Development of a Highly Sensitive Cytotoxicity Assay System for CYP3A4-Mediated Metabolic Activation

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ABSTRACT:

Drug-induced hepatotoxicity, which is a rare but serious adverse reaction to a large number of pharmaceutical drugs, is sometimes associated with reactive metabolites produced by drug-metabolizing enzymes. P450, cytochrome P450; Nrf2, nuclear factor erythroid 2-related factor 2; GCLC, glutamate-cysteine ligase, catalytic subunit; P450 enzymes, especially CYP3A4, are involved in the reaction to a large number of pharmaceutical drugs (Boelsterli and Lim, 2007). Pharmacologically, if the reactive metabolites covalently bind to intracellular proteins, cellular dysfunctions are apparently produced, resulting in the loss of ionic gradients, a decline in ATP levels, actin disruption, cell swelling, and cell rupture (Beaune et al., 1987; Yun et al., 1993). Although drug-induced hepatotoxicity can be evaluated using laboratory animal models, species differences in drug-metabolizing enzymes or other factors between humans and laboratory animals have made the prediction of drug-induced cytotoxicity difficult. Several studies used human hepatocytes to evaluate the drug-induced cytotoxicity mediated by drug-metabolizing enzymes (Li et al., 1999; Gómez-Lechón et al., 2003), but this approach is not often applicable because of the poor availability of human liver, the high cost, the significant variability among human hepatocyte preparations, and, in particular, the unstable enzyme activities of human hepatocytes.

To date, several systems for evaluating drug-induced cytotoxicity mediated by drug-metabolizing enzymes have been developed. Yoshitomi et al. (2001) used HepG2 cells stably expressing human cytochrome P450 P450 and P450 enzymes, and the viabilities of the cells expressing CYP1A2, CYP2E1, and CYP3A4 were decreased in an acetaminophen concentration-dependent manner. Vignati et al. (2005) demonstrated, using HepG2 cells transiently transfected with CYP3A4, that reactive metabolites of various hepatotoxic drugs such as albendazole, flutamide, and troglitazone were produced by CYP3A4. Our previous study showed that benzodiazepines such as flunitrazepam and nimetazepam were metabolically activated by CYP3A4 by co-incubation with HepG2 cells and CYP3A4. Super-somes (Mizuno et al., 2009). Thus, several in vitro studies demonstrated that P450 enzymes, especially CYP3A4, are involved in the cytotoxicities of various kinds of drugs. However, transfection of P450 expression plasmids into cells generally could not achieve high expression of P450 enzymes. In addition, many studies used cells with GSH levels decreased by treatment with L-buthionine sulfoximine to easily detect the cytotoxicity. Indeed, the GSH-conjugating process is considered that cells with decreased nuclear factor erythroid 2-related factor 2 (Nrf2) are associated with the detoxification of the cytotoxicities mediated by CYP3A4. We constructed a highly sensitive cell-based system to detect the drug-induced cytotoxicity mediated by CYP3A4. This system would be beneficial in preclinical screening in drug development and increase our understanding of the drug-induced cytotoxicity associated with CYP3A4.
factor 2 (Nrf2) levels would also be useful to sensitively detect the cytotoxicity, because Nrf2 is a transcription factor that acts as a main regulator for the up-regulation of a group of genes coding antioxidant proteins and phase II drug-metabolizing enzymes, such as NADPH-quinone oxidoreductase 1, heme oxygenase-1, and glutamate-cysteine ligase, catalytic subunit (GCLC) and modifier subunit (GCLM) (Thimmulappa et al., 2002; Balogun et al., 2003; Nioi et al., 2003). In fact, it has been reported that Nrf2 knockout mice had increased susceptibility to acetaminophen (Chan et al., 2001).

In a previous study, we established an adenovirus that could overexpress human CYP3A4 (AdCYP3A4) (Hosomi et al., 2010). In this study, we developed a highly sensitive in vitro cell-based assay system using AdCYP3A4 and investigated whether CYP3A4 is involved in the cytotoxicity of 23 drugs that are known to cause hepatotoxicity in humans. In addition, we investigated whether treatment with siNrf2 exacerbates the drug-induced cytotoxicity mediated by CYP3A4.

Materials and Methods

Chemicals and Reagents. Acetaminophen, allopurinol, clozapine, corticosterone, cyclizine, dantrolene sodium, disulfiram, erythromycin, felbamate, 3-hydroxyacetanilide (3-HAA), meprobamate, nefazodone, nitramide, rosiglitazone, sulindac, tacrine, terbinafine, testosterone, tolcapone, testosterone, 17β-hydroxytestosterone, a metabolite of testosterone by CYP3A4, seven hepatoma cell lines (HepG2, Hep3B, HLE, HLF, Huh6, Huh7, and Fa2N4) were infected with AdCYP3A4 at MOI 10, and the testosterone 6β-hydroxylase activity was measured at a concentration of 100 μM testosterone, the concentration of 6β-hydroxylase activity was measured at a

**CYP3A4 Enzyme Activity.** AdCYP3A4 was constructed in our previous study (Hosomi et al., 2010). HepG2 cells (3 × 10^4 cells/well) were seeded in 12-well plates. After a 24-h incubation, the cells were infected with AdCYP3A4 for 48 h at a multiplicity of infection (MOI) of 10 (MOI 10). Then, after a 1-h incubation with 100 μM testosterone, the concentration of 6β-hydroxysteroidosterone, a metabolite of testosterone by CYP3A4, in the medium was measured as described previously (Hosomi et al., 2010).

**Cytotoxicity Assay.** HepG2 cells (2.0 × 10^5 cells/well) were seeded in 96-well plates and, if necessary, were treated with siNrf2 or siScramble as described above. After a 24-h incubation, the cells were infected with AdCYP3A4 or the recombinant adenovirus vector expressing green fluorescence protein (AdGFP) constructed in our previous study (Hosomi et al., 2010). The titers of AdCYP3A4 and AdGFP were 6.4 × 10^8 and 2.1 × 10^8 plaque forming units/ml, respectively. Forty-eight hours after infection at MOI 10, the cells were treated with various drugs for 24 h. After incubation with the drugs, cell viability was quantified by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-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 bromide assay, was performed using Cell Counting Kit-8 (Wako Pure Chemicals). After incubation with the drugs, Cell Counting Kit-8 reagent was added, and absorbance of WST-8 formazan was measured at 405 nm. The ATP assay was performed using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). After incubation with the drugs, CellTiter-Glo Reagent was added, and the generation of luminescent signals was recorded by using a 1420 ARVO MX luminometer (PerkinElmer Life and Analytical Sciences-Wallace Oy, Turku, Finland).

**Statistical Analyses.** Comparisons of two and several groups were made with a unpaired, two-tailed Student’s t test and Dunnett’s test, respectively. P < 0.05 was considered statistically significant.

**Results**

**CYP3A4 Enzyme Activity in Various Hepatoma Cell Lines Infected with AdCYP3A4.** To determine which hepatoma cell line is suitable for the evaluation of drug-induced cytotoxicity mediated by CYP3A4, seven hepatoma cell lines (HepG2, Hep3B, HLE, HLF, Huh6, Huh7, and Fa2N4 cells) were infected with AdCYP3A4 at MOI 10, and the testosterone 6β-hydroxylase activity was measured at a concentration of 100 μM (Fig. 1). Among these cell lines, HepG2 cells showed the highest enzyme activity (670 pmol · min^-1 · mg protein^-1). Huh7 cells also showed moderate enzyme activity (233 pmol · min^-1 · mg protein^-1), but the other cells showed activity at less than 100 pmol · min^-1 · mg protein^-1. From this result, HepG2 cells were used in the subsequent studies.

**Cytotoxic Effects of CYP3A4 on Cell Viability of HepG2 Cells.** To investigate whether CYP3A4 is associated with the cytotoxicity of 23 drugs (acetaminophen, allopurinol, amiodarone, clozapine, cyclizine, dantrolene sodium, disulfiram, erythromycin, felbamate, 3-hydroxyacetanilide (3-HAA), meprobamate, nefazodone, nitramide, rosiglitazone, sulindac, tacrine, terbinafine, testosterone, tolcapone, testosterone, 17β-hydroxytestosterone, a metabolite of testosterone by CYP3A4, seven hepatoma cell lines (HepG2, Hep3B, HLE, HLF, Huh6, Huh7, and Fa2N4 cells) were infected with AdCYP3A4 at MOI 10, and the testosterone 6β-hydroxylase activity was measured at a concentration of 100 μM (Fig. 1). Among these cell lines, HepG2 cells showed the highest enzyme activity (670 pmol · min^-1 · mg protein^-1). Huh7 cells also showed moderate enzyme activity (233 pmol · min^-1 · mg protein^-1), but the other cells showed activity at less than 100 pmol · min^-1 · mg protein^-1. From this result, HepG2 cells were used in the subsequent studies.
CYP3A4 enzyme activity in various cell lines infected with AdCYP3A4. Cells were infected with AdCYP3A4 at MOI 10 for 48 h, and the testosterone 6β-hydroxylase activity was measured as described under Materials and Methods. Data are the means of two independent experiments.

Drug-induced hepatotoxicity is a rare but serious adverse reaction to a large number of pharmaceutical drugs (Boelsterli and Lim, 2007). Because drug-induced hepatotoxicity is sometimes associated with reactive metabolites produced by drug-metabolizing enzymes (Guengerich and MacDonald, 2007), it would be ideal to be able to predict the hepatotoxicity mediated by these enzymes using an in vitro system. In the present study, we constructed a highly sensitive cell-based system to detect the cytotoxicity of drugs using HepG2 cells expressing CYP3A4, which is a predominant P450 isoform in human liver that is responsible for more than 50% of drug metabolism (Guengerich, 2008).

In general, infection of cells with a recombinant adenovirus can lead to high expression of specific genes of interest. In our previous study, we introduced the overexpression of CYP3A4 protein in rat hepatoma cells, H4IIE cells, by the infection of AdCYP3A4 (Hosomi et al., 2010). Likewise, in this study, seven human hepatoma cell lines were infected with AdCYP3A4, and the testosterone 6β-hydroxylase activity was measured. Among them, HepG2 cells showed the highest enzyme activity (Fig. 1). This result might reflect the fact that HepG2 cells express high endogenous levels of NADPH-P450 reductase and cytochrome b5 (Aoyama et al., 1990) in addition to the high efficiency of infection. The testosterone 6β-hydroxylase activity of AdCYP3A4-infected HepG2 cells (670 pmol·min⁻¹·mg protein⁻¹ at 100 μM testosterone) was more than 2.5-fold higher than the Vₘₙₙ values of the activity in human hepatocytes reported by Donato et al. (1995) and Gómez-Lechón et al. (2001) (177 ± 98 and 253 ± 110 pmol·min⁻¹·mg protein⁻¹, respectively). Given the large interindividual variability in CYP3A4 protein expression (a 32-fold variability) (Westlind-Johnsson et al., 2003), the cells used in this study would mimic human hepatocytes that exhibit the highest CYP3A4 enzyme activity.

In this study, we investigated the CYP3A4-mediated cytotoxicity of 23 drugs that are known to cause hepatotoxicity in humans (Xu et al., 2008). The viability of cells infected with AdCYP3A4 was significantly decreased by treatment with amiodarone, desipramine, disulfiram, isoniazid, leflunomide, nefazodone, nitrofurantoin, tacrine, terbinafine, and tolcapone compared with those infected with AdGFP. It has already been reported that the hepatotoxicity of some drugs is associated with the metabolic activation mediated by CYP3A4. The major metabolite of amiodarone, desethylamiodarone, was reported to...
cause cytotoxicity in HepG2 cells and rat hepatocytes at lower concentrations than the parent drug amiodarone (McCarthy et al., 2004). Desethylamiodarone is produced mainly by CYP3A4 and by CYP2C8 in humans (Ohyama et al., 2000). Erythromycin-associated hepatic injury is usually cholestatic and may mimic obstructive jaundice (Klatskin, 1975). Although CYP3A4 is involved in erythromycin

FIG. 2. Cytotoxic effects of CYP3A4 expression on cell viability of HepG2 cells treated with various drugs. HepG2 cells were infected with AdCYP3A4 (●) or AdGFP (○) at MOI 10 for 48 h and treated with 23 drugs for 24 h. Cell viability was measured by the WST-8 assay. Each point represents the mean ± S.D. (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared with the AdGFP group at each concentration of the drug.
duced hepatotoxicity has been reported, although most of these drugs were metabolized to hydroxynefazodone, followed by the formation of quinone-imine and 2-chloro-1,4-benzoquinone, which would be associated with allopurinol (Al-Kawas et al., 1981; Arellano and Uetrecht, 2003). However, these kinds of adverse effects were not demonstrated to be potent at 50 μM desipramine and nitrofurantoin. The cytotoxicity of parent drugs was demonstrated to be potent at 50 μM desipramine and nitrofurantoin and amiodarone (Fig. 2, ○). Thus, the effect of metabolic activation by CYP3A4 could not be detected at 50 μM concentrations of the drugs (Fig. 2), indicating that it should be considered the cytotoxicity of active metabolite(s) as well as that of the parent drugs at the various concentrations of drugs used in this in vitro assay system.

Nrf2 is the main regulator of the up-regulation of genes associated with detoxification reactions and is known to be constitutively and ubiquitously expressed in several tissues and cell lines. In the absence of CYP1A2, which is reported to be associated with the formation of iminoquinone from tacrine and tolcapone (Balson et al., 1995; Smith et al., 2003). Furthermore, CYP2E1 is considered to be associated with isoniazid-induced hepatotoxicity by metabolizing acetylamidizone, one of the metabolites of isoniazid, to N-acetylamidizone (Huang et al., 2003). Further studies on the involvement of CYP3A4 in the metabolic activation of these drugs are needed. Although pharmacological drug interactions were reported in cyclizine, a metabolic activation pathway would not be involved.

Nitroaromatic drugs such as tacrine and nitrofurantoin have been associated with liver injury (Boelsterli et al., 2006). In the reductive pathways from nitro to the fully reduced amine catalyzed mainly by cytochrome P450 reductase, several reactive metabolites including nitroso and N-hydroxylamine derivatives could be produced. The reductive metabolite of tacrine is produced by cytochrome P450 reductase in HepG2 cells, and the enhanced reactive oxygen species production results in cytotoxic effects (Osseni et al., 1999). Cytochrome P450 reductase is also involved in nitrofurantoin-induced redox cycling and cytotoxicity (Wang et al., 2008). There is no report suggesting the involvement of CYP3A4 in the metabolic activation of tacrine and nitrofurantoin. From these lines of evidence, the positive cytotoxic effects of tacrine and nitrofurantoin in Fig. 2 would be due to the cytochrome P450 reductase activity, which is highly expressed in tumor cells compared with hepatocytes. Flutamide is known to induce severe hepatic dysfunction. Obubichi et al. (2009) suggested that CYP3A4 catalyzed the N-oxidation of the amino metabolite of flutamide, which had hepatotoxic effects. Dantrolene was also implicated in the generation of reactive oxygen species via cytochrome P450 reductase. However, we previously reported that CYP3A4-mediated cytotoxicity was not involved in the effects of the nitroaromatic drugs, flutamide and dantrolene, which we investigated using CYP3A4-expressing rat BRL3A cells (Yoshikawa et al., 2009), supporting our present data in Fig. 2. Although the cytochrome P450 reductase-mediated redox reactions were suggested in flutamide- and dantrolene-induced hepatotoxicities, the underlying mechanism for metabolic activation is likely to be different for different drugs, producing different cytotoxic outcomes, an issue that needs to be clarified.

Felbamate induces the activity of CYP3A4, which accounts for the in vivo drug interactions in human (Glue et al., 1997). It was reported that felbamate heteroactivates the CYP3A4-mediated pathway (Egnell et al., 2003). However, these kinds of adverse effects were not detected in the present assay system, because only the cytotoxic effect by the CYP3A4-mediated metabolic activation of drug could be assessed. In addition, hepatotoxicity and hypersensitivity syndromes are associated with allopurinol (Al-Kawas et al., 1981; Arellano and Sacristán, 1993). Although the involvement of the CYP3A4-mediated reaction was still not clear, this kind of hepatotoxicity was also not detected in the present system.

A cytotoxic effect of metabolic activation of drug by CYP3A4 (Fig. 2, ◇) was clearly demonstrated in a dose-dependent manner at 10 and 25 μM desipramine and nitrofurantoin. The cytotoxicity of parent drugs was demonstrated to be potent at 50 μM desipramine and nitrofurantoin and amiodarone (Fig. 2, ◇). Thus, the effect of metabolic activation by CYP3A4 could not be detected at 50 μM concentrations of the drugs (Fig. 2), indicating that it should be considered the cytotoxicity of active metabolite(s) as well as that of the parent drugs at the various concentrations of drugs used in this in vitro assay system.
of cellular stress, Nrf2 is localized in the cytosol by binding with Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1. When cells are exposed to oxidative stress, the newly synthesized Nrf2 accumulates in the nucleus (Kobayashi et al., 2006). However, it has been reported that Nrf2 is localized in the nucleus in HepG2 cells under nonstressed conditions (Nguyen et al., 2005). In the present study, HepG2 cells with decreased Nrf2 expression were used to detect the drug-induced cytotoxicity more sensitively. The Nrf2 mRNA level in HepG2 cells could be decreased to less than 10% by siNrf2 (Fig. 3A). Given that the Nrf2 mRNA level in HepG2 was 1.6-fold higher than that in human hepatocytes (data not shown), the HepG2 cells used in this study had approximately 20% of the Nrf2 mRNA expression as that in human hepatocytes.

The present study was conducted as shown in Fig. 4 with four drugs (troglitazone, flutamide, acetaminophen, and clozapine) that did not show cytotoxicity in AdCYP3A4-infected HepG2 cells without siNrf2 and two drugs that did show cytotoxicity (desipramine and terbinafine). The viabilities of cells transfected with siNrf2 were significantly decreased by treatment with the drugs compared with those transfected with siScramble (Fig. 4). These results suggest that the genes regulated by Nrf2 play important roles in the detoxification of the drug-induced cytotoxicities activated by CYP3A4. It has been reported that troglitazone is metabolized to quinone-type forms, which may be the reactive metabolites produced by CYP3A4 and CYP2C8 (Yamazaki et al., 1999). Although it has been reported that a postulated reactive metabolite of clozapine was produced by myeloperoxidase, leading to depletion of the cellular GSH level (Williams et al., 1997), there is no information about the association of CYP3A4 with the cytotoxicity of clozapine. Flutamide is reported be oxidized into reactive metabolites that covalently bind to microsomal proteins metabolized by CYP3A4 and CYP1A2 (Berson et al., 1993; Fau et al., 1994). In addition, acetaminophen is metabolized to N-acetyl-p-benzoquinone imine, a postulated ultimate reactive metabolite, by CYP2E1 and CYP3A4 in humans. These reactive metabolites could be trapped with GSH in a process that is the major detoxification pathway. Because GCLC, GCLM, and glutathione peroxidase, which are important factors for maintaining the cellular GSH level, are regulated by Nrf2 (Thimmulappa et al., 2002), the decreased viability of AdCYP3A4-infected cells with siNrf2 by these drugs may be due to the decreased level of cellular GSH. The reactive metabolite of
terbinafine, TBF-A, is known to be trapped with GSH (Iverson and Uetrecht, 2001). The viability of AdCYP3A4-infected HepG2 cells was decreased by treatment with terbinafine even without siNrf2 transfection. This result was different from those by the treatment with troglitazone, flutamide, and acetaminophen. In addition, information about the association of CYP3A4/Nrf2 with desipramine-induced hepatotoxicity is limited, although epoxide formation and nitrosou- lane species were suggested to be related to the toxicity.

The WST-8 assay is a modified 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide assay, a conventional cytotoxicity as- say, that uses a highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator. The WST-8 assay was used to measure the mitochondrial NADH enzymes as an indicator of cell viability. The ATP assay was used to measure the cellular concentration of ATP, which is produced in mitochondria. Thus, in both assays, the cell viability was evaluated by measuring the mitochondrial activity, but the sensitivity of the ATP assay is lower than that of the WST-8 assay (Fig. 4), and the result should therefore be carefully evaluated. 

Many researchers have used cells transfected with P450 expression plasmids to investigate the drug-induced cytotoxicity mediated by P450s, but this system generally could not induce high expression of P450 enzymes. However, our established adenosivirus-using system induced the overexpression of CYP3A4 in HepG2 cells, resulting in more than 2.5-fold higher enzyme activity than that of human hepa- tocytes. In addition, the transfection of siNrf2 to HepG2 cells could decrease to approximately 20% the Nrf2 expression level in human hepatocytes. Thus, the combination of AdCYP3A4 infection with the transfection of siNrf2 would enable sensitive detection of the drug- induced cytotoxicity mediated by CYP3A4.

In conclusion, we constructed a highly sensitive cell-based system to detect the drug-induced cytotoxicity mediated by CYP3A4. Fur- thermore, we found that a decrease in the level of Nrf2 could increase the sensitivity of detection for drug-induced cytotoxicity. It is gener- ally known that very complicated mechanisms are involved in drug- induced liver injury and the basic mechanism is likely to be different for different drugs. Therefore, this in vitro assay system is not likely to predict in vivo drug-induced liver injury. However, this system would be beneficial in the preclinical screening in drug development and increase our understanding of the drug-induced cytotoxicity asso- ciated with CYP3A4.

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Authorship Contributions
Participated in research design: Hosomi, Fukami, Nakajima, and Yokoi.
Conducted experiments: Hosomi and Iwamura.
Performed data analysis: Hosomi, Fukami, and Iwamura.
Wrote or contributed to the writing of the manuscript: Hosomi, Fukami, and Yokoi.

References
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