of most UGTs, except for a slight reduction of UGT2B1 activity.

Effect of DDB and SLE on TAA Disposition in Vitro

To determine whether SLE and DDB could influence TAA disposition, the NADPH dependent metabolic depletion of TAA was determined in microsomal incubation systems in vitro. The influences of DDB and schizandrol A on the metabolic elimination of TAA were very limited. SLE and its lignan component deoxyschizandrin at high concentrations exhibited slight inhibitory effects on the metabolic elimination of TAA in microsomes; however, such a high concentration would be difficult to achieve at in vivo conditions (Fig. 9).

Discussion

As a typical hepatotoxicant, TAA induces a progressive liver fibrosis and cirrhosis in experimental animals mimicking the pathological processes and consequences of liver cirrhosis in human beings. The present study contributes to disclose that the mRNA and protein expression levels of the major UGT isoforms in rats including UGT1A1, UGT 1A6, and UGT 2B1 are adaptively up-regulated in a TAA induced rat liver fibrosis model, whereas the enzyme activities of UGTs remain largely unchanged. The dissociation of protein expression and enzyme activity is potentially attributed to the inactivating effects of TAA upon NADPH dependent bioactivation on UGTs. The hepatoprotective agents SLE and DDB elicit a significant counteracting effect on the adaptive change of UGT expressions, accompanied with powerful hepatoprotective effects as evidenced from the examinations of serum biomarkers and histopathology.

In accordance with previous reports (Karantonis et al., 2010; Shaker et al., 2010), the present study confirmed that TAA treatment resulted in a significant retardation of natural body growth, liver hypertrophy, elevated serum biomarkers of liver function, and the progressive liver fibrosis. As expected, both SLE and DDB treatments exhibited powerful hepatoprotective effects, supported by the significant recovery of most indexes determined. The hepatoprotective effect of SLE (and its lignans components), a well-known herbal medicine not only widely used in China, but also served as a component of Kampo medicines and dietary supplements (Mu *et al.*, 2006), had been previously proven in many other experimental models of liver injury. DDB, an

intermediate produced from the chemical synthesis of schisandrin C, had been clinically

used for more than 30 years in the treatment of viral and chemical hepatitis (Gao and

Kang, 2006; Abdel-Hameid, 2007). The model-independent hepatoprotective effects of

SLE and DDB may be explained by their powerful anti-oxidative effects (Chiu et al.,

2003; Ko and Chiu, 2005).

Although the pathological processes and underlying mechanisms of TAA induced liver

fibrosis/cirrhosis have been extensively studied, little is known about the potential

dysregulation of drug metabolizing enzymes in this model. The present study found that

the major rat UGT isoforms including UGT1A1, UGT1A6, and UGT2B1 were

transcriptionally up-regulated after a consecutive 8 weeks TAA intoxication.

Correspondingly, the protein levels of all the three isoforms were increased in parallel

with the mRNA upregulation. The references in the current literature concerning the

transcriptional regulation of UGTs in liver injury are largely controversial. Some reports

showed that most UGT isoforms were transcriptionally down-regulated in the liver

samples from humans with liver diseases, which was found correlated with inflammation

but not with fibrosis scores (Congiu et al., 2002), and also from the liver samples of mice

treated with lipopolysaccharides or bacterial infections (Richardson et al., 2006).

However, another set of reports witnessed an upregulation of most UGT isoforms in the

cirrhotic human (Debinski et al., 1995) and rat liver samples (Debinski *et al.*, 1996), as

well as in regenerated rat liver tissues after partial hepatectomy (Pellizzer *et al.*, 1996).

Such discrepancies across different researches may be explained by the diverse severity

and property of liver diseases, various pathological factors, and different animal models

applied. The large individual variability of UGT isoforms in human beings caused by potential genetic and environmental factors, and the exposures to drugs and dietary components, further complicated the determination of liver injury *per se* on regulating UGTs.

It is important to note that most previous studies concerning UGT regulations in liver injury have not performed a concomitant assay of mRNA, protein, and enzyme activity, which may constitute another cause for the controversial results obtained in different researches. We found that, in spite of a 2-3-fold upregulation of mRNA and protein levels after 8 weeks TAA treatment, the enzyme activities of all the three UGT isoforms remain unchanged. Similar phenomenon had been found in a previous study, in which the influence of octachlorostyrene treatment on inducing UGT mRNA levels was much more prominent than that on the glucuronidation activity towards 1-naphthol (Yanagiba *et al.*, 2009). It is now well known that TAA is bioactivated mainly by CYP2E1 to produce reactive intermediates and the metabolite di-S-oxide, which is highly reactive against macromolecules by covalent binding and/or oxidative modifications (Kang *et al.*, 2008). We hypothesized that the enzyme activity of UGTs may be inactivated by the reactive metabolites and/or reactive oxygen species generated from the process of TAA bioactivation. To test this hypothesis, microsomes obtained from healthy rats were pre-incubated with either TAA or hydrogen peroxide for 0.5 h, and then the remaining enzyme activities of UGTs were determined. The results confirmed that the enzyme activity of all the three UGT isoforms was dramatically inactivated by TAA treatment, providing a good explanation on TAA induced unparallel change between the expressions

and activities of UGTs. It is important to note that the inactivating effect of TAA on the enzyme activities of UGTs is NADPH dependent, suggesting that the metabolic bioactivation is necessary for TAA to inactivate UGTs. Hydrogen peroxide can also inactivate the enzyme activities of UGTs; however, a relatively high concentration is necessary to take effect. This evidence suggests that the reactive metabolites produced from TAA bioactivation may play an important role on inactivating UGTs. However, the contribution of TAA induced oxidative stress cannot be excluded, considering that both GSH and DDB pretreatment partially abolished TAA induced inactivation of UGTs. Since DDB has little effect on the metabolic depletion of TAA in liver microsomes (Fig.9), its effect on protecting against TAA inactivating effect on UGTs is unlikely resulted from its influence on TAA bioactivation. It deserves further research to determine whether such effects of DDB and GSH are attributed to their anti-oxidant activities and/or capacities of trapping TAA reactive metabolites.

The potential influences of single dose treatment of TAA to rats were further evaluated to determine whether the chronic TAA intoxication induced transcriptional upregulation of UGTs was associated with the liver fibrosis progression or merely an acute effect of TAA. The results showed that single dose treatment of TAA had little effect on regulating both the mRNA levels and enzyme activities of UGTs. It seems controversial between the in vivo and in vitro assay in terms of the inactivating effect of TAA on UGTs. However, it would be understandable considering that some in vivo protective machinery such as antioxidant and the trapping of reactive metabolites might prevent the enzyme from inactivation by a single dose of TAA treatment. Together, these results suggest that the

transcriptional upregulation of UGTs after chronic TAA treatment is more likely an adaptive response from the progression of liver fibrosis, and that repeated doses of TAA intoxication may be necessary to inactivate UGTs in vivo. The major limitation of this study is that the underlying molecular mechanisms of TAA induced upregulation of UGTs have not been addressed. TAA induced liver injury was typically characterized with oxidative stress and lipid peroxidation (Low et al., 2004; Natarajan et al., 2006; Aller et al., 2008), and the antioxidant treatment was very effective to prevent TAA induced liver injury (Hyoudou et al., 2007; Tsai et al., 2010). In addition, we found in the present study that SLE and DDB, both proven as good antioxidants, could significantly attenuate TAA induced liver injury and transcriptional upregulation of UGTs. These lines of evidence suggest that TAA triggered oxidative stress might be an underlying factor contributing to the transcriptional upregulation of UGTs. Therefore, it will be of interest to determine in future studies whether Nrf2, an oxidative stress responsive transcription factor, is involved in TAA induced upregulation of UGTs, considering that some recent reports documented an important role of Nrf2 on regulating UGTs (Enomoto et al., 2001; Umemura et al., 2006; Yeager et al., 2009).

In conclusion, TAA induced liver fibrosis leads to a transcriptional upregulation of UGTs; however, the enzyme activities of UGTs remain largely unchanged. The dissociation between enzyme expression and activity is possibly attributed to the UGT inactivating effects of TAA upon bioactivation. Both SLE and DDB can largely abolish TAA induced transcriptional upregulation of UGTs, in spite of both the hepatoprotective agents themselves possessing little effect on regulating UGTs. Results obtained from the present

study may provide novel explanations on the preserved UGT activities in liver injury, and

may shed a light on understanding the influence of hepatoprotective agents on regulating

UGTs in liver injury.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Hao, Wang

Conducted experiments: Hao, Zhang, Jiang, Sun, Xie, Zhou

Performed data analysis: Hao, Zhang, Jiang, Gong

Wrote or contributed to the writing of the manuscript: Hao, Zhang, Jiang, Wang

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Footnotes

H.H and L.Z contributed equally to this work.

Legends for Figures

Fig. 1. Biochemical parameters of liver function after 8 weeks TAA intoxications. The serum level of T-Bil, ALT, AST, AP, γ-GT, and ALB for control group is of 2.96±0.25 μmol/L, 44.40±3.21 IU/L, 149.25±18.01 IU/L, 191.80±10.66 U/L, 1.20±0.45 U/L, and 19.80±0.84 g/L, respectively. Data are expressed as percentage (%) of control (mean ± SD, n=6). * $P < 0.05$, ** $P < 0.01$, compared with the control group; △ $P < 0.05$, △△ $P < 0.01$, compared with TAA group.

Fig. 2. RT-PCR analysis of the mRNA levels of UGT1A1, UGT1A6, and UGT2B1. The mRNA levels of UGTs were normalized to that of GADPH in the same preparation and quantitated by densitometry. Data are expressed as percentage (%) of control, and bars represent mean ± SD of six individual liver samples. * $P < 0.05$, ** $P < 0.01$, compared with the control group; △ $P < 0.05$, △△ $P < 0.01$, compared with TAA group.

Fig. 3. Immunoblot analysis of the protein levels of UGT1A1, UGT1A6, and UGT2B1. The protein levels of UGTs were normalized to that of β-actin in the same preparation and quantitated by densitometry. Each of the UGTs protein blots is a representative of six liver samples. Densitometry data are expressed as percentage (%) of the control, and bars represent mean ± SD of six liver samples. * $P < 0.05$, ** $P < 0.01$, compared with the

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control group; △ P < 0.05, △△ P < 0.01, compared with TAA group.

Fig. 4. Enzyme activities of UGT1A1, UGT1A6, and UGT2B1. Bilirubin (320 μM), 4-MU (500 μM), and TES (75 μM) was used as the typical substrate for UGT1A1, UGT1A6, and UGT2B1, respectively. Data are expressed as percentage (%) of control; bars represent mean ± SD of five replicates. * P<0.05, ** P <0.01, compared with the control group; △ P < 0.05, △△ P < 0.01, compared with TAA group.
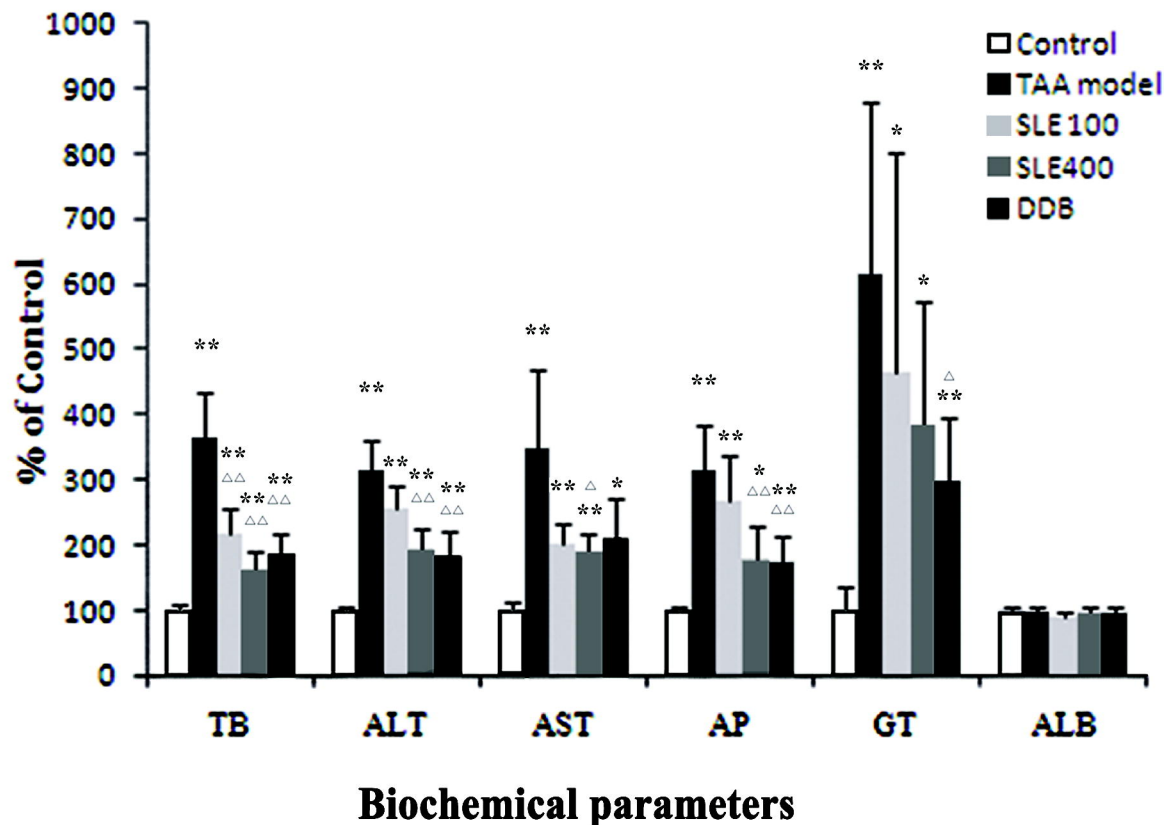
Fig. 5. The inactivating effects of TAA on the enzyme activities of UGT1A1, UGT1A6, and UGT2B1. TAA was pre-incubated in liver microsomes with the addition of NADPH regenerating system for 0.5 h before the enzyme activity assay of UGTs. The pre-incubation of TAA without the addition of NADPH had little effect on UGT activities. Data are expressed as percentage (%) of control, and bars represent mean ± SD of five incubations. * P<0.05, ** P <0.01, compared with the control group.

Fig. 6. The inactivating effects of hydrogen peroxide on UGT1A1, UGT1A6, and UGT2B1. Hydrogen peroxide (200 μM, 1mM, and 5 mM) was pre-incubated with microsomes for 0.5 h before the enzyme activity test of UGTs. Data are expressed as percentage (%) of control; bars represent mean ± SD five incubations. * P<0.05, ** P <0.01, compared with the control group.

Fig. 7. The protective effects of DDB and GSH against the inactivating effect of TAA on

UGT1A6. NADPH regenerating system was included in all the groups of samples except

TAA 200 μM (-) group. Data are expressed as mean ± SD of five incubations. * P<0.05,

** P <0.01, compared with TAA 200 μM (-) group; △△ P <0.01, compared with TAA 200

μM (+) group.

Fig. 8. Acute effects of TAA on regulating mRNA and enzyme activities of UGTs in rats.

a, mRNA levels of UGT1A1, UGT1A6, and UGT2B1. The mRNA levels of UGTs were

normalized to that of GADPH in the same preparation and quantitated by densitometry. b,

enzyme activities of UGTs. Data are expressed as percentage (%) of control; bars

represent mean ± SD of triplicate samples. * P<0.05, ** P <0.01, compared with the

control group.

Fig. 9. Effects of DDB and SLE and its major components on the metabolic depletion of

TAA in microsomal incubation systems. TAA (0.1mM) was incubated in liver micrsomes

incubation systems containing NADPH regenerating system for 0.5 h at 37 °C. Velocity

of TAA metabolic depletion was calculated by measuring the loss of TAA in the

incubation media. Data are expressed as mean ± SD of five incubations. *P<0.05,

compared with control incubations (without the addition of hepatoprotective agents).

Table 1. Liver fibrosis scores of rats with TAA intoxication for 8 weeks

Groups	Fibrosis grading scores (number of rats)					Mean ± SD
	0	1	2	3	4	
TAA	0	0	1	5	0	2.83±0.41
SLE 100	0	1	3	2	0	2.17±0.75
SLE 400	0	3	1	2	0	1.83±0.98△
DDB	0	2	3	1	0	1.83±0.75△

The extent of fibrosis was graded according to the semi-quantitative METAVIR scoring system and scored as 0, 1, 2, 3, and 4, respectively. The liver fibrosis for rats of normal control was graded as zero. △$P < 0.05$, compared with TAA group.

Fig. 1

Fig. 2

a

b

Fig. 3

a

	Control	TAA	SLE100	SLE400	DDB

54 kDa bilirubin UGT (UGT1A1)

53 kDa phenol UGT (UGT1A6)
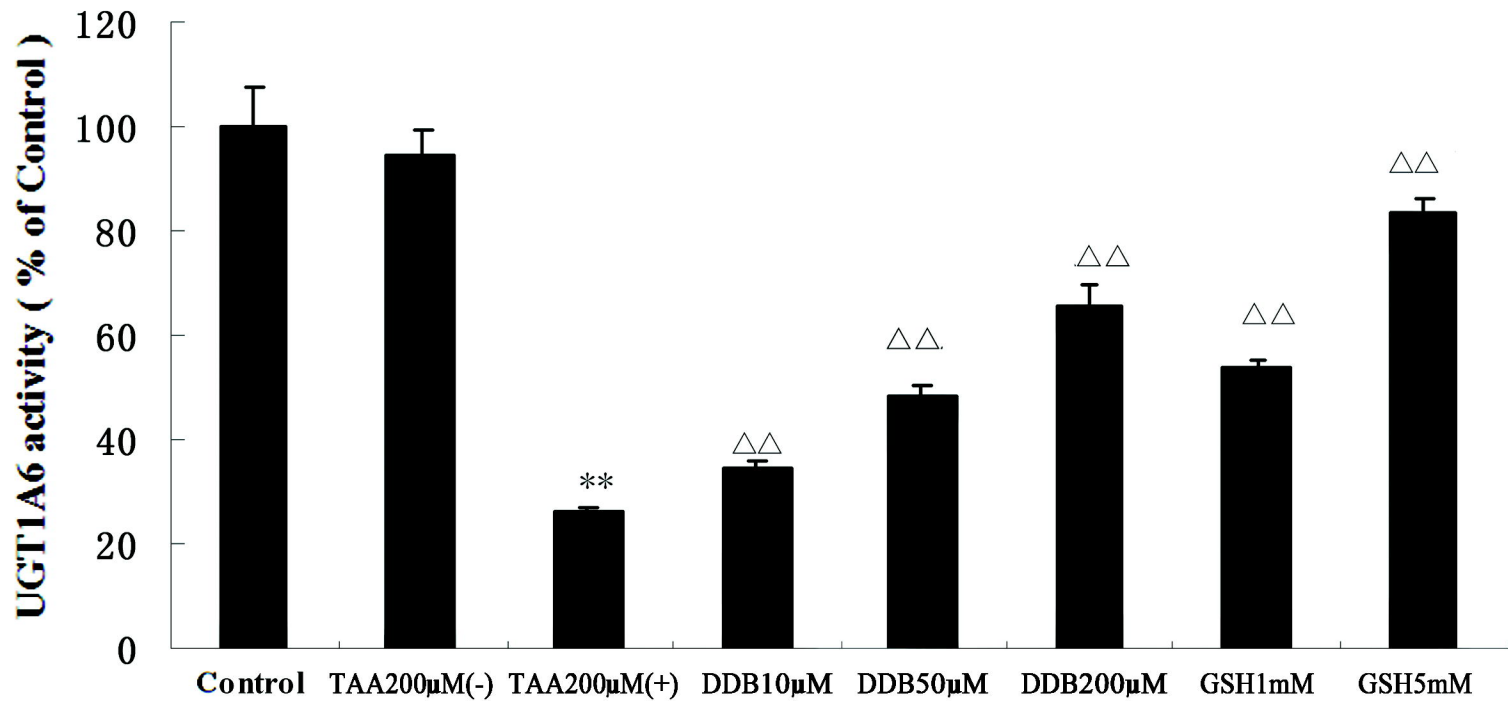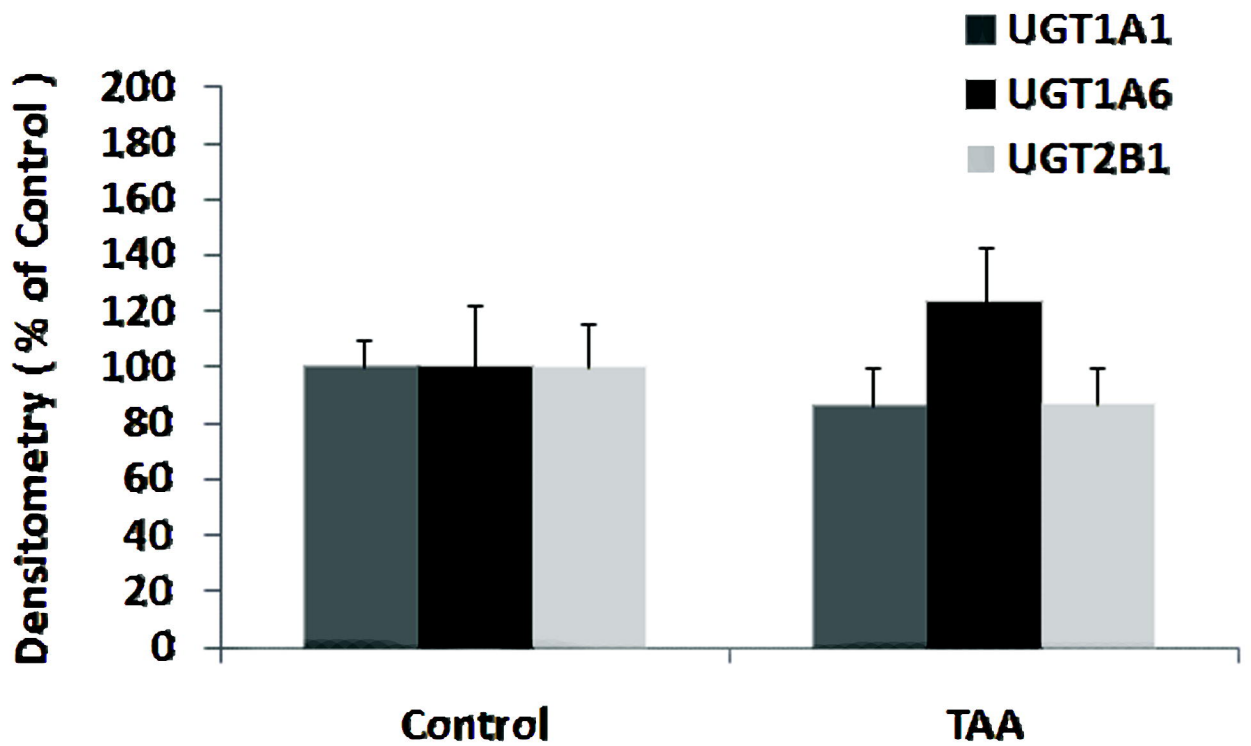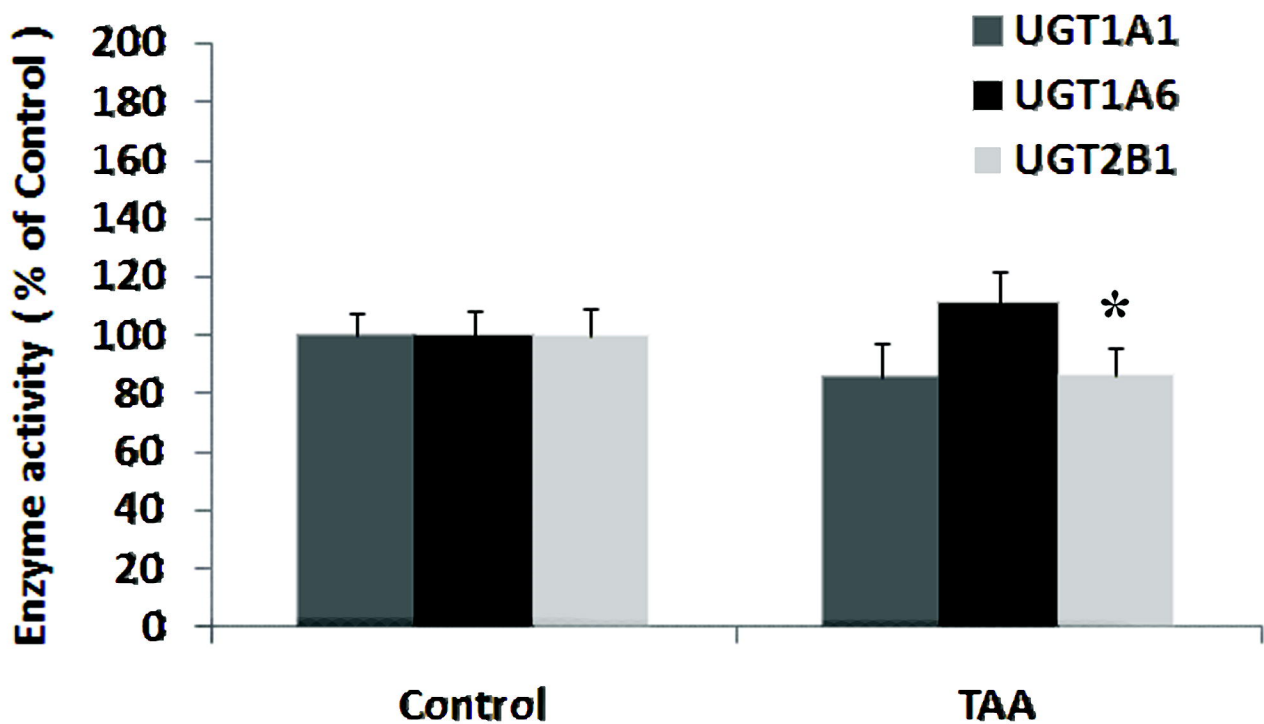
50-52 kDa isoforms of UGT2B family

43 kDa β-actin
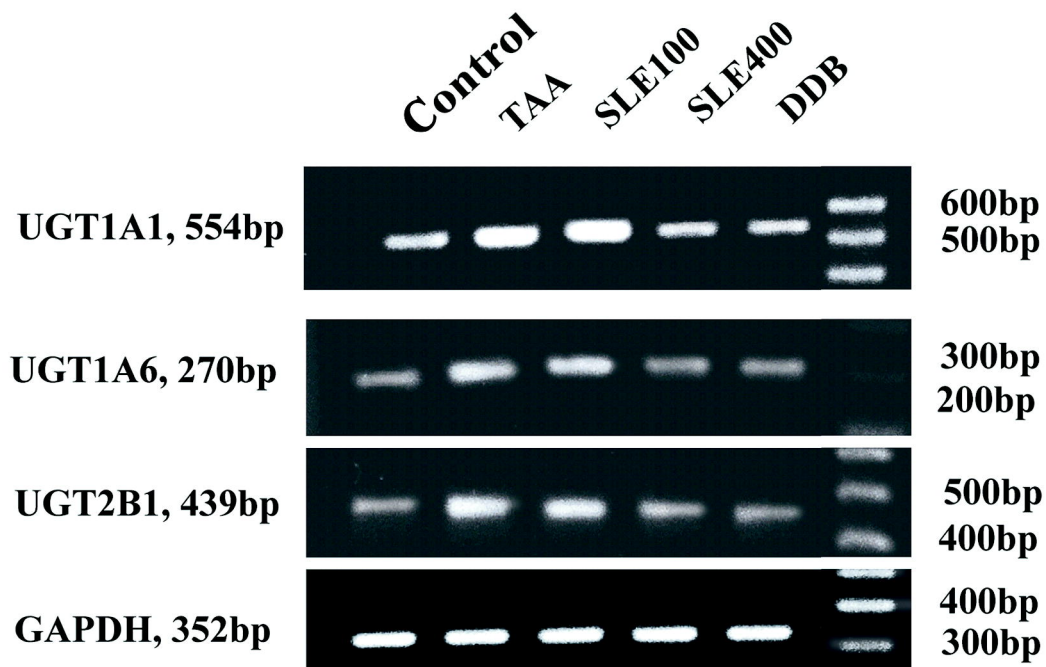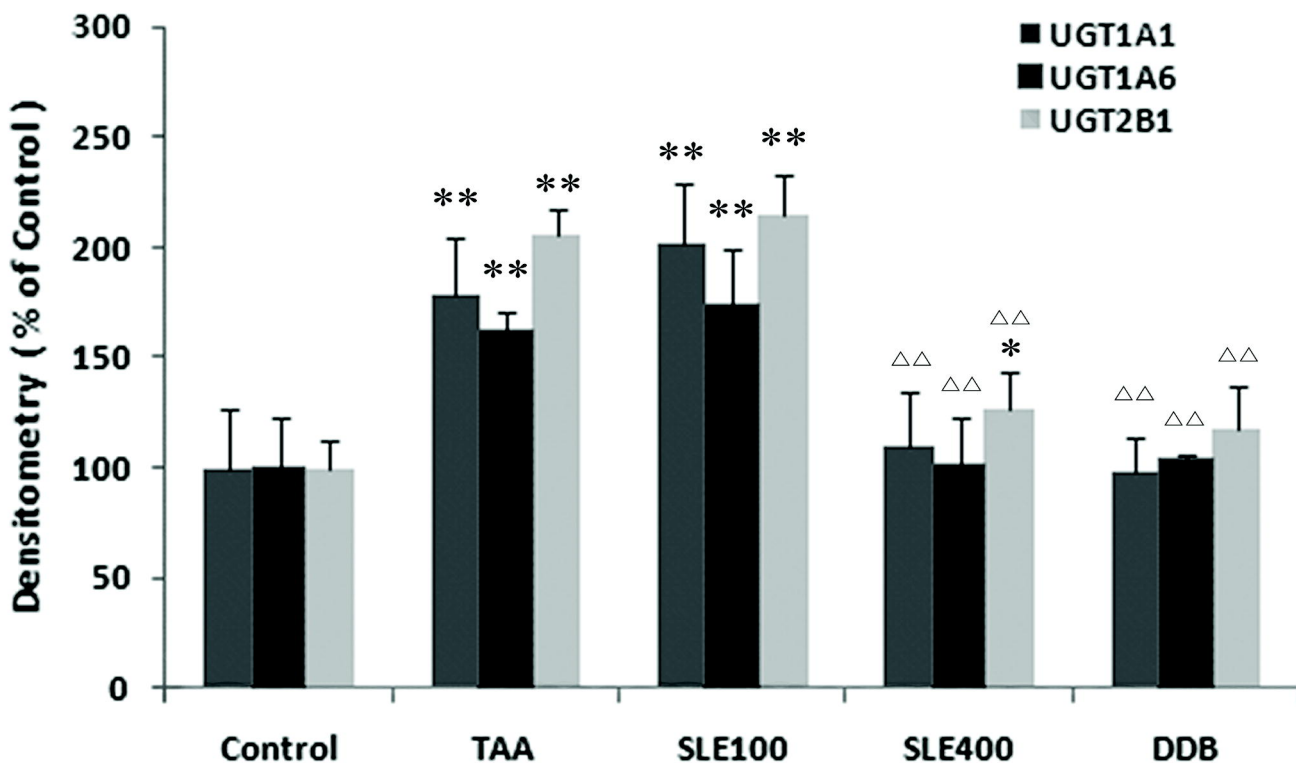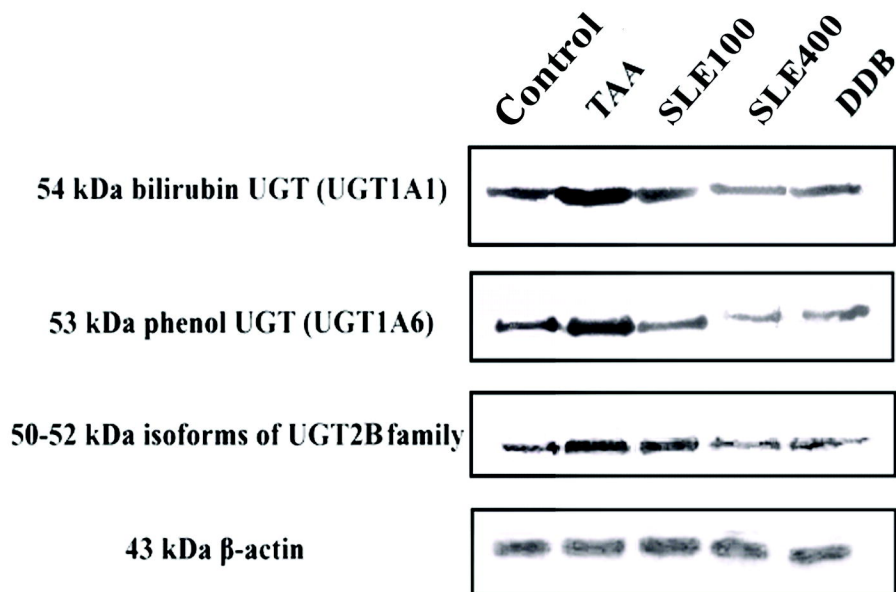
b
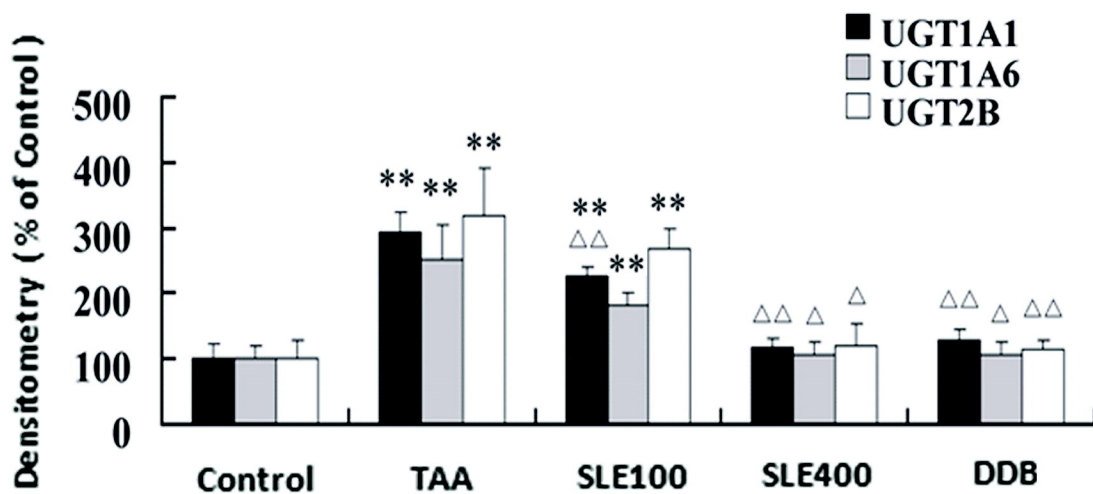
Fig. 4
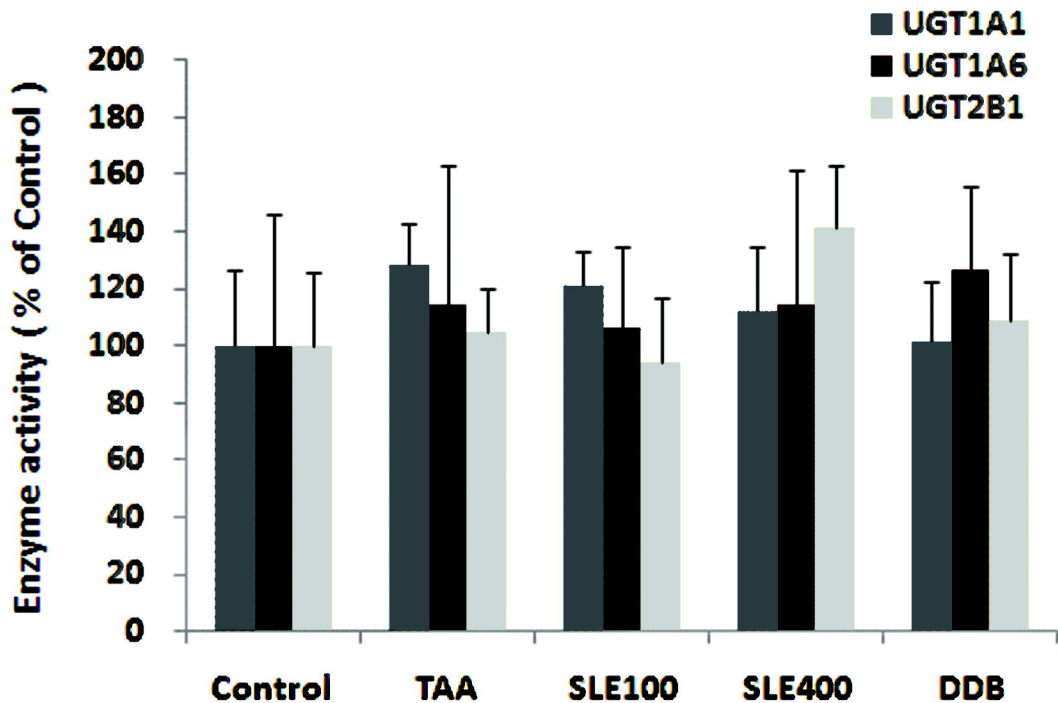
Fig. 5

Fig. 6

Fig. 7

Fig. 8

a

b

Fig. 9

a

b