CYP2C9-mediated Metabolic Activation of Losartan Detected by A Highly Sensitive Cell-based Screening Assay

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Abbreviations:
ALT, alanine aminotransferase; BSO, buthionine sulphoximine; CYP, cytochrome P450;
FBS, fetal bovine serum; GFP, green fluorescence protein; HPLC, high performance-liquid chromatography; LC, liquid chromatography; MS/MS tandem mass spectrometry; MS, mass spectrometry; LCMS-IT-TOF, liquid chromatography ion trap time-of-flight mass spectrometry; MOI, multiplicity of infection; Nrf2, NF-E2 p-45-related factor; PFU, plaque forming unit
Abstract

Drug-induced hepatotoxicity is a major problem in drug development, and reactive metabolites generated by CYPs are suggested to be one of the causes. CYP2C9 is one of the major enzymes in hepatic drug metabolism. In the present study, we developed a highly sensitive cell-based screening system for CYP2C9-mediated metabolic activation using an adenovirus vector expressing CYP2C9 (AdCYP2C9). Human hepatocarcinoma HepG2 cells infected with our constructed AdCYP2C9 for 2 days at MOI 10 showed significantly higher diclofenac 4’-hydroxylase activity than human hepatocytes. AdCYP2C9-infected cells were treated with several hepatotoxic drugs, resulting in a significant increase of cytotoxicity by treating with losartan, benzbromarone and tienilic acid. Metabolic activation of losartan by CYP2C9 has never been reported, although the metabolic activations of benzbromarone and tienilic acid have been reported. To identify the reactive metabolites of losartan, the semicarbazide adducts of losartan were investigated by liquid chromatography-tandem mass spectrometry. Two CYP2C9-specific semicarbazide adducts of losartan (S1 and S2) were detected. S2 adduct suggested a reactive metabolite was produced from the aldehyde metabolite E3179, but a possible metabolite from S1 adduct formation was not produced via E3179. In conclusion, a highly sensitive cell-based assay to evaluate CYP2C9-mediated metabolic activation was established and we first found that CYP2C9 is involved in the metabolic activation of losartan. This cell-based assay system would be useful for evaluating drug-induced cytotoxicity caused by human CYP2C9.
Introduction

Drug-induced hepatotoxicity is a serious problem in drug development and clinical practice. In the United States, it accounts for more than 50% of cases of acute liver failure, and more than 600 drugs have been associated with hepatotoxicity (Lee, 2003; Park et al., 2005). That is why some drugs that were launched on the market were later withdrawn. Therefore, the prediction of drug-induced hepatotoxicity before clinical trials is quite important in drug development, and multiple cell-based assays have been developed for evaluation of drug-induced hepatotoxicity (Greer et al., 2010). Sometimes, drug-induced hepatotoxicity is associated with reactive metabolites produced by drug-metabolizing enzymes (Guengerich, 2008). However, species differences in drug metabolizing enzymes or other factors between human and laboratory animals are a major problem in predicting the hepatotoxicity.

Cytochrome P450 (CYP) enzymes are the most studied drug-metabolizing enzymes, accounting for ~75% of the metabolism of clinical drugs (Guengerich, 2008). Among them, CYP3A4 is the predominant isoform expressed in human liver, accounting for up to 60% of the total hepatic P450 protein and responsible for more than 50% of drug metabolism (Guengerich, 2008). To date, many researchers have tried to predict drug-induced hepatotoxicity in vitro using human hepatocarcinoma HepG2 cells, but the low expression levels of CYP enzymes in HepG2 cells may be responsible for the fact that 30% of the compounds were falsely classified as non-toxic (Hewitt and Hewitt, 2004; Rodriguez-Antona et al., 2002). Recently, useful in vitro cell-based assays were established with HepG2 cells, leading to the improved evaluation of drug-induced cytotoxicity. For example, our previous study found that benzodiazepines such as flunitrazepam and nimetazepam were metabolically activated by CYP3A4 by
co-incubation with HepG2 cells and CYP3A4 Supersomes (Mizuno et al., 2009). Vignati et al. (2005) demonstrated that various hepatotoxic drugs such as flutamide and troglitazone were activated by CYP3A4 using HepG2 cells transiently transfected with CYP3A4. Thus the activation of hepatotoxic drugs by CYP3A4 has been well evaluated, but the contribution of other CYP enzymes remains to be evaluated. CYP2C is the second most highly expressed CYP subfamily in human liver and CYP2C9 is the most highly expressed isoform in this family (Edwards et al., 1998). CYP2C9 is responsible for the metabolism of various pharmaceutical drugs and appears to be partially involved in the generation of reactive metabolites as is CYP3A4 (Li, 2002). For example, benzbromarone is metabolized via 6-hydroxybenzbromarone to catechol by CYP2C9, following by the oxidization of the catechol to a reactive ortho-quinone metabolite (McDonald and Rettie, 2007). Tienilic acid is metabolized to reactive intermediates, the thiophene sulfoxide or the thiophene epoxide, by CYP2C9 (Koenigs et al., 1999). Recently, we developed useful in vitro cell-based assays using adenovirus to sensitively evaluate the involvement of CYP3A4 and superoxide dismutase 2 in drug-induced cytotoxicity (Hosomi et al., 2010; Yoshikawa et al., 2009). In the present study, a highly sensitive cytotoxicity assay system for CYP2C9-mediated metabolic activation was established in a similar way, and the drug-induced cytotoxicity was evaluated with the established assay system. Drugs investigated in this study were hepatotoxic drugs that are known to be CYP2C9 substrates (flutamide, fluvastatin, losartan, terbinafine, valproic acid, and zolpidem) and those that are known to be metabolically activated by CYP2C9 (benzbromarone and tienilic acid). As a result, we first found that the cytotoxicity of losartan was enhanced by CYP2C9, and then performed additional studies to identify the structures of the reactive metabolites.
Materials and Methods

Chemicals and Reagents. Diclofenac, fluvastatin, and tienilic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Losartan and terbinafine were obtained from LKT laboratories (St.Paul, USA). Benzbromarone, flutamide, valproic acid, zolpidem were obtained from Sigma (St. Louis, MO). Candesartan, eprosartan, irbesartan, telmisartan, and valsartan were obtained from Toronto Research Chemicals (Ontario, Canada). Olmesartan was kindly provided by Daiichi-Sankyo (Tokyo, Japan). 4’-Hydroxydiclofenac, human CYP2C9 and CYP3A4 Supersomes (recombinant cDNA-expressed P450 enzymes prepared from a baculovirus insect cell system) were purchased from BD Gentest (Woburn, MA). The Adenovirus Expression Vector kit (Dual Version) and Adenovirus genome DNA-TPC were obtained from Takara Bio (Shiga, Japan). The QuickTiter Adenovirus Titer Immunoassay kit was from Cell Biolabs (Tokyo, Japan). Stealth Select RNAi for Nrf2 (Accession NM_006164) and Stealth RNAi Negative Control Medium GC Duplex #2 were obtained from Invitrogen (Grand Islanda, NY). Dulbecco’s modified eagle’s medium (DMEM) was from Nissui Pharmaceutical (Tokyo, Japan). Restriction enzymes were from New England Biolabs (Beverly, MA) and Takara Bio. All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Other chemicals were of analytical or the highest grade commercially available.

Cell Culture. Human embryonic kidney 293 cells and human hepatocarcinoma HepG2 cells were obtained from American Type Culture Collection (Manassas, VA). The 293 and HepG2 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) (Invitrogen), 3% glutamine, 16% sodium bicarbonate and 0.1 mM non-essential amino
acids (Invitrogen) in a 5% CO₂ atmosphere at 37°C. Cells were infected with the adenovirus in medium containing 5% FBS.

**Recombinant Adenovirus.** A recombinant adenovirus expressing CYP2C9 (AdCYP2C9) was constructed using the cosmid-terminal protein complex (COS-TPC) method according to the manufacturer’s instructions. CYP2C9 cDNA prepared by reverse transcription-polymerase chain reaction using total RNA from human liver obtained at autopsy was inserted into the Swa I site of the pAxcwtit vector. The use of the human liver was approved by the Ethics Committees of Kanazawa University (Kanazawa, Japan) and Iwate Medical University (Morioka, Japan). The nucleotide sequences of CYP2C9 were confirmed by DNA sequence analysis (Long-Read Tower DNA sequencer; GE Healthcare, Little Chalfont, Buckinghamshire, UK). This vector and the parental adenovirus DNA terminal protein complex were co-transfected into 293 cells by lipofectoamine 2000 (Invitrogen, Carlsbad, CA). The recombinant adenovirus was isolated and propagated into the 293 cells. In a similar way, the recombinant adenovirus vector expressing a green fluorescence protein (GFP) was generated in the previous study (Hosomi et al., 2010). Viral titers were determined by a QuickTiter Adenovirus Titer Immunoassay kit. The titers of AdCYP2C9 and AdGFP were $8.6 \times 10^8$ PFU/ml and $2.1 \times 10^8$ PFU/ml, respectively.

**Immunoblot Analyses of Human CYP2C9 and Nrf2.** SDS-polyacrylamide gel electrophoresis and immunoblot analyses of human CYP2C9, Nrf2 and GAPDH were performed. For human CYP2C9, total cell homogenates from adenovirus-infected HepG2 cells (5 µg) were separated on 7.5% polyacrylamide gels and electrotransferred...
onto polyvinylidene difluoride membrane, Immobilon-P (Millipore Corporation, Billerica, MA). The membrane was probed with polyclonal rabbit anti-human CYP2C9 antibody (Daiichi Pure Chemicals, Tokyo, Japan). Biotinylated anti-rabbit IgG and VECTASTAIN ABC kit (Vector laboratories, Burlingame, CA) were used for diaminobenzidine staining. For human Nrf2, total cell homogenates from siRNA-transfected and adenovirus-infected HepG2 cells (25 µg) were separated on 7.5% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membrane, Immobilon-P. The membrane was probed with polyclonal rabbit anti-human Nrf2 antibody (Santa Cruz Biotechnologies, San Diego, CA), and the corresponding fluorescent dye-conjugated second antibody and an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) were used for detection. For human GAPDH, SDS-polyacrylamide gel electrophoresis and immunoblot analysis was performed according to Hosomi et al (submitted).

Diclofenac 4'-Hydroxylase Activity. HepG2 cells (3 × 10^5 cells/well) were seeded in 12 well plates. After 24-hr incubation, cells were infected with AdCYP2C9 or AdGFP for 1, 2, 3 or 5 days. Then, after 1-hr incubation with 100 µM diclofenac, the medium was subjected to high-performance liquid chromatography (HPLC) to measure the concentration of 4'-hydroxydiclofenac, a metabolite of diclofenac catalyzed by CYP2C9. The HPLC analysis was performed using an L-2130 pump (Hitachi, Tokyo, Japan), an L-2200 autosampler (Hitachi), and a D-2500 Chromato-Integrator (Hitachi) equipped with a Mightysil RP-18 C18 GP column (5-µm particle size, 4.6 mm i.d. × 150 mm; Kanto Chemical, Tokyo, Japan). The eluent was monitored at 280 nm. The mobile phase was 35% acetonitrile containing 20 mM sodium perchlorate (pH 2.5). The
flow rate was 1.0 ml/min. The column temperature was 35˚C. The retention times of 4’-hydroxydiclofenac and diclofenac were 8.1 and 22.8 min, respectively. The quantification of 4’-hydroxydiclofenac was performed by comparing the HPLC peak height with that of an authentic standard. The limit of quantification in the reaction mixture for 4’-hydroxydiclofenac was 250 nM with a coefficient of variation of < 2%.

**Cytotoxicity Assay.** Nrf2 is known to regulate cytoprotective genes such as glutathione S-transferase, heme oxygenase-1, NAD(P)H:quinone oxidoreductase, superoxide dismutase, and UDP-glucuronosyltransferase (Copple *et al.*, 2008). A previous study demonstrated that drug-induced cytotoxicity could be detected with high sensitivity by the knockdown of Nrf2 in HepG2 cells (Hosomi *et al.*, submitted). Similarly, the knockdown of Nrf2 was performed by siRNA transfection in this study. HepG2 cells were transfected with Stealth Select RNAi for Nrf2 (siNrf2) and Stealth RNAi Negative Control Medium GC Duplex #2 (siScramble) by Lipofectamine™RNAiMAX Reagent (Invitrogen). According to the manufacturer’s protocol, RNAi duplex-Lipofectamine™RNAiMAX complexes were prepared and added to each well before the HepG2 cells were seeded (1.0 × 10^4 cells/well). The concentrations of siNrf2 and siScramble were 10 nM. After 24-hr incubation, the cells were infected with AdCYP2C9 or AdGFP. Forty-eight hrs after infection, the cells were treated with benzbromarone, tienilic acid, flutamide, fluvastatin, terbinafine, valproic acid, zolpidem, or sartans (candesartan, eprosartan, irbesartan, losartan, olmesartan, telmisartan, and valsartan) for 24 hrs. After incubation with the drugs, cell viability was quantified by WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt) and ATP assays according to the
manufacturer's protocol. The WST-8 assay, which is a modified MTT assay, was performed using Cell Counting Kit-8 (CCK-8 kit) (Wako Pure Chemical Industries). After incubation with the drugs for 24 hrs, CCK-8 reagent was added and the absorbance of WST-8 formazan was measured at 450 nm. The ATP assay was performed using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). After incubation with the drugs for 24 hrs, CellTiter-Glo Reagent was added and the generation of a luminescent signal was recorded by using a 1420 ARVO MX luminometer (Perkin Elmer Wallac, Turku, Finland).

Detection of Semicarbazide Adducts. A typical reaction mixture (final volume of 0.5 ml) contained 100 nM human CYP2C9 or CYP3A4 Supersomes, 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 10 mM semicarbazide, and 20 μM \([14C]losartan\). The final concentration of ethanol in the reaction mixture was less than 1%. Incubation was carried out at 37˚C for 60 min and terminated by adding 2 ml of ice-cold methanol. After centrifugation at 15,000 g, the supernatant was subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS; 4000 QTRAP, Applied Biosystems, Foster City, CA). Agilent 1200 (Agilent technologies, Inc., CA) was used as the LC with an Inertsil ODS-3V column (5-μm particle size, 4.6 mm i.d. × 250 mm, GL Science, Inc., Tokyo, Japan). The column temperature was 40˚C. The mobile phase was 10 mM ammonium acetate buffer (A) and acetonitrile (B). The conditions for elution were as follows: 5-45% B (0-5 min), 45-70% B (5-55 min), 70-100 % B (55-60 min), and 5% B (60.01 -70 min). Linear gradients were used for all solvent changes. The flow rate was 1 ml/min. The LC was connected to a 4000 QTRAP mass spectrometer operated by the enhanced product ion under the positive mode. The turbo
gas was maintained at 450˚C. Air was used as the nebulizing and turbo gas at 50 psi. Nitrogen was used as the curtain gas at 30 psi. The declustering potential and collision energy was 50 and 20 V, respectively. The m/z 150-500 was scanned at the precursor ion (m/z 494.2; semicarbazide adducts of losartan hydroxide).

**Identification of Semicarbazide Adducts.** Liquid chromatography ion trap time-of-flight mass spectrometry (LCMS-IT-TOF) (Shimadzu, Kyoto, Japan) was used to identify the structures of the semicarbazide adducts of the losartan hydroxide. The incubation mixture was the same as described above. After centrifugation at 15,000 g for 5 min, the supernatant was subjected to LCMS-IT-TOF using the Inertsil ODS-3 analytical column (5-µm particle size, 4.6 mm i.d. × 250 mm). The LC conditions were the same as described earlier.

**Statistical Methods.** Data are expressed as mean ± SD. Statistical significance between two groups was determined by two-tailed Student’s t-test. A value of $P < 0.05$ was considered statistically significant.
Results

MOI- and Time-dependent Changes of Diclofenac 4’-Hydroxylase Activity and CYP2C9 Protein Level. To investigate the optimum multiplicity of infection (MOI), HepG2 cells were infected with AdCYP2C9 at MOI 0, 2.5, 5, 10, or 20 for 2 days. Diclofenac 4’-hydroxylase activity and CYP2C9 protein level were measured (Fig. 1A). The activity and CYP2C9 protein level were increased MOI-dependently in AdCYP2C9-infected cells, whereas they were not detected in AdGFP-infected cells at MOI 20. The highest activity and protein level were observed in cells infected with AdCYP2C9 at MOI 20, but the cells were slightly damaged (data not shown). At MOI 10, diclofenac 4’-hydroxylase activity was 0.957 ± 0.070 nmol/min/mg protein, which value was higher than those in human hepatocytes reported in other reports (Supplemental Table 1). With HepG2 cells infected with AdCYP2C9 at MOI 10 for 1, 2, 3, or 5 days, the highest activity was observed after 2-days infection although the protein levels appeared to be similar after 2 to 5-days infections (Fig. 1B). From these results, AdCYP2C9 infection to HepG2 cells was performed at MOI 10 for 2 days in the subsequent experiments.

Effect of SiNrf2 on Nrf2 Protein Expression in Adenovirus-infected HepG2 Cells.

Our recent study demonstrated that CYP3A4-induced cytotoxicities of several drugs such as acetaminophen and flutamide were sensitively detected by Nrf2 knockdown (Hosomi et al., submitted). This study also utilized HepG2 cells transfected with siNrf2. Nrf2 protein expression in HepG2 cells was efficiently decreased by transfection of siNrf2 (26.8 ± 1.1% and 27.1 ± 2.0%, respectively), and the effect of siNrf2 was not affected by CYP2C9 overexpression (Fig. 1C).
CYP2C9-induced Cytotoxicity in HepG2 Cells Transfected with siNrf2. To investigate the CYP2C9-mediated metabolic activation of 8 hepatotoxic drugs (benzbromarone, flutamide, fluvastatin, losartan, terbinafine, tienilic acid, valproic acid, and zolpidem), HepG2 cells infected with AdCYP2C9 at MOI 10 for 2 days were treated with drugs for 24 hrs. As a negative control, AdGFP was infected at MOI 10. To improve the sensitivity, HepG2 cells were transfected with siNrf2 24-hr prior to adenovirus infection. Cytotoxicity was evaluated by WST-8 and ATP assays (Figs. 2 and 3). In the WST-8 assay, the viabilities of AdCYP2C9-infected cells were significantly decreased compared with those of AdGFP-infected cells by treatment with benzbromarone (10 - 40 µM), tienilic acid (100 and 200 µM) and losartan (25 - 100 µM) (Fig. 2). On the other hand, the viabilities of AdCYP2C9-infected cells were not different from those of AdGFP-infected cells by treatment with flutamide, fluvastatin, terbinafine, valproic acid, and zolpidem, except when treated with the 100 µM fluvastatin. The ATP assay revealed a similar result as the WST-8 assay insofar as the viabilities of AdCYP2C9-infected cells were significantly decreased compared with those of AdGFP-infected cells by treatment with the benzbromarone (10 - 40 µM), tienilic acid (50 - 200 µM), and losartan (10 - 100 µM) (Fig. 3). These results suggested that the benzbromarone-, tienilic acid-, and losartan-induced cytotoxicities are caused by the metabolic activation of CYP2C9.

CYP2C9-induced Cytotoxicity in HepG2 Cells Transfected with siScramble. To investigate whether Nrf2-associated cytoprotective genes were involved in the benzbromarone-, tienilic acid-, and losartan-induced cytotoxicities mediated by
CYP2C9, the cytotoxicity was evaluated with HepG2 cells transfected with siScramble instead of siNrf2 (Fig. 4). Terbinafine was used as a negative control. With the drugs except terbinafine, the viabilities of AdCYP2C9-infected cells were significantly decreased compared with those of AdGFP-infected cells, but the differences in the viabilities between AdGFP- and AdCYP2C9-infected cells transfected with siScramble were less than those transfected with siNrf2.

Comparison of CYP2C9-mediated Cytotoxicity between Losartan and Various Sartans. Because the cell-based assay system revealed that the losartan-induced cytotoxicity involved the metabolic activation by CYP2C9, it was conceivable that other sartans with similar structures were also metabolically activated by CYP2C9. The viabilities of AdCYP2C9-infected cells were investigated by treatment with various sartans such as eprosartan, candesartan, irbesartan, olmesartan, telmisartan, and valsartan. However, no sartans other than losartan affected the cell viabilities (Fig. 5). Thus, among member of the sartan family, only losartan would be associated with CYP2C9-mediated cytotoxicity.

Detection of Semicarbazide Adducts of Losartan. The semicarbazide adducts of losartan were investigated by the positive ion mode of LC-MS/MS. It was reported that CYP3A4 is involved in the metabolism of losartan (Stearns et al., 1995). However, no cytotoxicity of losartan was induced when the cells were infected with AdCYP3A4 constructed previously (Hosomi et al., 2010) instead of AdCYP2C9 (data not shown). Therefore, to detect adducts specifically generated by CYP2C9, losartan was incubated with CYP2C9 or CYP3A4 (negative control) Supersomes. As shown in Fig. 6,
semicarbazide adducts of losartan (S1, S2 and S3) were detected in the presence of CYP2C9 Supersomes by precursor ion scan at m/z 494.2 ([M+H]^+). Because S3 was also detected when incubated with the CYP3A4 Supersomes, S3 was considered not to be involved in the CYP2C9-mediated cytotoxicity. Therefore, the subsequent study of S3 was not performed.

**Identification of Semicarbazide Adducts of Losartan.** The structures of S1 and S2 were estimated by the positive ion mode of LCMS-IT-TOF (Fig. 7). The product ion mass spectrum of losartan exhibited a major fragment ion at m/z 405.1513 (C_{22}H_{22}N_{6}Cl) (Fig. 7A). The fragment ion at m/z 405.1513 was [M + H - 18]^+ indicating the losses of H_2O from alcohol group of losartan. The product ion mass spectrum of S1 exhibited two major fragment ions at m/z 476.1606 (C_{23}H_{23}N_{9}OCl) and m/z 459.1312 (C_{23}H_{20}N_{8}OCl). The fragment ions at m/z 476.1606 and m/z 459.1312 were [M + H - 18]^+ and [M + H - 35]^+ indicating the losses of H_2O, and NH_3 and H_2O, respectively. On the other hand, the product ion mass spectrum of S2 exhibited a fragment ion at m/z 477.1477. The fragment ions at m/z 477.1477 were [M + H - 17]^+ indicating the loss of NH_3 from semicarbazide. Furthermore, the fragment ion at m/z 207.08 given from all three precursor ions indicated no conjugation with the biphenyl or tetrazole ring. The possible structures of S1 and S2 are shown in Figs. 7B and C. The fragment ions at m/z 207.08 and [M + H - 18]^+ detected in both losartan and S1 suggested that semicarbazide conjugated with somewhere in imidazole ring or the adjacent butyl side chain. In contrast, [M + H - 17]^+ instead of [M + H - 18]^+ given from S2 suggested that a reactive metabolite conjugated with semicarbazide is a hydroxylated form of E3179, an aldehyde metabolite of losartan.
Discussion

In this study, we constructed an *in vitro* cell-based assay system to evaluate the hepatotoxicity mediated by CYP2C9 and performed a cytotoxicity assay for drugs that have been known to cause hepatotoxicity. Benzbromarone and tienilic acid are converted to reactive metabolites by CYP2C9. In addition, six other hepatotoxic drugs, whose reactive metabolites generated by CYP2C9 have not been identified, although CYP2C9 is involved in their metabolism, were evaluated by our cell-based assay system. According to O’Brien et al. (2006), the cytotoxicity assay was performed within the drug concentration of 30 × the maximal efficacious concentration or 100 µM. We found that the viabilities of AdCYP2C9-infected cells were significantly decreased compared with those of AdGFP-infected cells by treatments with benzbromarone, tienilic acid, and losartan suggesting that the hepatotoxicity induced by these drugs would involve the metabolic activation by CYP2C9. Benzbromarone is metabolized via 6-hydroxybenzbromarone to the catechol by CYP2C9, followed by the oxidization of the catechol to a reactive *ortho*-quinone metabolite (McDonald and Rettie, 2007). Tienilic acid is metabolized via the sulfoxide to 5-hydroxytienilic acid by CYP2C9. This sulfoxide can form a covalent bond with CYP2C9 or other proteins (Jean et al., 1996). In rat, administration of tienilic acid in combination with the glutathione biosynthesis inhibitor, buthionine sulfoximine (BSO) induced a marked elevation of the serum alanine aminotransferase (ALT) level, but no increase in the serum ALT activity was observed in the presence of the CYP inhibitor, 1-aminobenzotioriazole (Nishiya et al., 2008). Thus, the mechanisms for the metabolic activation of these drugs have been well examined. However, to our knowledge, cell-based assays for assessment of the metabolic activations of these drugs have not been performed. The results
obtained in our cell-based assay system were in agreement with several reports that CYP2C9 is involved in the metabolic activations of benzbromarone and tienilic acid. However, there have been no reports of the involvement of CYP2C9 in the cytotoxicity of losartan. It has been reported that losartan could form protein or glutathione adducts by incubating with human liver microsomes and/or human hepatocytes, suggesting the metabolic activation of losartan (Usui et al., 2009; Gan et al., 2009). The present study firstly demonstrated that CYP2C9 was responsible for the metabolic activation of losartan. The concentrations at which the metabolic activation of losartan was observed were much higher than those in plasma in clinical practice. To predict the involvement of CYP2C9 in losartan-induced toxicity, the combination of our established cell based assay with other studies would be needed.

Sartans have generally been used as safe drugs in clinical practice, but there have been various reports of losartan-induced hepatotoxicity, which is categorized as hepatocellular injury (Tabak et al., 2002; Chang and Schiano, 2007). In some case reports, a rechallenge to losartan after ALT normalization occurred hepatotoxicities again (Bosch, 1997; Tabak et al., 2002). However, the contribution of immunological factors to losartan-induced hepatotoxicity was unknown. Losartan is metabolized to the carboxylic acid metabolite E3174, which is pharmacologically more active than the parent compound, via the aldehyde metabolite E3179, which is an intermediate in the oxidation of losartan. These biotransformations are catalyzed by CYP2C9 and CYP3A4. In addition to this pathway, the monohydroxylation of the butyl side chain is also catalyzed by CYP2C9 (Stearns et al., 1995). The viability of HepG2 cells was not decreased by treatment of E3179 and E3174 (Supplemental Fig. 1), suggesting that they may not show cytotoxicity. In this study, two CYP2C9-specific semicarbazide adducts
of losartan (S1 and S2) were detected (Figs. 6 and 7). From the fragment ions of S1 and S2, it was suggested that S2 was produced via E3179 but S1 was not (Fig. 8). The cytotoxicity of losartan induced by CYP2C9 was attenuated by the treatment with semicarbazide (Supplemental Fig. 2). Therefore, the possible reactive metabolites from S1 and S2 might be involved in the cytotoxicity. Furthermore, no significant decreases in cell viabilities were observed by treatment with various sartans other than losartan (irbesartan, valsartan, candesartan, olmesartan, telmisartan eprosartan). Taken together, it is suggested that the side chains or a chloro group beside the imidazole ring that is unique to losartan is important for the losartan-induced cytotoxicity mediated by CYP2C9.

The CYP2C9-induced cytotoxicities of benzbromarone, tienilic acid, and losartan were enhanced by Nrf2 knockdown suggesting that the genes regulated by Nrf2 are associated with detoxification of their cytotoxicities. In our recent study, CYP3A4-induced cytotoxicities of several drugs such as acetaminophen and flutamide were sensitively detected by Nrf2 knockdown (Hosomi et al., submitted). In addition, it was demonstrated that nrf2−/− mice are more vulnerable to acetaminophen-induced liver injury, due in part to lower cellular thiol levels and decreased expression of detoxification enzymes (Enomoto et al., 2001). Thus, Nrf2 is considered to play a quite important role in the detoxification of hepatotoxic drugs. Among genes regulated by Nrf2, there are various genes involved in glutathione synthesis such as glutamate cysteine ligase catalytic subunit, glutamate cysteine ligase regulatory subunit, and glutathione synthetase (Copple et al., 2008). Glutathione is an important intracellular peptide that detoxifies reactive metabolites by conjugation (Lu, 1999). In fact, reactive ortho-quinone metabolites of benzbromarone generated by CYP2C9 can be trapped...
with glutathione (McDonald and Rettie, 2007). In addition, the presence of glutathione markedly decreased the level of covalent binding of tienilic acid to microsomal proteins (Bonierbale et al., 1999). From these backgrounds, we considered whether the cytotoxicity of losartan could be clearly observed in a cytotoxicity assay with HepG2 cells transfected with siRNA for γ-glutamylcysteine synthetase heavy chain or treated with BSO instead of being transfected with siNrf2. However, no significant decreases in cell viabilities were observed by either transfection of siRNA for γ-glutamylcysteine synthetase heavy chain or treatment with BSO (data not shown). These results suggested that glutathione conjugation was not required for the detoxification of losartan-induced cytotoxicity, but that other detoxification enzymes regulated by Nrf2 would be involved. Therefore, semicarbazide was used as a trapping agent for the reactive metabolites of losartan in this study. Semicarbazide is hard nucleophiles, which will preferentially react with hard electrophiles such as aldehydes (Chauret et al., 1995). Indeed, cytotoxicity of losartan induced by CYP2C9 was attenuated by treatment with semicarbazide (Supplemental Fig. 2). Thus, it is conceivable that reactive metabolites trapped by semicarbazide are involved in the CYP2C9-induced cytotoxicity of losartan.

In the present study, CYP2C9-mediated metabolic activation was not observed with flutamide, fluvastatin, terbinafine, valproic acid, and zolpidem, which are suspected to be associated with hepatotoxicity (Thole et al., 2004; Chang and Schiano, 2007; Karsenti et al., 1999). Flutamide is hydrolyzed to 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1), which is further metabolized to N-hydroxy FLU-1, which can cause hepatotoxicity in rat (Ohbuchi et al., 2009). The N-hydroxylation of FLU-1 is catalyzed by CYP2C9 as well as CYP3A4 (Goda et al., 2006). In this study, flutamide-induced cytotoxicity could not be detected. One
possibility is that the intracellular concentration of FLU-1 was low due to the low
flutamide hydrolase activity in HepG2 cells, although it was not measured. Terbinafine
is known to be metabolized by a wide range of CYP enzymes including CYP2C9,
primarily through N-demetylation, deamination, and hydroxylation (Vickers et al.,
1999). Among its metabolites, 7, 7-dimethylhept-2-ene-4-ynal was considered to play a
role in the pathogenesis of hepatotoxicity (Iverson and Uetrecht, 2001), but this
metabolite is generated by CYP3A4, not by CYP2C9. That is why the cell viability was
not affected by treatment with terbinafine in AdCYP2C9-infected cells. For the
hepatotoxicity caused by valproic acid, the involvement of its reactive metabolites such
as 4-ene-valproic acid and 2, 4-diene-valproic acid was suggested (Baillie, 1988;
Kassahun et al., 1991; Tang et al., 1995). CYP2C9 played a role in the formation of
4-ene-valproic acid (Sadeque et al., 1997), but no involvement of CYP2C9 in the
cytotoxicity of valproic acid was observed in this study. It was reported that valproic
acid produced a high level of covalent binding in rat liver after oral administration,
although it did not bind to microsomal protein in vitro (Leone et al., 2007). Other
factors as well as CYP2C9 might be responsible for the hepatotoxicity of valproic acid.
The cytotoxicities of fluvastatin and zolpidem induced by CYP2C9 were not detected in
the present study. Until now, the mechanisms of their cytotoxicities have been unknown.
The present study suggested low involvement of CYP2C9 in their cytotoxicities,
although it is responsible for the metabolism of these drugs (Fischer et al., 1999; Von
Moltke et al., 1999).

In conclusion, we constructed a highly sensitive cell-based assay system to
evaluate CYP2C9-mediated cytotoxicity, and found for the first time that CYP2C9 is
involved in the metabolic activation of losartan. This cell-based assay system would be
useful in evaluating drug-induced cytotoxicity caused by human CYP2C9.
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AUTHORSHIP CONTRIBUTION

Participated in research design: Iwamura, Fukami, Nakajima and Yokoi.

Conducted experiment: Iwamura and Hosomi.

Contributed new reagents or analytic tools: Iwamura and Hosomi.

Performed data analysis: Iwamura and Fukami.

Wrote or contributed to the writing of the manuscript: Iwamura, Fukami and Yokoi.
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Footnotes

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A submitted article used in this study as a reference:

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Figure Legends.

Fig. 1. MOI- (A) and time- (B) dependent changes of diclofenac 4'-hydroxylase activity and CYP2C9 protein level in adenovirus (AdCYP2C9 or AdGFP)-infected HepG2 cells. HepG2 cells were infected with adenovirus for 2 days (A) or at MOI 10 (B). Diclofenac 4'-hydroxylase activity was measured as described in Materials and Methods. The CYP2C9 protein level was analyzed by immunoblotting using total cell homogenates from adenovirus-infected HepG2 cells and the representative bands were demonstrated. Data are mean ± SD (n = 3). ND: not detected. (C) Nrf2 protein level in HepG2 cells transfected with siScramble or siNrf2. HepG2 cells were infected with adenovirus (AdCYP2C9 or AdGFP) at MOI 10 for 2 days after 24-hr incubation with 10 nM siRNA. Relative band intensity of Nrf2 was normalized with the band intensity of GAPDH. Data are mean ± SD (n = 3). **P < 0.01 compared with AdGFP-infected groups transfected with siScramble.

Fig. 2. CYP2C9-induced cytotoxicity in HepG2 cells transfected with siNrf2 (WST-8 assay). HepG2 cells were infected with adenovirus at MOI 10 for 2 days after 24-hr incubation with 10 nM siNrf2. Cell viability was measured by WST-8 assay after 24-hr treatment of the test drugs. Cell viability is expressed as a percentage of cells without drug treatment. Data are mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with AdGFP-infected groups.

Fig. 3. CYP2C9-induced cytotoxicity in HepG2 cells transfected with siNrf2 (ATP assay). HepG2 cells were infected with adenovirus at MOI 10 for 2 days after 24-hr
incubation with 10 nM siNrf2. Cell viability was measured by ATP assay after 24-hr treatment of the test drugs. Cell viability is expressed as percentage of cells without drug treatment. Data are mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with AdGFP-infected groups.

**Fig. 4.** CYP2C9-induced cytotoxicity in HepG2 cells transfected with siScramble.

HepG2 cells were infected with adenovirus at MOI 10 for 2 days after 24-hr transfection with 10 nM siScramble. Cell viability was measured by WST-8 assay (A) and ATP assay (B) after 24-hr treatment of the test drugs. Cell viability is expressed as percentage of cells without drug treatment. Data are mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with AdGFP-infected groups.

**Fig. 5.** Comparison of CYP2C9-mediated cytotoxicity between losartan and various sartans. HepG2 cells were infected with adenovirus at MOI 10 for 2 days after 24-hr transfection with 10 nM siNrf2. Cell viability was measured by WST-8 assay after 24-hr treatment of the drugs. Cell viability is expressed as percentage of cells without drug treatment. Data are mean ± SD (n = 3). *P < 0.05, **P < 0.01 compared with AdGFP-infected groups.

**Fig. 6.** Ion chromatograms from LC-MS/MS analysis of the semicarbazide adducts of losartan at m/z 494.2 ([M+H]^+). (A) CYP2C9 Supersomes without semicarbazide; (B) CYP2C9 Supersomes with semicarbazide; (C) CYP3A4 Supersomes without semicarbazide; (D) CYP3A4 Supersomes with semicarbazide. Incubation and LC-MS/MS conditions were as described in Material and Methods.
Fig. 7. Predicted structures of semicarbazide adducts of losartan and MS/MS spectra of the product ion obtained by collision-induced dissociation of (A) losartan at $m/z$ 423.2 ([M+H]$^+$) and (B) S1 and (C) S2 at $m/z$ 494.2 ([M+H]$^+$). The precursor ion at $m/z$ 423.2 is semicarbazide adducts of losartan hydroxide. These spectra were scanned using LCMS-IT-TOF. Incubation and LCMS-IT-TOF conditions were as described in Material and Methods.

Fig. 8. Proposed metabolic pathways of losartan.
Figure 4

(A) Cell viability of benz bromarone and tienilic acid at different concentrations for AdGFP/siScr and AdCYP2C9/siScr.

(B) Cell viability of losartan and terbinafine at different concentrations for AdGFP/siScr and AdCYP2C9/siScr.