Carbamazepine-induced liver injury requires CYP3A-mediated metabolism and glutathione depletion in rats

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Abbreviations: ALT, alanine aminotransferase; APAP, acetaminophen; AST, aspartate aminotransferase; BSO, L-buthionine sulfoximine; CBZ, carbamazepine; CV, central vein; CYP, cytochrome P450; DEX, dexamethasone; G6P, glucose 6-phosphate; G6PDH, glucose 6-phosphate dehydrogenase; H&E, hematoxylin and eosin; HPLC, high-performance liquid chromatography; KTZ, ketoconazole; MDZ, midazolam; NAC, N-acetyl cysteine; NADPH-GS, nicotine adenine dinucleotide phosphate-generating system; NAPQI, N-acetyl-p-benzoquinoneimine; OXC, oxcarbazepine; RLM, rat liver microsomes; TAO,
troleandomycin; TOL, tolbutamide
ABSTRACT

Carbamazepine (CBZ) is widely used as an antiepileptic agent and causes rare but severe liver injury in humans. It has been generally recognized that reactive metabolites formed via the metabolic activation reaction contribute to the onset of liver injuries in several drugs. However, the role of CBZ metabolism in the development of liver injury is not fully understood. In this study, we developed a novel rat model of CBZ-induced liver injury and attempted to elucidate the associated mechanisms by focusing on the metabolism of CBZ. The repeated-administration of CBZ for five days in combination with L-buthionine sulfoximine (BSO), a glutathione (GSH) synthesis inhibitor, resulted in increases in the plasma alanine aminotransferase (ALT) levels and centrilobular necrosis in the liver that were observed at various degrees. The CBZ and 2-hydroxy-CBZ concentrations in the plasma after the last CBZ administration were lower in the rats with high plasma ALT levels compared with those with normal plasma ALT levels, showing the possibility that the further metabolism of CBZ and/or 2-hydroxy-CBZ is associated with the liver injury. Although a single administration of CBZ did not affect the plasma ALT levels, even when co-treated with BSO, pretreatment with dexamethasone, a CYP3A inducer, increased the plasma ALT levels. In addition, the rats co-treated with troleandomycin or ketoconazole, CYP3A inhibitors, suppressed the increased plasma ALT levels. In conclusion, reactive metabolite(s) of CBZ produced by CYP3A under the GSH-depleted condition might be involved in the development of liver injury in rats.
Introduction

Drug-induced liver injury is a potential complication with many drugs, and it is the most frequent reason for the withdrawal of an approved drug from the market (Björnsson and Olsson, 2005; Lee, 2003). More than the 600 drugs on the market have been associated with hepatotoxicity (Park et al., 2005). Carbamazepine (CBZ), an antiepileptic drug, is widely used for the treatment of partial seizures (Beghi and Perucca, 1995). However, CBZ is associated with idiosyncratic adverse effects, such as liver injury, aplastic anemia and agranulocytosis (Zaccara et al., 2007; Björnsson and Olsson, 2005). It is reported that patients who developed CBZ-induced serious liver injury were under drug treatment for an average of 30 weeks (Björnsson, 2008), suggesting that long-term CBZ treatment is a risk factor. It is known that long-term treatment with some drugs results in the induction of drug-metabolizing enzymes, such as cytochrome P450 (CYP) 3A4 (Oscarson et al., 2006). In fact, CBZ is known to induce CYP3A and/or other CYPs in humans and rats (Oscarson et al., 2006; Tateishi et al., 1999). However, the relationship between CYPs induction and the development of CBZ-induced liver injury is not fully understood.

It is generally recognized that reactive metabolites formed by hepatic drug-metabolizing enzymes, such as CYP enzymes, have chemical reactivity to endogenous proteins, and are thought in many cases to be the cause of idiosyncratic reactions (Park et al., 2005). Moreover, reactive metabolites that are soft electrophiles can react with soft nucleophiles, such as thiol molecules (e.g., glutathione (GSH), cysteine). For example, N-acetyl-p-benzoquinoneimine (NAPQI), which is known to be an electrophilic reactive metabolite of acetaminophen (APAP), is scavenged by GSH (Kaplowitz, 2005; Park et al., 2005). In the case of CBZ, previous reports showed that GSH or N-acetyl cysteine (NAC)
significantly decreased the irreversible binding of \[^{14}C\]CBZ to human and mouse liver microsomes (Pirmohamed et al., 1992b; Lillibridge et al., 1996). In addition, it was reported that an autoantibody directed against protein(s) with a molecular weight of 94 kDa in the liver was detected in the serum of patients who developed CBZ-induced liver injury (Pirmohamed et al., 1992a). Considering these studies, it was suggested that the generation of reactive metabolites, followed by adduct formation with endogenous proteins, is critical for the development of CBZ-induced liver injury.

Proposed metabolic pathways of CBZ in humans are described in Fig. 1. CBZ is mainly metabolized to CBZ-10,11-epoxide, which is a pharmacologically active metabolite, by CYP3A4 and CYP2C8 (Kerr et al., 1994). CBZ-10,11-epoxide is further metabolized to \textit{trans}-10,11-dihydroxy-CBZ by microsomal epoxide hydrolase (Mather and Levy, 2000). In addition, CBZ is metabolized to 2-hydroxy-CBZ and 3-hydroxy-CBZ by several CYPs, including CYP3A4 and CYP2C9 (Pearce et al., 2002). These hydroxyl metabolites are candidates to be the precursors of the reactive metabolites from the following viewpoints: 1) 2-Hydroxy-CBZ and/or 3-hydroxy-CBZ can be further metabolized to 2,3-dihydroxy-CBZ (Lertratanangkoon and Horning, 1982), which is proposed to form an \textit{o}-quinone metabolite via nonenzymatic rearrangement (Pearce et al., 2002). 2) 2-Hydroxy-CBZ is metabolized by CYP3A4 to 2-hydroxyiminostilbene (Pearce et al., 2005), which can be readily oxidized to an iminoquinone species (Ju and Uetrecht, 1999).

It is recognized that idiosyncratic drug reactions are caused by reactive metabolites and inflammatory reactions (Uetrecht, 2007). Some researchers have established animal models of drug induced-liver injury, such as trovafloxacin and sulindac, using lipopolysaccharide to study immune-mediated mechanisms (Shaw et al., 2007, Zou et al., 2009). In addition, it has been reported that GSH-depleted animal models using L-buthionine...
sulfoximine (BSO), which is an inhibitor of GSH synthesis, are useful in sensitization to liver injury induced by drugs via reactive metabolite formations, such as methimazole, tienilic acid, amodiaquine, and ticlopidine (Kobayashi et al., 2012a; Nishiya et al., 2008; Shimizu et al., 2009 and 2011).

Information about species differences in the mechanisms of drug-induced toxicity would prompt our understanding for the risk assessment of drug candidates in preclinical development (Bollard et al., 2005). Previously, we established the mouse model of CBZ-induced liver injury and suggested the relationship between 3-hydroxy-CBZ and hepatotoxicity (Higuchi et al., 2012). In the present study, to expand our knowledge about the role of CBZ metabolism on liver injury, we attempted to develop a novel rat model of CBZ-induced liver injury. Based on the present results, we discussed the different mechanisms of CBZ-induced liver injury between the rat and mouse.
Materials and Methods

Materials. CBZ, BSO, troleandomycin (TAO), tolbutamide (TOL), and dexamethasone (DEX) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Oxcarbazepine (OXC) and ketoconazole (KTZ) were purchased from LKT Laboratories (St Paul, MN). 4-Hydroxy-TOL was purchased from Cayman Chemical (Ann Arbor, MI). 2-Hydroxy-CBZ and 3-hydroxy-CBZ were purchased from Toronto Research Chemicals (Toronto, Canada). CBZ-10,11-epoxide and trans-10,11-dihydroxy-CBZ were kindly provided by Novartis Pharma Inc. (Basel, Switzerland). Midazolam (MDZ), 4-hydroxy-MDZ, and 1'-hydroxy-MDZ were kindly provided by Roche Diagnostic Japan (Tokyo, Japan). Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), and β-nicotinamide adenine dinucleotide phosphate oxidized form (NADP+) were purchased from Oriental Yeast (Tokyo, Japan). Fuji DRI-CHEM slides of GPT/ALT-PIII and GOT/AST-PIII used to measure plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST), respectively, were purchased from Fuji Film (Tokyo, Japan). All other chemicals used in this study were of the highest or analytical quality that could be obtained commercially.

Animals. Male F344 rats (9-week old, 170-190 g) were obtained from Japan SLC (Hamamatsu, Japan). The rats were housed in the institutional animal facility in a controlled environment (temperature 23 ± 1°C, humidity 50 ± 10%, and 12-h light/12-h dark cycle) with access to food and water ad libitum. The rats were acclimated prior to their use in our experiments. Animal maintenance and treatment were conducted in accordance with the National Institutes of Health Guide for Animal Welfare of Japan, and the protocols were approved by the Institutional Animal Care and Use Committee of Kanazawa University,
Japan.

**CBZ and OXC administration.** It is reported that 344 rats are more sensitive to liver injury by several chemicals, such as 1,2-dichlorobenzene, carbon tetrachloride and cadmium, compared to SD rats (Stine et al., 1991; Steup et al., 1991; Harstad and Klaassen, 2002). Therefore, F344 rats were used in the present study. In the single administration study, rats were orally administered CBZ (dissolved in corn oil, 10 ml/kg) at a dose of 400 or 600 mg/kg, and BSO (dissolved in saline, 10 ml/kg) was intraperitoneally injected at a dose of 700 mg/kg 2 h before the CBZ administration. The rats were sacrificed at 24 h after the CBZ administration, and the plasma ALT levels were measured.

In the repeated administration study, rats were administered CBZ at a dose of 400 mg/kg once daily for 4 days and at a dose of 400, 600 or 800 mg/kg on the 5th day, and BSO was injected at a dose of 700 mg/kg 2 h before the last CBZ administration. The dosing regimen [CBZ (400 mg/kg) for 4 days, and CBZ (600 mg/kg) and BSO on the 5th day] is termed “Method A” in the following studies. As a negative control, rats were administered OXC following Method A. Plasma samples were collected for the measurement of plasma ALT and AST levels (at 0, 6, 12, 24, 48 and 72 h after the last CBZ administration) and plasma concentrations of CBZ and its metabolites (at 0, 1, 3, 6, 12 and 24 h). Livers were collected 24 h after the last CBZ administration, and a portion of each excised liver was fixed in 10% formalin neutral buffer solution and used for immunohistochemical staining. The degree of liver injury was assessed by histopathological staining with hematoxylin and eosin (H&E) and the plasma ALT and AST levels.

**Measurement of hepatic GSH contents.** Rats were divided into 4 groups according to the
dosing regimen as follows: (1) CBZ (400 mg/kg) for 4 days, and saline on the 5th day; (2) CBZ (400 mg/kg) for 4 days, and CBZ (600 mg/kg) on the 5th day; (3) CBZ (400 mg/kg) for 4 days, and BSO on the 5th day; (4) CBZ (400 mg/kg) for 4 days, and CBZ (600 mg/kg) and BSO on the 5th day (Method A). Livers were collected 6 h after the BSO treatment, which was the same time as 4 h after the last CBZ administration. As a control, rats were administered corn oil for 4 days, and liver samples were collected on the 5th day. The rat livers were homogenized in ice-cold 5% sulfosalicylic acid and centrifuged at 8,000g for 10 min. Total GSH and GSSG concentrations in the supernatant were measured as previously described (Tietze, 1969). The GSH contents were calculated from the difference between the total GSH and the GSSG concentrations.

**Plasma concentrations of CBZ and its metabolites.** The plasma concentrations of CBZ, CBZ-10,11-epoxide, *trans*-10,11-dihydroxy-CBZ, 2-hydroxy-CBZ and 3-hydroxy-CBZ were measured by high-performance liquid chromatography (HPLC). Plasma (50 μl) was transferred to a glass tube containing 10 μl of internal standard solution (bromazepam, 100 μM in mobile phase), 50 μl of mobile phase (20% acetonitrile) and 50 μl of water. The mixture was extracted with 5 ml of chloroform on a horizontal shaker for 5 min. After centrifugation at 570g for 10 min, the organic phase was transferred to a clean tube and evaporated under nitrogen flow at 40°C. The residue was dissolved in 100 μl of mobile phase (20% acetonitrile), and a 50-μl portion of the resulting solution was subjected to HPLC. The HPLC analysis was performed using an L-2130 pump (Hitachi, Tokyo, Japan), an L-2200 autosampler (Hitachi), an L-2400 UV detector (Hitachi), and a D-2500 chromatointegrator (Hitachi) equipped with a Cosmosil C18-MS-II column (5-μm particle size, 4.6 mm i.d. × 250 mm: Nacalai Tesque, Kyoto, Japan). The eluent was monitored at 235 nm with a noise-base
clean Uni-3 (Union, Gunma, Japan), which can reduce the noise by integrating the output and increase the signal 3-fold by differentiating the output and 5-fold by further amplification with an internal amplifier, resulting in a maximum 15-fold amplification of the original signal. The flow rate of the mobile phase was 1.0 ml/min. The column temperature was set at 35°C. The quantification of CBZ and its metabolites was performed by comparing the HPLC peak heights with that of an authentic standard.

**Preparation of rat liver microsomes (RLM).** RLM were prepared according to our previous study (Kobayashi et al., 2012b). The protein concentrations were determined according to the method of Bradford (Bradford, 1976) using \( \gamma \)-globulin as the standard.

**Immunoblot analysis.** SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to our previous study (Kobayashi et al., 2012b). RLM (10 \( \mu \)g) were separated on 7.5% polyacrylamide gels and electrotransferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA). The membrane was probed with monoclonal goat anti-rat CYP3A2 serum (Sekisui Medical, Ibaraki, Japan) and the corresponding fluorescent dye-conjugated secondary antibody. An Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) was used for the detection of protein bands and to quantitate the band intensities. The relative expression level of each protein band was determined by the intensity of the band. Immunoblot analysis was performed across the linear range of band intensity with respect to the amount of protein.

**MDZ hydroxylase activity.** MDZ 4- and 1’-hydroxylase activities were assessed as marker activities for rat CYP3A and CYP3A/CYP2C, respectively (Kotegawa et al., 2002). The
activities were determined according to a previous study (Emoto et al., 2000) as follows: a typical incubation mixture (final volume of 0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4), RLM (0.1 mg/ml) and MDZ (2.5 μM) dissolved in dimethyl sulfoxide (≤ 1% v/v final concentration). In a preliminary study, we confirmed that the MDZ 4- and 1’-hydroxylase activities were linear with respect to protein concentration (≤ 0.4 mg/ml) and incubation time (≤ 15 min). The reaction was initiated by the addition of a nicotine adenine dinucleotide phosphate-generating system (NADPH-GS) after preincubation at 37°C for 2 min. After a 10-min incubation, the reactions were terminated by the addition of 200 μl of ice-cold methanol. After removal of the protein by centrifugation at 20,380 g for 5 min, an 80-μl portion of the supernatant was subjected to HPLC. The HPLC apparatus was the same as described above, except that the column was a Cosmosil Cholesterol (5-μm particle size, 4.6 mm i.d. × 150 mm: Nacalai Tesque). The eluent was monitored at 220 nm. The mobile phase was 30% methanol/20% acetonitrile/5 mM potassium phosphate buffer (pH 7.4). The flow rate was 1.0 ml/min. The column temperature was set at 35°C. The quantification of 4-hydroxy-MDZ and 1’-hydroxy-MDZ was performed by comparing the HPLC peak heights with that of an authentic standard.

**Treatment with the CYP3A inducer.** A previous report showed that intraperitoneal treatment with DEX at a dose of 80 mg/kg once daily for 3 days to male F344 rats resulted in a 7.4-fold increase in testosterone 6β-hydroxylase activity, which is catalyzed by CYP3A1/2, compared with vehicle treatment (Gardner et al., 1997). The induction protocol from that report was followed, briefly, rats were intraperitoneally injected with DEX (dissolved in corn oil, 10 ml/kg) at a dose of 80 mg/kg for 3 days and administered CBZ at a dose of 400 mg/kg on the 4th day. BSO was intraperitoneally injected in the rats at a dose of 700 mg/kg 2 h before the
CBZ administration. The plasma samples were collected at 0, 3, 6 and 12 h after the CBZ administration.

**Treatment with CYP3A or CYP3A/CYP2C inhibitors.** One and a half hours before the last CBZ administration in Method A, TAO (300 mg/kg dissolved in corn oil, 10 ml/kg) or KTZ (50 mg/kg dissolved in corn oil, 10 ml/kg) was intraperitoneally injected into the rats. Plasma samples were collected 0, 1, 3 (for analysis of the plasma concentration of CBZ and its metabolites) or 24 h (for measurement of the ALT levels) after the last CBZ administration. Livers were collected 24 h after the last CBZ administration for immunohistochemical staining and 1.5 h after the KTZ treatment to examine inhibitory effects of KTZ on CYP3A and CYP2C enzyme activities.

**TOL hydroxylase activity.** TOL hydroxylase activity was assessed as a marker activity for rat CYP2C11 (Wang et al., 2010). The activity was determined as follows: a typical incubation mixture (final volume of 0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4), RLM (0.2 mg/ml) and TOL (500 μM) dissolved in methanol (≤1% v/v final concentration). In a preliminary study, we confirmed that the TOL hydroxylase activity was linear with respect to protein concentration (≤0.8 mg/ml) and incubation time (≤60 min). The reactions were initiated by the addition of an NADPH-GS after preincubation at 37°C for 2 min. After a 45-min incubation, the reactions were terminated by the addition of 10 μl of perchloric acid (60%). After removal of the protein by centrifugation at 20,380g for 5 min, a 50-μl portion of the supernatant was subjected to HPLC. The HPLC apparatus was the same as described above, except that the column was a Cosmosil Cholesterol (5-μm particle size, 4.6 mm i.d. × 150 mm: Nacalai Tesque). The eluent was monitored at 230 nm. The mobile phase was 18%
acetonitrile/0.04% phosphoric acid. The flow rate was 1.0 ml/min. The column temperature was set at 35°C. The quantification of 4-hydroxy-TOL was performed by comparing the HPLC peak heights with that of an authentic standard.

**Statistical analyses.** The statistical analyses of multiple groups were performed using a one-way ANOVA with the Dunnett’s *post hoc* test or Tukey’s *post-hoc* test to determine the significance of the differences between the individual groups. Comparisons between two groups were carried out using a two-tailed Student’s *t*-test. A value of *P* < 0.05 was considered statistically significant.
Results

Establishment and Evaluation of a CBZ-Induced Liver Injury Rat Model. To establish a rat model for CBZ-induced liver injury, we first performed the single administration study. Rats were orally administered CBZ at a dose of 400 or 600 mg/kg, then blood samples were collected 24 h later to measure the plasma ALT levels, resulting in no change in the plasma ALT levels (Fig. 2A). Many researchers have applied GSH-depleted animal models using BSO to evaluate the hepatotoxic potential of several drugs (Kobayashi et al., 2012a; Nishiya et al., 2008; Shimizu et al., 2009, 2011). Therefore, BSO (700 mg/kg) was intraperitoneally injected 2 h prior to the oral CBZ administration, however the plasma ALT levels were not increased (Fig. 2A). The mortality of the rats within 24 h after single administrations of CBZ were 0% (CBZ 400 mg/kg and saline), 0% (CBZ 400 mg/kg and BSO), 71% (CBZ 600 mg/kg and saline) and 75% (CBZ 600 mg/kg and BSO), suggesting that the pharmacological activity of CBZ led to the high mortality in rats administered CBZ at a dose of 600 mg/kg.

Subsequently, we conducted a CBZ-repeated administration study. The administration method was determined by reference to our previous mouse study of CBZ-induced liver injury (Higuchi et al., 2012). Rats were orally administered CBZ at a dose of 400 mg/kg once daily for 4 days, then 400, 600 or 800 mg/kg on the 5th day. BSO at a dose of 700 mg/kg was injected 2 h prior to the last CBZ administration. At 24 h after the last CBZ or vehicle (corn oil) administration, mice co-treated with BSO showed the significant higher plasma ALT levels in the groups treated with 600 mg/kg (ALT = 9,986 ± 5,627 U/l, n = 6) or 800 mg/kg (ALT = 20,700 ± 2,938 U/l, n = 3) of CBZ compared with the vehicle-treated group (Fig. 2B). However, without BSO treatment, no increases in the plasma ALT levels were observed at 24 h after the last CBZ administration at a dose of 600 or 800 mg/kg (Fig.
There were no increases in the plasma ALT levels in vehicle- or BSO alone-treated rats (Fig. 2B).

The plasma ALT levels were increased in a dose-dependent manner in rats treated with CBZ at a dose of 400, 600, or 800 mg/kg on the 5th day. In the group treated with CBZ at a dose of 800 mg/kg, 5 out of 8 rats died within 24 h after the last CBZ administration, whereas only 1 out of 7 rats died at a dose of 600 mg/kg. Thus, we adapted a dose of 600 mg/kg CBZ on the 5th day in the subsequent experiments. This dosing regimen was named “Method A”. We categorized the rats treated with Method A into 2 groups according to their plasma ALT levels as follows: “high responder (ALT ≥ 2,000 U/l)” and “low responder (ALT < 2,000 U/l)”, due to the large interindividual variability in the susceptibility to hepatotoxicity (Fig. 2B).

The hepatic GSH contents in rats administered CBZ and/or BSO were assessed (Fig. 2C). The contents were significantly decreased by the repeated administration of CBZ compared with the vehicle alone-treated group. In addition, the contents in rats treated with BSO alone or the combination of CBZ and BSO on the 5th day were significantly lower compared with those with vehicle treatment on the 5th day. Thus, it was confirmed that hepatic GSH was depleted by the repeated administration of CBZ for 4 days and BSO on the 5th day.

Time-dependent changes in the plasma ALT and AST levels were analyzed in rats administered CBZ with Method A (Fig. 2D). In high responders (n = 4), the plasma ALT and AST levels were time-dependently increased until 24 h (the peak time point, 24 h ALT = 1,7193 ± 2,818 U/l) after the last CBZ administration. Although the plasma ALT and AST levels in the high responders declined from 24 to 72 h after the last CBZ administration, the levels were still higher (48 h ALT = 3,845 ± 950 U/l; 72 h ALT = 579 ± 124 U/l) than those in
the low responders (n = 6, 24 h ALT = 270 ± 57 U/l; 48 h ALT = 370 ± 212 U/l; 72 h ALT = 99 ± 33 U/l) and CBZ alone-treated rats (n = 3, 24 h ALT = 158 ± 22 U/l; 48 h ALT = 95 ± 12 U/l; 72 h ALT = 73 ± 8 U/l) (Fig. 2D). The plasma ALT levels were higher than the AST levels in the high responders at 12 to 72h (Fig. 2D), suggesting that liver damage was predominantly induced by CBZ administration. In this study, OXC, the 10-keto analogue of CBZ, was used as a negative control because case reports showing mild to moderate liver injury are limited in spite of a similar pharmacological activities to CBZ (Fox et al., 2003; Ahmed and Siddiqi, 2006; Björnsson, 2008). Rats administered OXC according to the Method A resulted in no increase in the plasma ALT and AST levels until 72 h after the last OXC administration (Fig. 2D), suggesting that the pharmacological activity did not contribute to CBZ-induced liver injury in rats.

The histopathological analyses of the livers revealed massive focal necrosis around the central vein (CV) 24 h after the last CBZ administration in the high responder (ALT = 21, 800 U/l), whereas no histopathological changes were observed in the low responder (ALT = 176 U/l) or a rat treated with Method A without BSO treatment (ALT = 105 U/l) (Fig. 2E).

**Plasma concentrations of CBZ and its Metabolites.** Plasma concentrations of CBZ and its metabolites were measured to estimate which metabolites were critical for the development of liver injury. The plasma ALT levels 24 h after the last CBZ administration were 1,7193 ± 2,818 U/l in the high responders (n = 4), 271 ± 57 U/l in the low responders (n = 6), and 123 ± 15 U/l in non-BSO-treated rats (n = 5). In these rats, the maximum plasma concentrations of CBZ, 2-hydroxy-CBZ were observed 1 h after the last CBZ administration (Fig. 3), and those of CBZ-10,11-epoxide and trans-10,11-dihydroxy-CBZ were observed 3 h and 3 to 12 h after the last CBZ administration, respectively (Fig. 3). The peak time points of CBZ and its
metabolites appeared prior to that of the plasma ALT levels (Figs. 2D and 3). The plasma concentration of CBZ in the high responders was significantly lower than that in the low responders and non-BSO-treated rats 1 h after the last CBZ administration, suggesting that high responders have a high ability to metabolize CBZ. In addition, the plasma concentration of 2-hydroxy-CBZ in the high responders was lower than that in the low responders 1 - 24 h after the last CBZ administration. The maximum plasma concentration of 3-hydroxy-CBZ was observed 24 h after the last CBZ administration in non-BSO-treated rats (Fig. 3). Plasma concentrations of CBZ-10,11-epoxide and 3-hydroxy-CBZ in the high responders also tended to be lower than those in the low responders. Considering the comparison between high and low responders in plasma CBZ and its metabolite concentrations, lower plasma concentrations of 2-hydroxy-CBZ and 3-hydroxy-CBZ in high responders might be due to the up-regulated metabolism of 2- or 3-hydroxy-CBZ. In addition, the plasma concentrations of CBZ metabolites including 2-hydroxy-CBZ in non-BSO-treated rats were higher than that in the low and high responders, suggesting that BSO treatment would affect in CBZ metabolism.

Effect of CYP3A Induction against CBZ-induced Liver Injury. Considering the results shown in Fig. 3, the up-regulated metabolism of CBZ appears to be involved in the development of liver injury. CBZ is well-known to induce CYP3A in humans and rats (Oscarson et al., 2006; Tateishi et al., 1999). To confirm CYP3A induction by the administration of CBZ in this study, we assessed CYP3A2 protein levels and MDZ 4-hydroxylase activities in the hepatic microsomes prepared from rats administered CBZ or OXC at a dose of 400 mg/kg once daily for 4 days. Because CYP activities were remarkably affected in response to inflammatory cytokines when liver tissue damage occurred (e.g., after BSO and CBZ treatment on the 5th day) (Renton, 2001), we assessed CYP contents and
activity after the 4th CBZ administration when the liver injury did not occur. The microsomal CYP3A2 protein levels in the CBZ- and OXC-administered rats were 1.8- and 1.9-fold increased compared with the vehicle-administered rats, respectively (Fig. 4A). Similarly, MDZ-4-hydroxylase activities were increased 2.7- and 2.5-fold compared with the vehicle-administered rats by the CBZ or OXC administration, respectively (Fig. 4B). These results suggest that hepatic CYP3A2 protein levels and CYP3A activities were induced by the CBZ or OXC administration in the rats.

Furthermore, we examined the effect of the CYP3A inducer on CBZ-induced liver injury. To induce the hepatic CYP3A enzyme, DEX, which is known as a potent CYP3A inducer (Lake et al., 1998), was used. Although rats single-administered CBZ at a dose of 600 mg/kg did not show the increase in plasma ALT levels even when co-treated with BSO (Fig. 2A), rats pretreated with DEX for 3 days followed by a single administration of CBZ at a dose of 400 mg/kg in combination with BSO treatment showed significant increases in plasma ALT levels (Fig. 4C). These observations revealed that CYP3A induction was critical for the development of CBZ-induced liver injury in rats.

Effects of CYP3A Inhibitors on CBZ-Induced Liver Injury. To investigate the involvement of CYP3A-mediated metabolisms in CBZ-induced liver injury served by Method A, rats were co-treated with TAO (300 mg/kg) or KTZ (50 mg/kg), CYP3A inhibitors, 1.5 h prior to the last CBZ administration. The increased levels of plasma ALT were completely suppressed by TAO or KTZ co-treatment (Fig. 5A). In addition, the histopathological analyses revealed the absence of massive focal necrosis and the loss of hepatocytes around CV in livers from TAO or KTZ co-treated rats (Fig. 5B).

Although TAO is known as a potent and specific CYP3A inhibitor (Kostrubsky et al.,
1997; Crincoli et al., 2008), KTZ has been reported to inhibit potently not only CYP3A1/2 activities but also CYP2C11 activity by the study using vaculovirus-infected insect cells expressing each rat P450s (Kobayashi et al., 2003). To examine whether KTZ treatment affected hepatic CYP3A and CYP2C enzyme activities in the rats, MDZ and TOL hydroxylase activities were measured in the microsomes prepared from livers collected 1.5 h after the KTZ administration in rats administered CBZ at a dose of 400 mg/kg once daily for 4 days. As results, MDZ-4-hydroxylase and 1'-hydroxylase activities were significantly decreased by KTZ-treatment. In contrast, TOL hydroxylase activities were not decreased (Fig. 5C). These results indicate that KTZ specifically inhibited hepatic CYP3A enzyme activity in the present condition.

Effects of CYP3A Inhibitors on the Plasma Concentrations of CBZ and its Metabolites. As shown in Fig. 3, an increased activity of CBZ metabolism, especially the enhanced metabolism of 2-hydroxy-CBZ and 3-hydroxy-CBZ, seems to be involved in the development of liver injury. To assess CBZ, 2-hydroxy-CBZ and 3-hydroxy-CBZ formations under the TAO or KTZ co-treated condition, we measured their plasma concentrations 1 h and 3 h after the last CBZ administration. There were no significant differences in the plasma concentrations of CBZ, 2-hydroxy-CBZ and 3-hydroxy-CBZ 1 h after the last CBZ administration between each group (Fig. 6A). At 3 h after the last CBZ administration, the plasma concentration of CBZ showed a tendency to increase in TAO co-treated rats and significantly increased in KTZ co-treated rats compared with vehicle co-treated rats (Fig. 6B), indicating that CYP3A is mainly involved in CBZ metabolism in rats. In addition, the plasma concentration of 2-hydroxy-CBZ was significantly increased 3 h after the last CBZ administration in rats co-treated with TAO or KTZ compared with the high responders.
Plasma concentrations of 3-hydroxy-CBZ also tended to be higher in rats co-treated with TAO or KTZ compared with the high responders (Fig. 6B). There were no significant differences in the plasma concentrations of CBZ-10,11-epoxide and \textit{trans}-10,11-dihydroxy-CBZ 1 h and 3 h after the last CBZ administration among the 4 groups (data not shown). These results suggest that CYP3A has a critical role for CBZ metabolism and 2-hydroxy-CBZ metabolism.
Discussion

In this study, we demonstrated that the GSH-depleted condition and increased CYP3A levels sensitized the development of CBZ-induced liver injury in rats. A previous in vitro study reported that GSH significantly suppressed the metabolism-dependent cytotoxicity of CBZ in human peripheral blood mononuclear leukocytes and the irreversible binding of $[^{14}\text{C}]$CBZ to human liver microsomes, suggesting that reactive metabolites, which are trapped by GSH, were generated from CBZ (Pirmohamed et al., 1992b). In this study, hepatic GSH contents in rats repeatedly administered CBZ were significantly lower than those in rats administered vehicle (Fig. 2C). This result indicates that hepatic GSH is consumed by CBZ administration. In this study, BSO was used to deplete GSH to develop CBZ-induced liver injury in rats because BSO is known to be a specific inhibitor of $\gamma$-glutamylcysteine synthetase, a rate-limiting enzyme of GSH synthesis (Griffith and Meister 1979), and can decrease the hepatic GSH content in rats (Gao et al., 2010). Many researchers have applied the GSH-depleted animal model using BSO to evaluate hepatotoxic potential for several drugs that are known to generate electrophilic reactive metabolites, such as methimazole, tienilic acid, amodiaquine and ticlopidine (Kobayashi et al., 2012a; Nishiya et al., 2008; Shimizu et al., 2009 and 2011). In this study, several rats administered CBZ at a dose of 600 mg/kg or 800 mg/kg in combination with BSO on the 5th day showed severe hepatotoxicity, whereas rats administered CBZ alone showed no hepatotoxicity (Figs. 2B and D). Hepatic GSH contents were depleted in the rats with BSO treatment on the 5th day (Fig. 2C). These results suggest that GSH depletion is essential for developing CBZ-induced liver injury in rats.

Previously, we developed a mouse model of CBZ-induced liver injury without BSO treatment as follows: mice were administered CBZ at a dose of 400 mg/kg once daily for 4 days and 800 mg/kg on the 5th day (Higuchi et al., 2012). Although we applied this dosing regimen,
only slight changes in the plasma ALT levels were observed (Fig. 2B), suggesting that GSH plays an important role in the protection from CBZ-induced liver injury in rats. The hepatic GSH contents in rats (approximately 7 μmol/g tissue) are similar to those in mice (approximately 8 μmol/g tissue) (Allameh et al., 1997, Watanabe et al., 2003). There is a possibility that rats have a poor ability to form reactive metabolites compared to mice or that rats have higher GSH conjugation activity. These presumable species differences in formation and/or detoxification of reactive metabolites may account for the requirements of BSO treatment to develop CBZ-induced liver injury in rats.

At 24 h after the last CBZ administration, there is large interindividual variability in the plasma ALT levels in rats (Fig. 2B). To determine the causal factor for the large variation in plasma ALT levels, we investigated the interindividual variability in the hepatic GSH contents. It has been reported that hepatic GSH content is significantly decreased in rats at 6 to 9 h after a single BSO treatment and were recovered to a normal level at 24 h (Gao et al., 2010). In the present study, when hepatic GSH contents were measured at 6 h after BSO treatment on the 5th day, moderate interindividual variability in hepatic GSH contents was observed (BSO-treated group, 0.37 - 1.92 μmol/g liver; CBZ co-treated with BSO group, 0.28 - 2.02 μmol/g liver). Hepatic GSH levels treated with CBZ for 4 days and with vehicle on the 5th day showed moderate variability (2.09 - 5.18 μmol/g liver) (Fig. 2C). Therefore, the degree of hepatic GSH depletion might be involved in the large variation of the plasma ALT levels.

In the histopathological analysis, massive necrosis of hepatocytes around the CV was observed in the high responders (Fig. 2E). CBZ is known to induce several CYP enzymes, which are mainly expressed in hepatocytes around the CV, including CYP3A in humans and rats (Bühler et al, 1992; Clayton et al., 2007; Oscarson et al., 2006; Tateishi et al., 1999), and patients who develop CBZ-induced serious liver injury undergo long-term (average 30 weeks)
drug administration (Bjornsson, 2008), suggesting that CYP3A auto-induction contributes to the development of liver injury. In the present study, hepatic CYP3A contents and enzyme activities were significantly increased by the repeated administration of CBZ (Figs. 4A and B). The single administration of CBZ, even in combination with BSO treatment, failed to cause liver injury (Fig. 2A), whereas the repeated administration of CBZ with BSO treatment (Method A) showed a marked increase in the plasma ALT levels and massive necrosis in the hepatocytes (Figs. 2B and D). These results suggest that CYPs auto-induction is essential for developing liver injury.

In the present study, rats treated with DEX at a dose of 80 mg/kg once daily for 3 days followed by a single administration of CBZ at a dose of 400 mg/kg in combination with BSO treatment resulted in an increase in the plasma ALT levels (Fig. 4C). This observation supports the presumption that CYPs auto-induction before the exposure to a hepatotoxic amount of CBZ might be critical for the generation of reactive metabolites. We considered that the difference in the CYP3A auto-induction level may be involved in the interindividual variability of the plasma ALT levels, but there is small interindividual variability in CYP3A auto-induction levels after the repeated administration of CBZ (protein levels, 1.48 - 2.24-fold compared with the vehicle-administered group; MDZ 4-hydroxylase activity, 236 - 338 pmol/min/mg protein) (Figs. 4A and B). Thus, a causal factor that can explain the interindividual variability in plasma ALT level is still unknown. Although the CYP3A induction level due to the repeated administration of OXC was comparable with that by CBZ (Figs. 4A and B), rats administered OXC with Method A showed no hepatotoxicity (Fig. 2C). These results suggest that CYP3A auto-induction itself does not lead to the development of liver injury, however the enhancement of the CYP3A-mediated metabolic activation of CBZ is important.

In this study, TAO or KTZ co-treatment with Method A resulted in the protection from
liver injury in rats (Figs. 5A and B). This result is consistent with a previous study that KTZ significantly reduced the metabolism-dependent irreversible binding of radiolabelled CBZ to human liver microsomes (Pirmohamed et al., 1992b). In contrast, our previous study reported that co-treatment with TAO or KTZ exacerbated the CBZ-induced liver injury in mice (Higuchi et al., 2012). This controversial result may be attributed to the species differences in the CYP-mediated metabolism of CBZ between the mouse and rat.

Low responders showed a higher plasma concentration of CBZ than the high responders, suggesting that the enhancement of CBZ metabolism is important for the development of liver injury (Fig. 3). In addition, the plasma concentrations of 2-hydroxy-CBZ in the low responders and rats co-treated with TAO or KTZ were higher compared with those in the high responders (Figs. 3 and 6B). Therefore, we considered that the metabolic pathway via 2-hydroxy-CBZ is involved in the development of CBZ-induced liver injury. There are two possibilities why the plasma concentration of 2-hydroxy-CBZ in the high responders was lower than that in the low responder and in the non-BSO-treated rats; 1) a decrease in the formation of 2-hydroxy-CBZ and 2) the enhancement of the 2-hydroxy-CBZ metabolism. We considered that the latter reason is conceivable because the formation of reactive metabolites from 2-hydroxy-CBZ was demonstrated as follows: the metabolic reaction of 2-hydroxy-CBZ to 2-hydroxyiminostilbene is mainly catalyzed by CYP 3A4 in humans (Pearce et al., 2005), and 2-hydroxyiminostilbene can be readily oxidized to an iminoquinone species (Ju and Uetrecht, 1999). The iminoquinone can react with GSH or NAC to form conjugates in vitro due to instability (Ju and Uetrecht, 1999; Pearce et al., 2005). In addition, Ju and Uetrecht (1999) detected a glucuronide conjugate of 4-methylthio-2-hydroxyiminostilbene, which is a probable metabolite of the iminoquinone-GSH conjugate, by liquid chromatography/mass spectrometry in urine from a patient who had received CBZ. Furthermore, the previous in vitro report showed
that catechol-\(\cdot\)-methyltransferase, which would methylate a catechol that might otherwise be converted to a quinone, significantly reduced the metabolism-dependent irreversible binding of radiolabelled CBZ to mouse liver microsomes (Lillibridge et al., 1996). This study suggests that CBZ-\(\cdot\)-quinone, which is proposed to form via oxidation of the catechol, 2,3-dihydroxy-carbamazepine, is related to the development of liver injury. Thus, the metabolic pathway of CBZ via 2-hydroxy-CBZ might be involved in the formation of the reactive metabolites in rats, although the possibilities of other metabolic pathways cannot be denied.

Plasma concentrations of CBZ and its metabolites in non-BSO-treated rats showed higher than those in the low- and high-responders (Fig. 3). Therefore, it is conceivable that BSO treatment affected in CBZ metabolism. However, repeated CBZ administration with or without BSO treatment reduced GSH levels to a similar degree (Fig. 2C), and yet repeated CBZ administration without BSO treatment did not produced liver injury (Fig. 2B). Taken together, it was suggested that the potentiation effect of BSO is not limited to GSH depletion.

The present study suggested that CYP3A auto-induction was involved in CBZ-induced liver injury. GSH-depleted condition is essential for developing CBZ-induced liver injury in rats (Fig. 2). However, there was small interindividual variability of changes of the expression levels of proteins (e.g. CYP3A and GSH) and CYP enzymes activities (Figs. 2, 4 and 5). Therefore, these results suggest that other factor(s) would be involved in the large interindividual variability of the plasma ALT levels.

In conclusion, we established a rat model for CBZ-induced liver injury under the GSH-depleted condition with the auto-induction of CYP3A. The present study suggested that the biotransformation of reactive metabolite(s) (conceivably a quinone-metabolite) from 2-hydroxy-CBZ by CYP3A, and the detoxification by hepatic GSH was involved in the
development of CBZ-induced liver injury. The results obtained in this study may help us to understand the mechanism of CBZ-induced liver injury in humans.
Authorship contributions

Participated in research design: Iida, Fukami, Nakajima and Yokoi

Conducted experiments: Iida, Sasaki, Yano and Tsuneyama

Contributed new reagents or analytic tools: Iida, Fukami and Yokoi

Performed data analysis: Iida and Yokoi

Wrote or contributed to the writing of the manuscript: Iida, Sasaki, Fukami and Yokoi
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Footnotes

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Figure legends

Fig. 1. Proposed metabolic pathways of CBZ in the human. The structure within the brackets is inferred from the products.

Fig. 2. Procedure for the establishment of CBZ-induced liver injury. (A) Effects of a single administration of CBZ and BSO co-administration. Rats were administered a single dose of CBZ at 400 or 600 mg/kg, and BSO was intraperitoneally injected at a dose of 700 mg/kg 2 h before the CBZ administration. Plasma ALT levels were measured 24 h after the CBZ administration. NT indicates the non-treatment group, and “-” indicates vehicle treatment (NT, CBZ 400 and CBZ 400 + BSO, n = 4; CBZ 600, n = 2; CBZ 600 + BSO, n = 1) (B) Dose-dependent changes in the plasma ALT levels following repeated administration of CBZ. Rats were administered CBZ at a dose of 400 mg/kg once daily for 4 days, then 400, 600, or 800 mg/kg on the 5th day. BSO was intraperitoneally injected at a dose of 700 mg/kg 2 h before the last CBZ administration. Plasma ALT levels were measured 24 h after the last CBZ administration. Each point indicates the plasma ALT level of the individual animal (vehicle alone and CBZ 600 + BSO, n = 6; BSO alone and CBZ 400 + BSO, n = 5; CBZ 600, n = 7; CBZ 800, n = 4; CBZ 800 + BSO, n = 3) *p < 0.05 and **p < 0.01 compared with the vehicle alone-administered group by Dunnett’s post hoc test. (C) Hepatic GSH contents after the administration of CBZ in combination with BSO. The rats were administered CBZ for 4 days and treated with vehicle, CBZ alone, BSO alone or CBZ in combination with BSO on the 5th day. Livers were collected 6 h after the BSO treatment, which was the same time as 4 h after the last CBZ administration. The group treated with vehicle alone was administered corn oil for 4 days, and their livers were collected on the 5th day. **p < 0.001 compared with the
control group by Tukey’s post-hoc test; $^{ §§ } P < 0.01$ compared with rats treated with vehicle on the 5th day by Tukey’s post-hoc test. (D) Time-dependent changes in the plasma ALT and AST levels in CBZ- or OXC-administered rats with or without BSO treatment. Rats were administered CBZ or OXC by Method A (400 mg/kg for 4 days, and 600 mg/kg and BSO on the 5th day). Plasma samples were collected at 0, 6, 12, 24, 48 and 72 h after the last CBZ administration to assess the plasma ALT and AST levels (high responders of CBZ and OXC-administered group, n = 4; low responders of CBZ, n = 6; CBZ alone-administered group, n = 4). $^{ ** } P < 0.01$ compared with each 0 h group by Dunnett’s post hoc test. $^{ § } P < 0.05$, $^{ §§ } P < 0.01$ and $^{ §§ §§ } P < 0.001$ compared with the low responder group at individual time points by the two-tailed Student’s $t$-test. $^{ † } P < 0.05$, $^{ † † } P < 0.01$ and $^{ † † † } P < 0.001$ compared with CBZ alone-administered group at individual time points by the two-tailed Student’s $t$-test. (E) Histopathological analyses of the livers from rats administered CBZ and BSO. Livers were collected 24 h after the last CBZ administration, and the liver sections were stained with H&E. Control indicates the vehicle-treated group, and CV indicates the central vein. The data shown are the mean ± S.E.

Fig. 3. Time-dependent changes in the plasma concentrations of CBZ and its metabolites after the last CBZ administration in high responders and low responders. Rats were administered CBZ in Method A. Plasma samples were collected at 0, 1, 3, 6, 12 and 24 h after the last CBZ administration. Plasma concentrations of CBZ, CBZ-10,11-epoxide, trans-10,11-dihydroxy-CBZ, 2-hydroxy-CBZ and 3-hydroxy-CBZ were determined by HPLC (high responder, n = 4; low responder, n = 6; without BSO treatment, n = 5). $^{ * } P < 0.05$, $^{ ** } P < 0.01$, and $^{ *** } P < 0.001$ compared with high responder group at individual time points by
Tukey’s post-hoc test. $^§P < 0.05$, $^§§P < 0.01$ and $^§§§P < 0.001$ compared with low responder group at individual time points by Tukey’s post-hoc test. The data shown are the mean ± S.E.

Fig. 4. Effects of CBZ or OXC administration on (A) the expression level of CYP3A2 protein and (B) MDZ 4-hydroxylase activity in RLM, and (C) the effects of a CYP3A inducer on plasma ALT levels by a single CBZ administration in rats. (A, B) Rats were administered CBZ or OXC at a dose of 400 mg/kg once daily for 4 days, and their livers were collected at 24 h after the last drug administration to prepare microsomes. The CYP3A2 protein level and MDZ 4-hydroxylase activities were determined by immunoblotting and HPLC, respectively. “−” indicates vehicle administration (vehicle and CBZ, n = 4; OXC, n = 5). **$P < 0.01$ compared with the vehicle-administered group. (C) Rats were intraperitoneally treated with DEX at a dose of 80 mg/kg once daily for 3 days, then CBZ at a dose of 400 mg/kg on the 4th day. Two hours before the CBZ administration, BSO was intraperitoneally injected at a dose of 700 mg/kg. Plasma samples were collected at 0, 3, 6 and 12 h after the CBZ administration (DEX with CBZ, n = 4; DEX without BSO, n = 2; vehicle with CBZ, n = 5). *$P < 0.05$ and **$P < 0.01$ compared with the 0 h group by Dunnett’s post hoc test. The data shown are the mean ± S.E.

Fig. 5. Effects of CYP3A inhibitors on CBZ-induced liver injury and CYP enzyme activities. (A) Vehicle (corn oil), TAO (300 mg/kg) or KTZ (50 mg/kg) was intraperitoneally injected 1.5 h before the last CBZ administration in Method A. Plasma ALT levels were measured 24 h after the last CBZ administration. “−” indicates vehicle treatment (vehicle, n = 4; TAO or KTZ, n = 5). (B) The liver sections from rats in Fig. 5A were stained with H&E. (C) Rats were administered CBZ at a dose of 400 mg/kg for 4 days and KTZ (50 mg/kg) was
intraperitoneally treated on the 5th day. The livers were collected 1.5 h after the KTZ treatment to prepare microsomes. CYP3A, CYP3A/CYP2C, and CYP2C11 enzyme activities were determined by MDZ 4-hydroxylase activities, MDZ 1’-hydroxylase activities and TOL hydroxylase activities, respectively (vehicle treatment, n = 4; KTZ treatment, n = 6). *P < 0.05 and ***P < 0.001 compared with control group by two-tailed Student’s t-test. The data shown are the mean ± S.E.

Fig. 6. Effects of CYP3A inhibitors on the plasma concentrations of CBZ and its metabolites. TAO (200 mg/kg) or KTZ (50 mg/kg) was treated 1.5 h before the last CBZ administration in Method A. Plasma samples were collected (A) 1 h and (B) 3 h after the last CBZ administration (n = 3 to 6). Plasma concentrations of CBZ, 2-hydroxy-CBZ and 3-hydroxy-CBZ were determined by HPLC. Each plot indicates the plasma concentrations of CBZ or its metabolites in the individual animal. *P < 0.05 and **P < 0.01 by Tukey’s post-hoc test.
Fig. 4

(A) Relative expression of CYP3A2.

(B) MDZ 4-hydroxylase activity (pmol/min/mg protein).

(C) Time course of ALT (U/L) levels.

- (Vehicle for 4 days)
- CBZ for 4 days
- OXC for 4 days

DEX with CBZ
DEX without CBZ
Vehicle with CBZ
Fig. 5

(A) ALT (IU/L)

(B) Images:
- Low responder
- High responder
- TAO-treated
- KTZ-treated

(C) Bar graphs:
- MDZ 4-hydroxylase activity (pmol/min/mg protein)
- MDZ 1'-hydroxylase activity (pmol/min/mg protein)
- TOL hydroxylase activity (pmol/min/mg protein)

Inhibitor:
- -
- TAO
- KTZ

**Notes:**
- Significant differences indicated by asterisks: **p < 0.001**
(A) 1 h after the last CBZ administration

(B) 3 h after the last CBZ administration