CYP2C9-Mediated Metabolic Activation of Losartan Detected by a Highly Sensitive Cell-Based Screening Assay

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ABSTRACT:

Drug-induced hepatotoxicity is a major problem in drug development, and reactive metabolites generated by cytochrome P450s 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 multiplicity of infection 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 in cytotoxicity by treatment 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 formation suggested that 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 found for the first time 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 That is why some drugs that were launched on the market were later withdrawn.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 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 humans and laboratory animals are a major problem in predicting the hepatotoxicity.

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ABBREVIATIONS: P450, cytochrome P450; Nr2, nuclear factor-E2 p-45-related factor; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; HPLC, high-performance liquid chromatography; WST-8, 2-(2-methoxy-4-nitropheno)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt; LC, liquid chromatography; MS/MS, tandem mass spectrometry; LCMS-IT-TOF, liquid chromatography ion trap time-of-flight mass spectrometry; MOI, multiplicity of infection; BSO, buthionine sulfoximine; ALT, alanine aminotransferase; FLU-1, 4-nitro-3-[(trifluoromethyl)phenylamine.

Cytochrome P450 (P450) 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 isofrom 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 P450 enzymes in HepG2 cells may be responsible for the fact that 30% of the compounds were falsely classified as nontoxic (Rodríguez-Antona et al., 2002; Hewitt and Hewitt, 2004). Useful in vitro cell-based assays have been established with HepG2 cells, leading to improved evaluation of drug-induced cytotoxicity. For example, our previous study showed that benzodiazepines such as flunitrazepam and nimetazepam were metabolically activated by CYP3A4 by coinucubation with HepG2 cells and CYP3A4 Superomics (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 P450 enzymes remains to be evaluated. CYP2C is the second most highly expressed P450 subfamily in human liver, and CYP2C9 is the most highly expressed isofrom in this family.
CYP2C9-MEDIATED LOSARTAN CYTOTOXICITY

(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-hydroxybenzbrorone to a catechol by CYP2C9, followed by the oxidation of the catechol to a reactive ortho-quinone metabolite (McDonald and Rettie, 2007). Tiennisic acid is metabolized to reactive intermediates, the thioephene sulfoxide or the thioephene epoxide, by CYP2C9 (Koenigs et al., 1999). In recent studies, 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 (Yoshikawa et al., 2009; Hosomi et al., 2010). 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, terfinbine, valproic acid, and zolpidem) and those that are known to be metabolically activated by CYP2C9 (benzbromaron and tiennisic acid). As a result, we found for the first time 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 tiennisic acid were obtained from Wako Pure Chemicals (Osaka, Japan). Losartan and terfinbine were obtained from LKT Laboratories (St. Paul, MN). Benzbromaron, flutamide, valproic acid, and zolpidem were obtained from Sigma-Aldrich (St. Louis, MO). Canesartan, eprosartan, irbesaran, telmisartan, and valsartan were obtained from Toronto Research Chemicals (Ontario, ON, Canada). Olmesaran was kindly provided by Daiichi-Sankyo (Tokyo, Japan). Diclofenac 4'-hydroxylatenoc and 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 (Duval 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 number NM_006164) and Stealth RNAi Negative Control Medium GC Duplex #2 were obtained from Invitrogen (Carlsbad, CA). Dulbecco’s modified Eagle’s medium was from Nissui Pharmaceutical (Tokyo, Japan). Restriction enzymes were from New England Biolabs (Ipswich, 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 Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Invitrogen), 3% glutamine, 16% sodium bicarbonate, and 0.1 M nonessential amino acids (Invitrogen) in 5% CO2 atmosphere at 37°C. Cells were infected with the adenovirus in medium containing 5% fetal bovine serum.

**Recombinant Adenovirus.** A recombinant adenovirus expressing CYP2C9 (AdCYP2C9) was constructed using the cosmid-terminal protein complex 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 site of the pXefwt vector. The use of 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 sequence; GE Healthcare, Little Chalfont, Buckinghamshire, UK). This vector and the parental adenovirus DNA terminal protein complex were cotransfected into 293 cells by Lipo-fectamine 2000 (Invitrogen). 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 x 108 and 2.1 x 108 plaque-forming units/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 electrotransfered onto a polyvinilindene difluoride membrane, Immobilon-P (Millipore Corporation, Billerica, MA). The membrane was probed with a polyclonal rabbit anti-human CYP2C9 antibody (Daiichi Pure Chemicals, Tokyo, Japan). Biotinylated anti-rabbit IgG and a 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 electrotransfered onto a polyvinilindene difluoride membrane, Immobilon-P. The membrane was probed with polyclonal rabbit anti-human Nrf2 antibody (Santa Cruz Biotechnology, Inc., San Diego, CA), and the corresponding fluorescent dye-conjugated secondary 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 were performed according to H. Hosomi, T. Fukami, A. Iwamura, M. Nakajima, and T. Yokoi (manuscript submitted for publication).

**Diclofenac 4'-Hydroxylase Activity.** HepG2 cells (3 x 105 cells/well) were seeded in 12-well plates. After a 24-h incubation, cells were infected with AdCYP2C9 or AdGFP for 1, 2, 3, or 5 days. Then, after a 1-h 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 autosam- pler (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. x 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 quantitation of 4'-hydroxydiclofenac was performed by comparing the HPLC peak height with that of an authentic standard. The limit of quantitation 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 transferase, heme oxygenase-1, NADPH:quinone oxidoreductase, superoxide dismutase, and UDP-glucuronosyltransferase (Couple et al., 2008). Our recent study demonstrated that drug-induced cytotoxicity could be detected with high sensitivity by the knockdown of Nrf2 in HepG2 cells (H. Hosomi, T. Fukami, A. Iwamura, M. Nakajima, and T. Yokoi, manuscript submitted for publication). Likewise, 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 x 104 cells/well). The concentrations of siNrf2 and siScramble were 10 nM. After 24-h incubation, the cells were infected with AdCYP2C9 or AdGFP. Forty-eight hours after infection, the cells were treated with benzbromaron, tiennisic acid, flutamide, fluvastatin, terfinbine, valproic acid, zolpidem, or sartans (canesartan, eprosartan, irbesaran, losartan, olmesaran, telmisartan, or valsartan) for 24 h. After incubation with the drugs, cell viability was quantified by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphonyl)-2H-tetrazolium monosodium salt (WST-8) and ATP assays according to the manufacturer’s protocol. The WST-8 assay, which is a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, was performed using a Cell Counting Kit-8 (CCK-8 kit; Wako Pure Chemicals). After incubation with the drugs for 24 h, CCK-8 reagent was added and the absorbance of WST-8 formazan was measured at 450 nm. The ATP assay was performed using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). After incubation with the drugs for 24 h, CellTiter-Glo Reagent was added, and the generation of a luminescent signal...
CYP2C9 overexpression (Fig. 1C).

Detection of Semicarbazide Adducts. A typical reaction mixture (final volume of 0.5 ml) contained 100 nM human CYP2C9 or CYP3A4 Super-somes, 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 performed at 37°C for 60 min and terminated by addition of 2 ml of ice-cold methanol. After centrifugation at 15,000g, the supernatant was subjected to liquid chromatography (LC)-tandem mass spectrometry (MS/MS) (400 QTRAP; Applied Biosystems, Foster City, CA). An Agilent 1200 (Agilent Technologies, Santa Clara, CA) was used as the liquid chromatograph with an Inertsil ODS-3 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 to 45% B (0–5 min), 45 to 70% B (5–55 min), 70 to 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 liquid chromatograph 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 were 50 V and 20 V, respectively. The m/z 130 to 500 was scanned at the precursor ion (m/z 494.2; semicarbazide adducts of losartan hydroxide).

Identification of Semicarbazide Adducts. A liquid chromatography ion trap time-of-flight mass spectrometry (LCMS-IT-TOF) system (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 15,000g for 5 min, the supernatant was subjected to LCMS-IT-TOF using an 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 means ± S.D. Statistical significance between the two groups was determined by a two-tailed Student’s t test. 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. 1). 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, a value that 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 a 2-day infection, although the protein levels appeared to be similar after 2- to 5-day 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 (T. Fukami, A Iwamura, M. Nakajima, and T. Yokoi, manuscript submitted for publication). This study also used 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).

Fig. 1. MOI-dependent (A) and time-dependent (B) 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 under Materials and Methods. The CYP2C9 protein level was analyzed by immunoblotting using total cell homogenates from adenovirus-infected HepG2 cells, and the representative bands are demonstrated. Data are means ± S.D. (n = 3). 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 a 24-h incubation with 10 nM siRNA. The relative band intensity of Nrf2 was normalized with the band intensity of GAPDH. Data are means ± S.D. (n = 3). **, P < 0.01 compared with AdGFP-infected groups transfected with siScramble. ND, not detected.

CYP2C9-Induced Cytotoxicity in HepG2 Cells Transfected with siNrf2. To investigate the CYP2C9-mediated metabolic activity of eight hepatotoxic drugs (benzbromarone, flutamide, fluvastatin, losartan, terbinafine, tiencilic acid, valproic acid, and zolpidem), HepG2 cells infected with AdCYP2C9 at MOI 10 for 2 days were treated with drugs for 24 h. As a negative control, AdGFP was infected at MOI 10. To improve the sensitivity, HepG2 cells were transfected with siNrf2 24 h before 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), tiencilic 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 100 μM fluvastatin. The ATP assay revealed a result similar to that of the WST-8 assay in that the viabilities of AdCYP2C9-infected cells were significantly decreased compared with those of AdGFP-infected cells by treatment with the benzbromarone (10–40 μM), tiencilic 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 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 groups.

**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 a 24-h incubation with 10 nM siNrf2. Cell viability was measured by WST-8 assay after a 24-h treatment with the test drugs. Cell viability is expressed as a percentage of cells without drug treatment. Data are means ± S.D. (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 a 24-h incubation with 10 nM siNrf2. Cell viability was measured by ATP assay after a 24-h treatment with the test drugs. Cell viability is expressed as a percentage of cells without drug treatment. Data are means ± S.D. (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared with AdGFP-infected groups.
infected cells, but the differences in the viabilities between AdGFP- and AdCYP2C9-infected cells transfected with siScramble were less than those of cells 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 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 members of the sartan family, only losartan is 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, three semicarbazide adducts of losartan (S1, S2, and S3) were detected in the presence of CYP2C9 Supersomes by a 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 (C22H22N6Cl) (Fig. 7A). The fragment ion at $m/z$ 405.1513 was $[M+H]^{+}$, indicating the losses of H2O from alcohol group of losartan. The product ion mass spectrum of S1 exhibited two major fragment ions at $m/z$ 476.1606 (C23H23N9OCl) and $m/z$ 459.1312 (C23H2ON8OCl). The fragment ions at $m/z$ 476.1606 and $m/z$ 459.1312 were $[M+H-H\text{O}]^{+}$ and $[M-H-H\text{O}]^{+}$, indicating the losses of H2O and NH3 and H2O, respectively. On the other hand, the product ion mass spectrum of S2 exhibited fragment ions at $m/z$ 477.1477. The fragment ions at $m/z$ 477.1477 were $[M+H-17]^{+}$, indicating the loss of NH3 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 Fig. 7, B and C. $m/z$ 207.08 indicated no conjugation with the biphenyl or tetrazole ring, and $[M+H-18]^{+}$ indicated the losses of H2O from alcohol group of losartan. Therefore, these two fragment ions detected in both losartan and S1 suggested that semicarbazide conjugates with another position, that is, somewhere in the 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 times 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 involves metabolic activation by CYP2C9. Benzbromarone is metabolized via 6-hydroxy-benzbromarone to the catechol by CYP2C9, followed by the oxidation 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. In rat, administration of tienilic acid in combination with the gluthathione 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 P450 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 incubation with human liver microsomes and/or human hepatocytes, suggesting the metabolic activation of losartan (Gan et al., 2009; Usui et al., 2009). The present study demonstrated for the first time 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 is 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 caused hepatotoxicities again (Borsch, 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 (irbesartan, valsartan, candesartan, olmesartan, telmisartan, and eprosartan) other than losartan. Taken together, these results suggested that the side chains or a chloro group besides 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 (H. Hosomi, T. Fukami, A. Iwamura, M. Nakajima, and T. Yokoi, manuscript submitted for publication). 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 the genes regulated by Nrf2, there are various genes involved in glutathione synthesis, such as the glutamate cysteine ligase catalytic subunit, the 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-glutamlycysteine 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-glutamlycysteine 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 a hard nucleophile, which will preferentially react with hard electrophiles, such as aldehydes (Chauret et al., 1995). Indeed, the 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 (Karsenti et al., 1999; Thole et al., 2004; Chang and Schiano, 2007). Flutamide is hydrolyzed to 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1), which is further metabolized to N-hydroxy FLU-1, which can cause hepatoxicity in rat (Obuchi et al., 2009). The N-hydroxylation of FLU-1 is catalyzed by CYP2C9 as well as by 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 because of the low flutamide hydrolyase activity in HepG2 cells, although it was not measured. Terbinafine is known to be metabolized by a wide range of P450 enzymes including CYP2C9, primarily through N-demethylation, 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 cytotoxities of fluvastatin and zolpidem induced by CYP2C9 were not detected in the present study. Until now, the mechanisms of their cytotoxities have been unknown. The present study suggested low involvement of CYP2C9 in their cytotoxities, 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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References


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