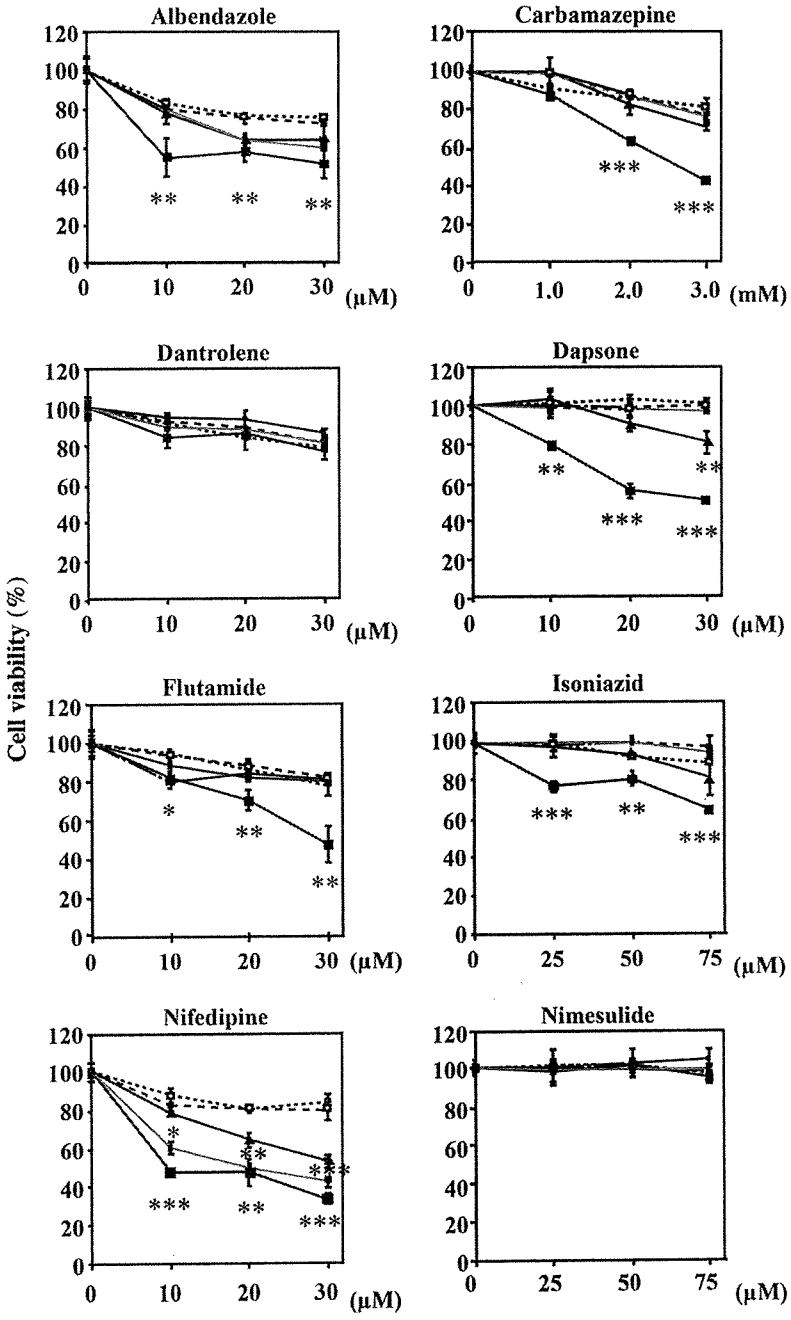

Fig. 5. Detection of SOD2- and CYP3A4-mediated cytotoxicity in BRL3A cells treated by various drugs. Data are mean ± SD ($n = 3$). Reproducibility of the data was confirmed by three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with AdLuc-shRNA infected cells. Statistical analyses were performed by ANOVA.

with both AdCYP3A4 and AdSOD2-shRNA were significantly increased compared to the other adenovirus infected groups. By treatment with carbamazepine (3.0 mM) or isoniazid (75 μM) for 24 h, ROS production was significantly increased in the groups infected with both AdCYP3A4 and AdSOD2-shRNA, but superoxide production was not changed. By treatment with flutamide (30 μM) or zidovudine (75 μM) for 24 h, ROS and superoxide productions in each group were not significantly different despite the significant decrease of the cell viabilities. By treatment with dantrolene (30 μM), nimesulide (75 μM), and rosiglitazone (75 μM) for 24 h, ROS or superoxide productions were not significantly changed, and these results were correlated with the cell viabilities. By treatment with drugs for 5 h, ROS productions were not significantly changed in each group (data not shown).

3.7. Changes of lipid peroxidation in adenovirus infected BRL3A cells

Lipid peroxidation was known as one of biomarkers of oxidative stress, thus its measurement was performed. BRL3A cells were infected with adenovirus for 2 days and then treated with the drugs used in the MTT assay for 24 h. The measurement of lipid peroxidation was conducted at the drug concentrations demonstrating the biggest differences in cell viabilities between co-infection of AdCYP3A4 and AdSOD2-shRNA and No Ad-treatment

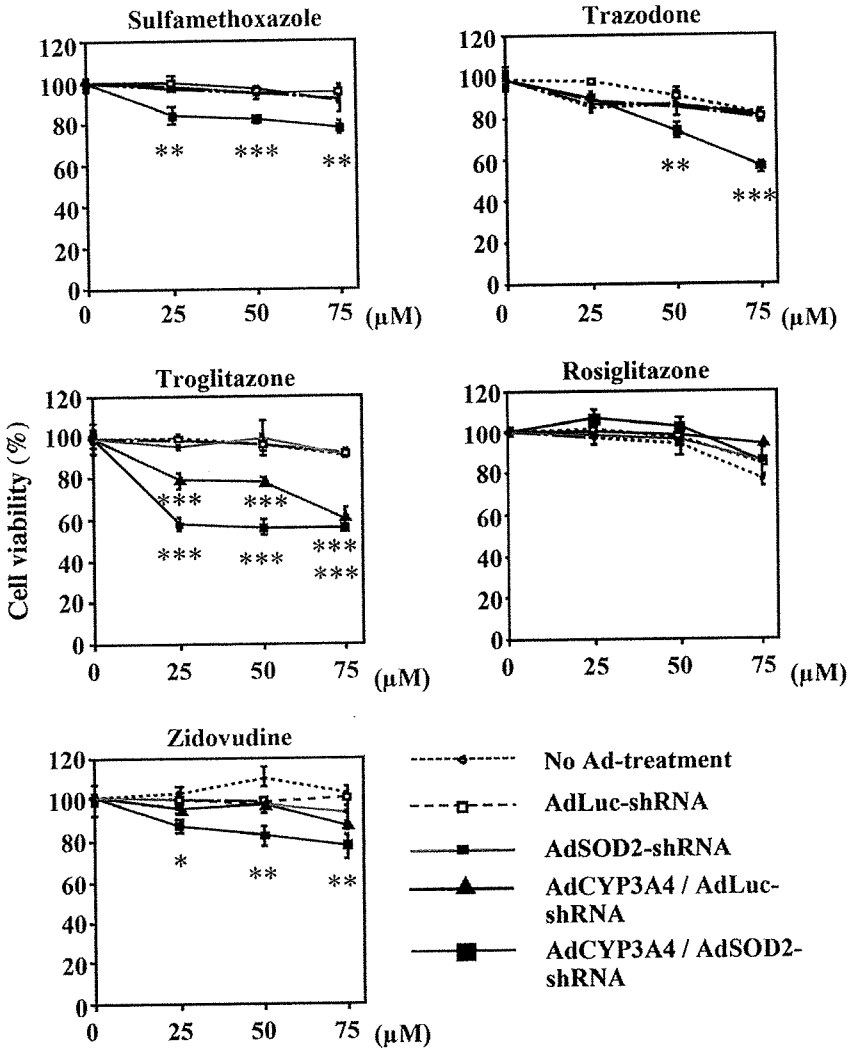

Fig. 5 (*continued*)

groups (Fig. 7). By treatment with albendazole (30 μM), carbamazepine (3.0 mM), dapsone (30 μM), flutamide (30 μM), nifedipine (30 μM), sulfamethoxazole (75 μM), trazodone (75 μM), or troglitazone (50 μM), lipid peroxidations in the groups infected with both AdCYP3A4 and AdSOD2-shRNA were significantly increased compared to the other adenovirus infected groups. By treatment with dantrolene (30 μM), isoniazid (75 μM), nimesulide (75 μM), rosiglitazone (75 μM), and zidovudine (75 μM), lipid peroxidations were not significantly changed.

4. Discussion

In this study, we constructed a recombinant adenovirus vector expressing shRNA-directed rat superoxide dismutase 2 (AdSOD2-shRNA). In BRL3A cells, the SOD2 mRNA was decreased MOI dependently (Fig. 1). At MOI 200, some cells floated and SOD2 mRNA was decreased, but glyceraldehydes-3-phosphate (GAPDH) mRNA was not changed, suggesting slight toxicity by the adenovirus itself was appeared. Thus, infection of AdSOD2-shRNA at MOI 100 was an appropriate condition to suppress the SOD2 mRNA without cytotoxic effects. On the other hand, SOD1, other isoform of cellular SOD, was not changed by infection of AdSOD2-shRNA (data not shown). In addition, co-infection with AdCYP3A4 did not affect on the expression of SOD2 and on the ability of AdSOD2-shRNA to reduce SOD2 expression (data not shown).

The target sequence of the rat SOD2-shRNA differs from those of mouse (63% homology) and human (58% homology). In AdSOD2-shRNA infected BRL3A cells, SOD2 mRNA was significantly decreased compared with the AdLuc-shRNA control group (Fig. 2). In H4IIE cells, SOD2 mRNA was not significantly decreased compared with the control group, and the cells became unhealthy. In Hepa1–6 and HLE cells, SOD2 mRNA was not changed because of low homologies of the target sequence of shRNA in mouse and human. The SOD2 mRNA, protein and activity were significantly suppressed after 3 days of AdSOD2-shRNA infection (Fig. 3), which was similar to our previous study using adenovirus expressing shRNA against rat γ-glutamylcystein synthetase heavy chain subunit (AdGCSh-shRNA) (Akai et al., 2007).

In general, since cultured cell lines are known to hardly express CYP enzymes, CYP3A4 in BRL3A cells was expressed. CYP3A4 is the dominant CYP3A enzyme in the liver and intestine (Rendic and Di Carlo, 1997; Shimada et al., 1994), and contributes to the metabolism of more than 50% of clinically used drugs (Rendic and Di Carlo, 1997; Nelson, 1999; Guengerich, 2001). In addition, metabolic activations of some drugs by CYP3A4 are thought to induce

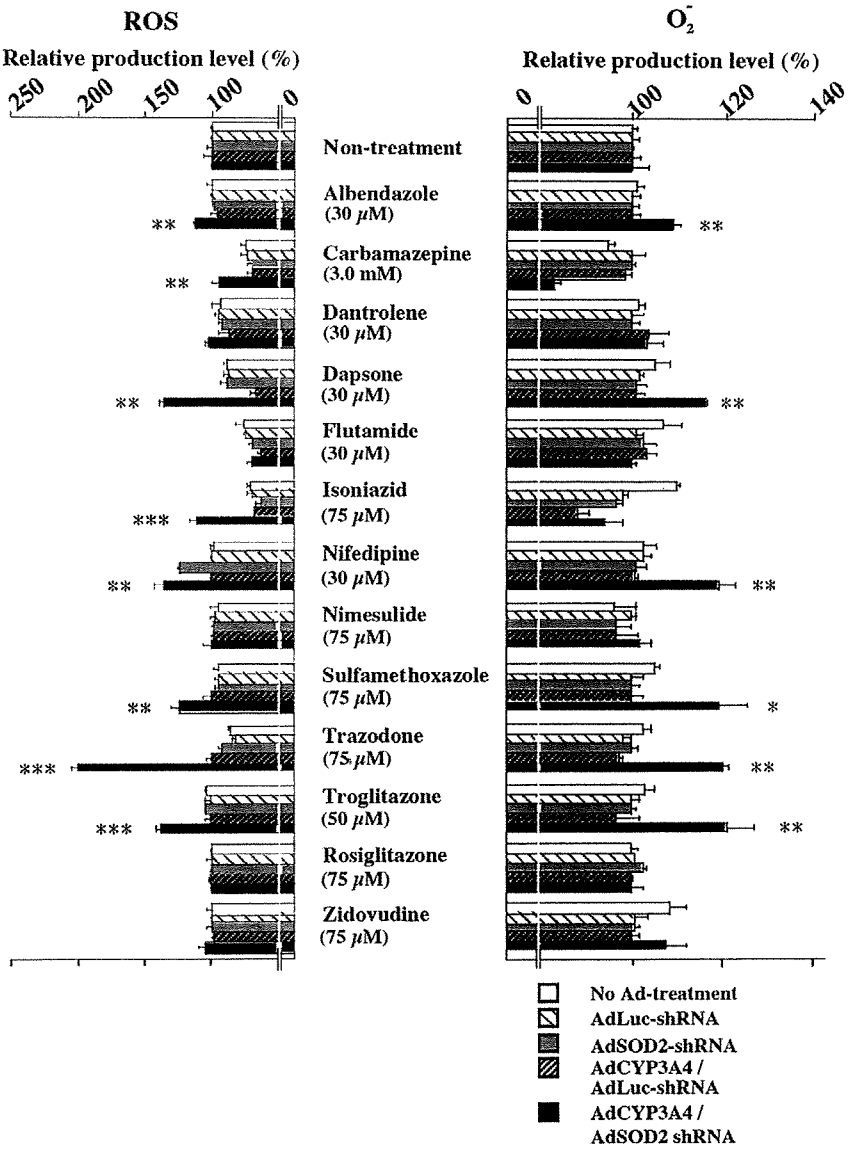

Fig. 6. Changes of SOD2- and CYP3A4-mediated ROS and superoxide productions in BRL3A cells treated by various drugs. Data are mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with AdLuc-shRNA infected cells. Statistical analyses were performed by ANOVA.

hepatotoxicity (Vignati et al., 2005). Therefore, CYP3A4 was thought to be more important than other CYP isoforms for investigating the drug-induced cytotoxicity. In the present study, testosterone 6β-hydrolation, CYP3A4 activities, (50 pmol/min/mg protein) were nearly equal to those of human hepatocytes (30–100 pmol/min/mg protein) (Venkataramanan et al., 2000: Runge et al., 2000), and co-infection of AdSOD2-shRNA and AdCYP3A4 did not affect the CYP3A4 activities (Fig. 4).

In the cell viability study, albendazole, carbamazepine, dapsone, flutamide, isoniazid, nifedipine, sulfamethoxazole, trazodone, troglitazone, and zidovudine demonstrated significant decreases of the cell viabilities in the group co-infected with AdCYP3A4 and AdSOD2-shRNA compared with the group co-infected with AdCYP3A4 and AdLuc-shRNA (Fig. 5). To our knowledge, these 13 drugs, except nifedipine and rosiglitazone, were reported to induce hepatotoxicity *in vitro* and/or *in vivo* in human (Vignati et al., 2005: Jayyosi et al., 1993: Vyas et al., 2006: Durham et al., 1984: Kalgutkar et al., 2005: Corcuera et al., 1996). Furthermore, these 13 drugs, except nimesulide and rosiglitazone, were reported to be metabolized by CYP3A4 (Vignati et al., 2005: Jayyosi et al., 1993: Kenworthy et al., 1999: Kalgutkar et al., 2005: Veal and Back, 1995). Among them, flutamide and troglitazone were reported to be bioactivated by CYP3A4 (Goda et al., 2006: He et al., 2001). In the present study, we found that both SOD2 and CYP3A4 were involved in the cytotoxicity of flutamide, and the cytotoxicity of troglitazone in CYP3A4-overexpressing cells was significantly increased by knockdown of SOD2. The cytotoxicities demonstrated in the 10 drugs in Fig. 5, except nifedipine, were mediated by co-infection of AdSOD2-shRNA and AdCYP3A4. With nifedipine, cytotoxicity was observed only by knockdown of SOD2, and co-infection of AdSOD2-shRNA and AdCYP3A4 increased the cytotoxicity. This result is interesting and mechanism should be established. In the present study, three times independent experiments in Fig. 5 were repeated, and the each data was <10% variance in all three experiments. This is the first report that the knockdown of SOD2 with CYP3A4-overexpression increased the cytotoxicities in these 10 drugs. However, we should investigate the cytotoxicity-related metabolites in detail in the near future.

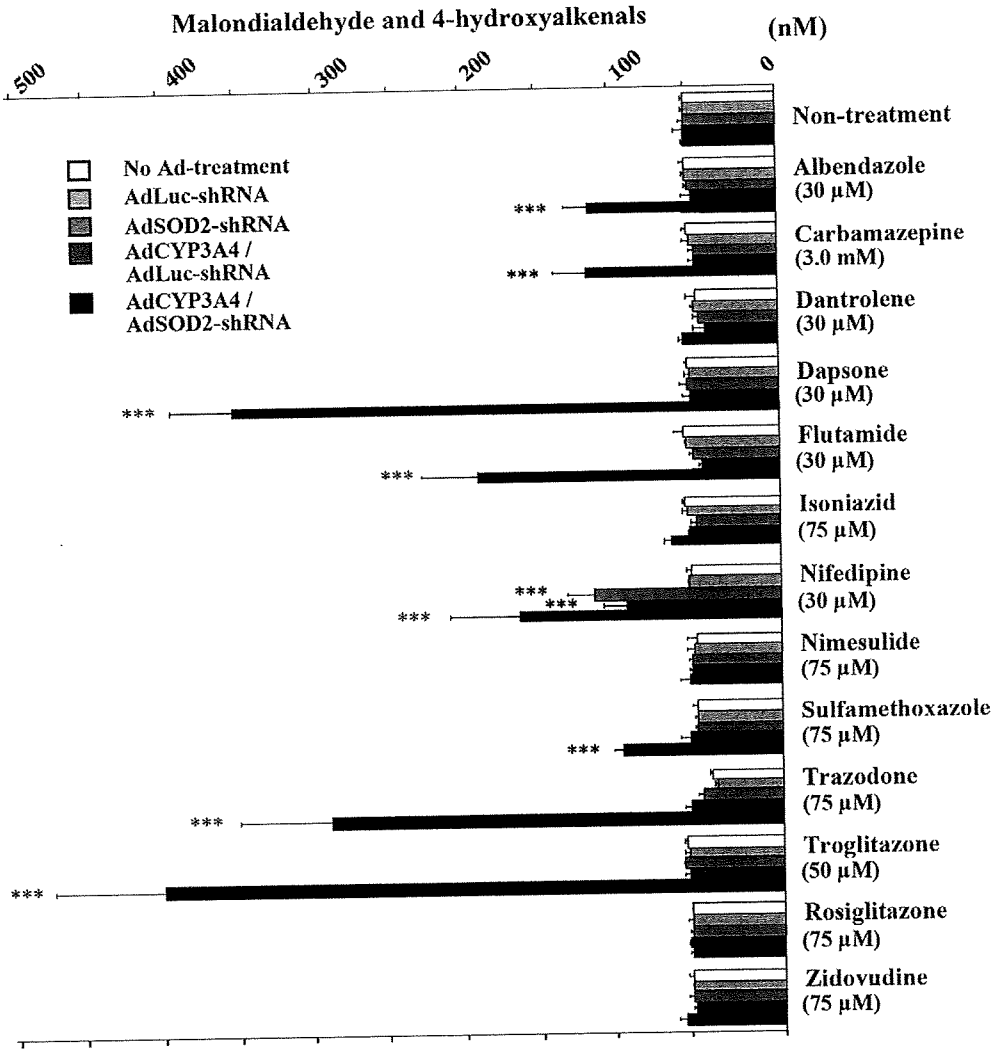

Fig. 7. Changes of SOD2- and CYP3A4-mediated lipid peroxidation in BRL3A cells treated by various drugs. Data are mean ± SD ($n = 3$). $^{***}P < 0.001$ compared with AdLuc-shRNA infected cells. Statistical analyses were performed by ANOVA.

In the study of ROS and superoxide productions (Fig. 6) and lipid peroxidations measurements (Fig. 7), albendazole, dapsone, nifedipine, sulfamethoxazole, trazodone, and troglitazone demonstrated similar cell viabilities (Fig. 5). With carbamazepine, ROS production and lipid peroxidation were increased, suggesting that oxidative stress was occurred. With isoniazid, only ROS production was increased, suggesting that the production of superoxide was lower than with albendazole, dapsone, nifedipine, sulfamethoxazole, trazodone, or troglitazone. These results suggested that the production of the active metabolites by CYP3A4 resulted in mitochondrial toxicity, and then superoxide production was increased. In flutamide, ROS and superoxide productions were not significantly increased, but lipid peroxidation was increased, suggesting that oxidative stress was involved in its hepatotoxicity, but not involved in ROS and superoxide production. The hepatotoxicity of flutamide was reported to be induced by superoxide production *in vitro* (Boelsterli et al., 2006), thus the mechanism of hepatotoxicity in our study was different. In zidovudine, ROS and superoxide productions and lipid peroxidation were not significantly increased, suggesting that oxidative stress is not likely to be the direct cause of cytotoxicity. For zidovudine, the mechanism of the cytotoxicity was unknown. In the present study, ROS and superoxide productions and cytotoxicity were measured after 24 h of drug exposure. The rate of active metabolite production will differ in each drug, and half-life of superoxide is very short. Thus, cytotoxicity and ROS and superoxide productions were not correlated in some drugs, and cytotoxic effects in relation to ROS and superoxide productions will not be able to discuss quantitatively in this study. However, the mechanism of cytotoxicity of drugs was supported by the present cell system. By the nimesulide and rosiglitazone treatments, ROS and superoxide productions and lipid peroxidations showed no difference. Rosiglitazone is known to be metabolized mainly by CYP2C8 rather than CYP3A4 (Baldwin et al., 1999), and have a low risk for cytotoxicity (Werner and Travaglini, 2001).

We previously constructed glutathione knockdown rats and demonstrated acetaminophen hepatotoxicity with high sensitivity (Akai et al., 2007). In the near future, we will apply AdSOD2-shRNA to rat to investigate the SOD2-mediated drug-induced hepatotoxicity *in vivo*.

In conclusion, this is the first report of an SOD2-knockdown cell model constructed by using AdSOD2-shRNA. We constructed an SOD2-knockdown and CYP3A4-expressed cell system, and successfully applied it to evaluate the SOD2- and CYP3A4-mediated cytotoxicity in several clinically used drugs with high sensitivity and

with small intra- and inter-laboratory difference. This *in vitro* cytotoxicity model would be a useful tool in preclinical drug development.

Conflict of Interest Statement

None of the authors has any conflicts of interest related to this manuscript.

Acknowledgments

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Drug-induced hepatotoxicity test using γ-glutamylcysteine synthetase knockdown rat

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ABSTRACT

Idiosyncratic drug-induced liver injury (DILI) is a major clinical problem for drug development. It is generally known that DILI is mainly caused by hepatic glutathione (GSH) depletion. The glutathione *S*-transferase activity of rodent is higher than that of human, which could make the prediction of DILI more difficult. Recently, we reported that an experimental rat model of GSH-depletion displayed high susceptibility to acetaminophen-induced hepatotoxicity. To deplete GSH, we used an adenovirus vector with short hairpin RNA against γ-glutamylcysteine synthetase heavy chain subunit (AdGCSh-shRNA). In this study, we further investigated the usefulness of this rat model for determining drug-induced sensitive acute and subacute toxicity. Rats were administered diclofenac and flutamide which have been reported as idiosyncratic hepatotoxic drugs. In the acute (6 or 24 h) or subacute (7 days) toxicity tests, rats were administered the drugs once or once a day for a week, respectively. Plasma biochemical markers for hepatotoxicity were measured. The 6 and 24 h toxicity test of diclofenac, and the 24 h and 7 days toxicity tests of flutamide showed significant ALT elevations. Additionally, the 24 h toxicity test of flutamide showed a slight bilirubin elevation, and histological hepatotoxicity. The 7 days toxicity test of flutamide also demonstrated histological hepatotoxicity. In conclusion, this rat model would contribute to evaluating acute and subacute DILI in preclinical drug development.

1. Introduction

Drug-induced liver injury (DILI) rarely appears but is a serious adverse event induced by a large number of clinical drugs (Makarova, 2008). In drug development, unpredictable DILI is currently the major cause for discontinuations of new candidates in development or for withdrawals of successfully launched drugs from the market (Kaplowitz, 2005). The choice of an appropriate animal for toxicological studies should be based on comparison of the pharmacokinetics and metabolism of the test compound in different experimental animals and human. Rat is suitable for the drug development process because of their body weight and ease of sampling blood or urine (Gad, 2007; Johnson, 2007). However, it is known that there is a considerable species difference between human and rat in pharmacokinetics and metabolism. Thus, it is often difficult to predict toxicity to human from experimental animal studies.

Glutathione (5-L-glutamyl-L-cysteinylglycine, GSH) serves an important function in protecting tissues against the degenerating effects of oxidative damage by scavenging free radicals from endogenous or exogenous compounds (Reed, 1986). The activity of hepatic glutathione *S*-transferase (GST) is 10–20 times higher in rodent than that of humans (Grover and Sims, 1964). Therefore, an active metabolite produced *in vivo* in rat would be quickly detoxified by GSH conjugation. γ-Glutamylcysteine synthetase (γ-GCS) (Meister and Anderson, 1983) is a rate-limiting step in GSH synthesis, and GSH is a feedback inhibitor of γ-GCS activity. γ-GCS is a heterodimeric enzyme composed of a catalytic subunit (heavy chain) and a modulatory subunit (light chain) (Huang et al., 1993a,b). In mice, embryos homozygous for the γ-GCS heavy chain (GCSh) mutation fail to survive until gestation (Dalton et al., 2000). We previously established an AdGCSh-short hairpin RNA (shRNA)-mediated GSH depletion rat model (Akai et al., 2007). GSH content of liver was decreased and that of extra hepatic tissues were not changed. Using this γ-GCS-knockdown rat model, acetaminophen-induced hepatotoxicity was shown to be significantly potentiated compared with normal rat (Akai et al., 2007). Why rat is more resistant to DILI than human would largely result from rapid detoxification by GSH conjugation. Thus, we thought that this rat model could sensitively detect DILI in human.

To date, hepatotoxicity has been reported in many clinical drugs. Diclofenac and flutamide are known to be major idiosyncratic hepatotoxic drugs (Kaplowitz, 2005). Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) frequently prescribed for the

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treatment of arthritis, ankylosing spondylitis, and acute muscle pain (Davies and Anderson, 1997). Flutamide is a nonsteroidal antiandrogen drug widely used in the treatment of prostate cancer. There are many reports that these drugs evoked DILI (García-Cortés et al., 2001; Aithal and Day, 2007).

There is no animal model to evaluate diclofenac- and flutamide-induced hepatotoxicity. We hypothesized that GSH would be mainly involved in the hepatotoxicity of these drugs in rat, and intended to demonstrate that the present γ-GCS-knockdown rat model could sensitively detect the hepatotoxic effects of these drugs and clarify the involvement of GSH in hepatotoxicity of these drugs.

2. Materials and methods

2.1. Materials

Flutamide and GSH were obtained from Wako Pure Chemical Industries (Osaka, Japan). Diclofenac sodium salt was from Sigma–Aldrich (St. Louis, MO). β-NADPH and glutathione reductase were from Oriental Yeast (Tokyo, Japan). The Adenovirus Expression Vector kit (Dual version) was from Takara (Osaka, Japan). The QuickTiter Adenovirus Titer Immunoassay kit was from Cell Biolabs (Tokyo, Japan). Dulbecco's modified Eagle's medium was from Nissui Pharmaceutical (Tokyo, Japan).

2.2. Animals

Male Fisher 344 rats (7 weeks old, 130–150 g) were obtained from SLC Japan (Hamamatsu, Japan). The animals were housed in a controlled environment (temperature 25 ± 1 °C, humidity 50 ± 10%, and 12 h light/12 h dark cycle) in the institutional animal facility with access to food and water ad libitum. Animals were acclimatized for a week before use in the experiments. Animal maintenance and treatment were conducted in accordance with the National Institutes of Health Guide for Animal Welfare of Japan, as approved by Institutional Animal Care and Use Committee of Kanazawa University, Japan.

2.3. Recombinant adenovirus

The adenovirus containing shRNA of rat γ-glutamylcysteine synthetase heavy chain subunit (γ-GCSh) (AdGCSh-shRNA) and shRNA of Luciferase (AdLuc-shRNA) were previously constructed in our laboratory (Akai et al., 2007). The titer was determined by a QuickTiter Adenovirus Titer Immunoassay kit. The viral stock solution was concentrated with the Amicon Ultra-15 filtration system (Millipore, Billerica, MA) and the titers of AdGCSh- or AdLuc-shRNA were adjusted to 1.5×10^{11} plaque forming unit (pfu)/ml for the *in vivo* study.

2.4. Adenovirus infection and drug administration in rats

To prepare the γ-GCS-knockdown rat, we used AdGCSh-shRNA as described previously (Akai et al., 2007). The optimum knockdown effect of GSH was obtained 14 days after infection. Therefore, in the 6 or 24 h toxicity test, 14 days after an intravenous injection of AdGCSh-shRNA or AdLuc-shRNA at 1.5×10^{11} pfu/ml/body, the rats were intraperitoneally administered diclofenac dissolved in saline (0, 50, 100 mg/kg body weight) or orally administered flutamide suspended in 0.5% carboxymethylcellulose (CMC) (0, 1000, 1500 mg/kg body weight). Blood samples were collected at 0, 6 and 24 h after the diclofenac or flutamide administration. The rats were sacrificed 24 h after administration, and the liver samples were collected. In the 7 days toxicity test, 10 days after an intravenous injection of adenovirus at 1.5×10^{11} pfu/ml/body, the rats were intraperitoneally administered diclofenac dissolved in saline (0, 5, 10 mg/kg body weight) or orally administered flutamide suspended in 0.5% CMC (0, 100, 500 mg/kg body weight) once a day for a week. The rats were sacrificed, and blood and the liver samples were collected at 6 h after the last diclofenac administration or at 24 h after the last flutamide administration. Plasma samples were collected for assessment of the transaminase, bilirubin and alkaline phosphatase (ALP) levels. Transaminases, bilirubin and ALP levels were determined by using transaminase CII-testwako kit (Wako), bilirubin BII-testwako kit (Wako) and FUJI DRI-CHEM system (FUJIFILM), respectively. A part of the liver was fixed in buffered neutral 10% formalin. The fixed samples were embedded in paraffin and sectioned at a thickness of 2 μm and stained with hematoxylin-eosin for microscopic examination. The histological changes were evaluated whether there is degenerated and dropped-out hepatocyte and hepatic necrosis around the central vein or not. In all experiments, the rats were not treated by fasting prior to the drug administration or sacrifice.

2.5. GSH levels

Rat liver was homogenized with glass homogenizer on ice cold 5% sulfosalicylic acid and centrifuged at 8000 × g at 4 °C for 10 min. The GSH concentration in the supernatant was measured as described previously (Tietze, 1969).

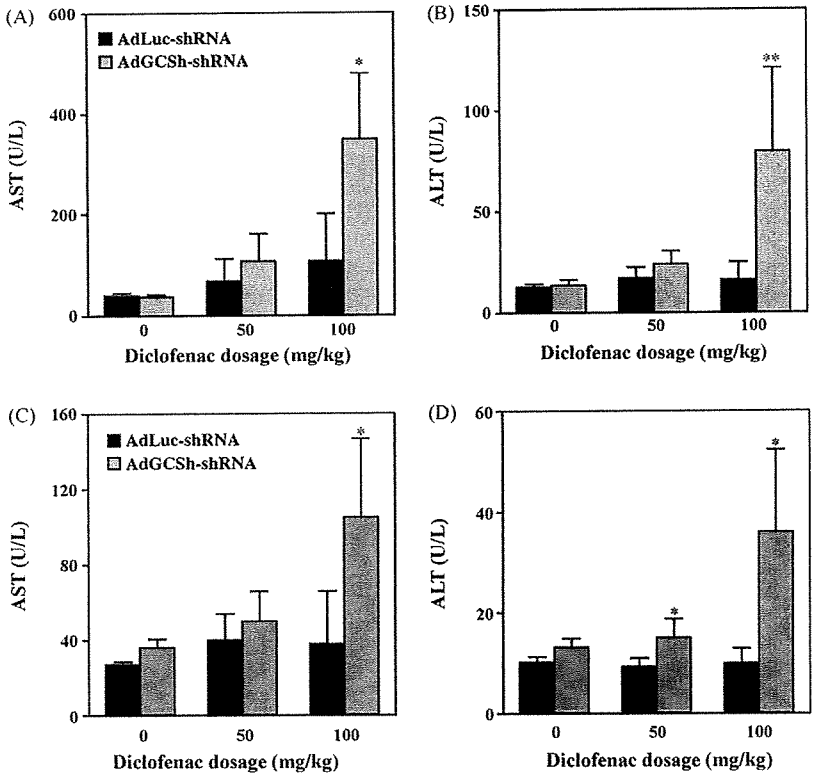

Fig. 1. Acute hepatotoxic effect of diclofenac in γ-GCS-knockdown rats. Diclofenac was i.p. administered. After 6 h (A and B) and 24 h (C and D), AST (A and C) and ALT (B and D) were measured. Data are mean ± SD ($n = 3$–5). *$P < 0.05$, and **$P < 0.01$ compared with each AdLuc-shRNA-infected group.

2.6. Statistical analyses

Statistical analyses were performed with the GraphPad Instat version 2.0 computer program (GraphPad Software, San Diego, CA). Comparison of two groups was made with two-tailed Student's *t*-test. Comparison of multiple groups was made with analysis of variance followed by Dunnett's post hoc test.

3. Results

3.1. Diclofenac-induced hepatotoxicity in γ-GCS-knockdown rat

Although it is preferable that the drug administration route should be the same as in human, diclofenac has severe gastrointestinal adverse effects. Thus, diclofenac was intraperitoneally administered in the present study to focus on the hepatotoxicity. Six hours after single diclofenac administration, the serum AST and ALT levels in the 50 mg/kg administered groups showed no change (Fig. 1A and B). In contrast, the γ-GCS-knockdown rats administered with 100 mg/kg diclofenac demonstrated a significant increase of AST (349 ± 131 U/L) and ALT (79 ± 41 U/L) compared with AdLuc-shRNA-infected control rats (Fig. 1A and B). In addition, 24 h after administration, the γ-GCS-knockdown rats administered with 100 mg/kg diclofenac demonstrated lower AST and ALT compared with those at 6 h, but demonstrated a significant increase of AST (105 ± 42 U/L) and ALT (36 ± 16 U/L) compared with AdLuc-shRNA-infected control rats (Fig. 1C and D). Degenerated or dropped-out hepatocytes and hepatic necrosis around the central vain were not observed in 100 mg/kg diclofenac-administered rats by histological examination (data not shown).

On the other hand, diclofenac did not produce hepatotoxicity in 7 days subacute study (data not shown).

3.2. Flutamide-induced acute hepatotoxicity in γ-GCS-knockdown rat

Six hours after single flutamide administration, AST and ALT were not changed in both rat groups (data not shown). Twenty-four hours after administration, the γ-GCS-knockdown rats administered with 1000 or 1500 mg/kg flutamide demonstrated a significant increase of AST (431 ± 120 or 420 ± 192 U/L) and ALT (157 ± 76 or 161 ± 79 U/L) compared with AdLuc-shRNA-infected control rats (Fig. 2A and B). The bilirubin level was slightly increased in 1000 and 1500 mg/kg flutamide administered γ-GCS-knockdown rats (0.18 ± 0.08 mg/dL and 0.17 ± 0.07 mg/dL). This increase was not significant compared with AdLuc-shRNA-infected control rats (0.09 ± 0.03 mg/dL and 0.08 ± 0.05 mg/dL). The ALP levels in the 1000 and 1500 mg/kg administered groups showed no change (data not shown). The results of the histological examination in 1500 mg/kg flutamide-administered rats are shown in Fig. 2C. Remarkably degenerated and dropped-out hepatocytes, especially around the central vein, were observed, consistent with the elevation of AST and ALT. However, cholestasis was not observed by histology in γ-GCS-knockdown rats. There was no histological change in the other groups.

3.3. Flutamide-induced subacute hepatotoxicity in γ-GCS-knockdown rat

Twenty-four hours after the last flutamide administration, the γ-GCS-knockdown rats administered with 500 mg/kg flutamide demonstrated a significant increase of ALT (40 ± 23 U/L) compared with AdLuc-shRNA-infected control rats. The γ-GCS-knockdown

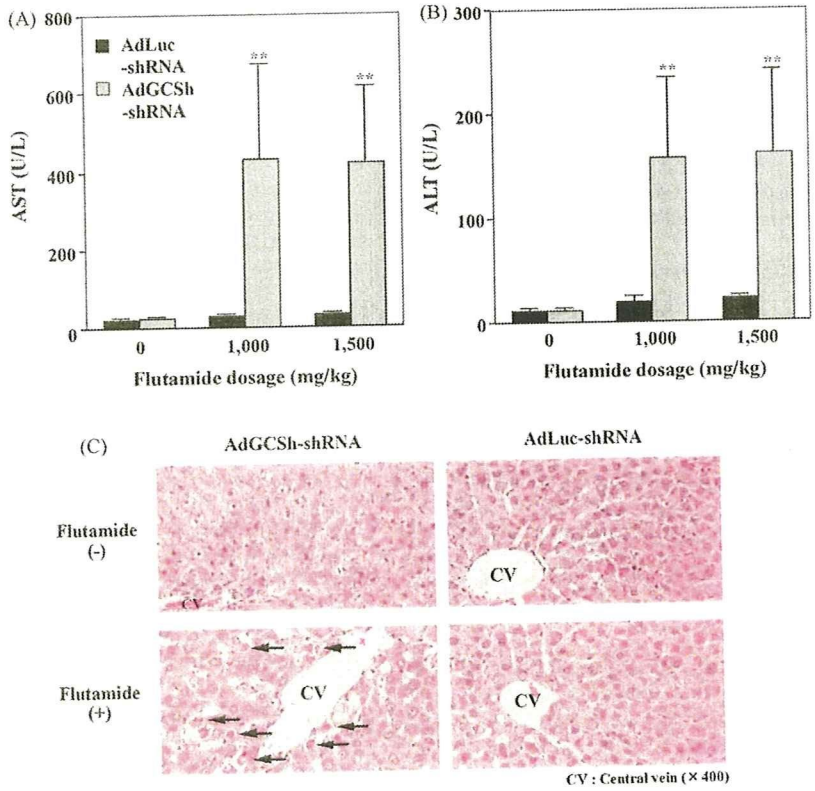

Fig. 2. Acute hepatotoxic effect of flutamide in γ-GCS-knockdown rats. Flutamide was p.o. administered. After 24 h, AST (A) and ALT (B) were measured, and hematoxylin-eosin staining (C) was performed in sections of rat liver. Degenerated hepatocytes shown by arrowheads were observed only in flutamide-administered γ-GCS-knockdown rats. Data are mean ± SD ($n = 4$–5). $^{**}P < 0.01$ compared with each AdLuc-shRNA-infected group.

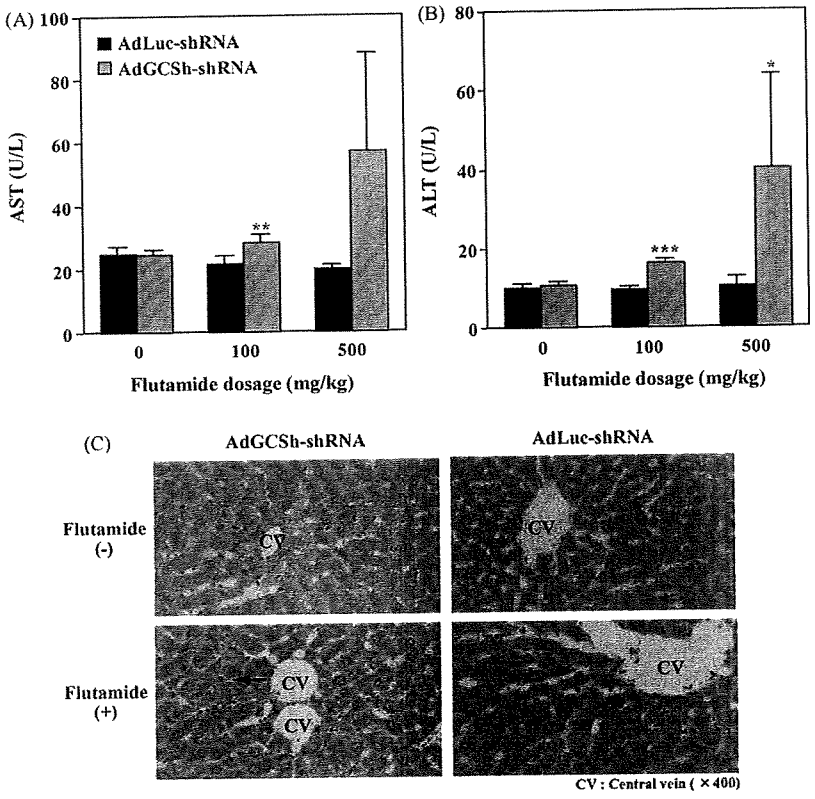

Fig. 3. Subacute hepatotoxic effect of flutamide in γ-GCS-knockdown rats. Flutamide was p.o. administered once a day for a week. Twenty-four hours after the last administration, serum AST (A) and ALT (B) were measured, and hematoxylin-eosin staining (C) was performed in sections of rat liver. Degenerated hepatocyte shown by arrowhead was observed only in flutamide-administered γ-GCS-knockdown rats. Data are mean ± SD ($n = 3–5$). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with each AdLuc-shRNA-infected group.

rats administered with 100 mg/kg flutamide demonstrated a significant increase of AST and ALT, but the increases were small (Fig. 3A and B). Histological examination revealed degenerated and dropped-out hepatocytes in γ-GCS-knockdown rats given 500 mg/kg flutamide, consistent with the elevation of AST and ALT. There was no histological change in the other groups.

3.4. Confirmation of γ-GCS-knockdown in diclofenac- or flutamide-administered rats

Fourteen days after an intravenous injection of 1.5×10^{11} pfu/ml/body AdGCSh-shRNA, hepatic GSH was depleted by 80% (Akai et al., 2007). To determine whether such GSH depletion also occurred in the present study, the hepatic GSH level was measured (Fig. 4). Hepatic GSH was significantly decreased by AdGCSh-shRNA-infection compared with AdLuc-shRNA-infection. In addition, hepatic GSH was significantly increased by the administration of flutamide in AdLuc-shRNA-infected control rats compared with no drug administered group.

4. Discussion

We previously established the AdGCSh-short hairpin RNA (shRNA)-mediated GSH depletion rat model (Akai et al., 2007). This γ-GCS-knockdown rat model showed significantly potentiated drug-induced hepatotoxicity by the administration of acetaminophen (Akai et al., 2007). Diclofenac and flutamide are now widely used for medical treatment. However, occasionally, their hepatotoxicity in human has been reported. There is no appropriate animal model to evaluate diclofenac- and flutamide-induced hepatotoxicity. We hypothesized that GSH would be mainly involved in the hepatotoxicity of these drugs in rat, and the γ-GCS-knockdown rat model could demonstrate the hepatotoxic effects of these drugs.

In the diclofenac-induced 6 h hepatotoxicity study, only in 100 mg/kg diclofenac administered γ-GCS-knockdown rats was hepatotoxicity observed (Fig. 1). However, no typical histological change was observed. Gastrointestinal and cardiovascular events, not only hepatotoxicity, are major adverse events of diclofenac (Hippisley-Cox et al., 2005; Waksman et al., 2007). Thus, diclofenac was intraperitoneally administered in the present study to avoid gastrointestinal effects. In diclofenac administered rats, the AST and ALT levels were increased in the present study. The AST level is associated with hepatotoxicity, but also can be elevated in association with other diseases, for example, heart and skeletal muscle injury (Burhop et al., 2004; Lott and Landesman, 1984). In the present study, involvement of cardio- or muscle-toxicity can not be excluded. The AST and ALT increases were attenuated after 24 h, compared with those after 6 h. In the present study, we were not able to demonstrate subacute hepatotoxicity by 7 days study. There has been no report of subacute hepatotoxicity in a rat model, but the present study firstly demonstrated that the γ-GCS-knockdown rat is sensitive to diclofenac-induced acute hepatotoxicity by 6 and 24 h study.

Regarding diclofenac, two hypotheses of the mechanism by which hepatotoxicity develops have been proposed. The first implicates the reactive acylglucuronide of diclofenac as a potential causative agent (Pumford et al., 1993; Hargus et al., 1994). The second is based upon the observation of an NADPH-dependent covalent modification of proteins by incubation of the drug with

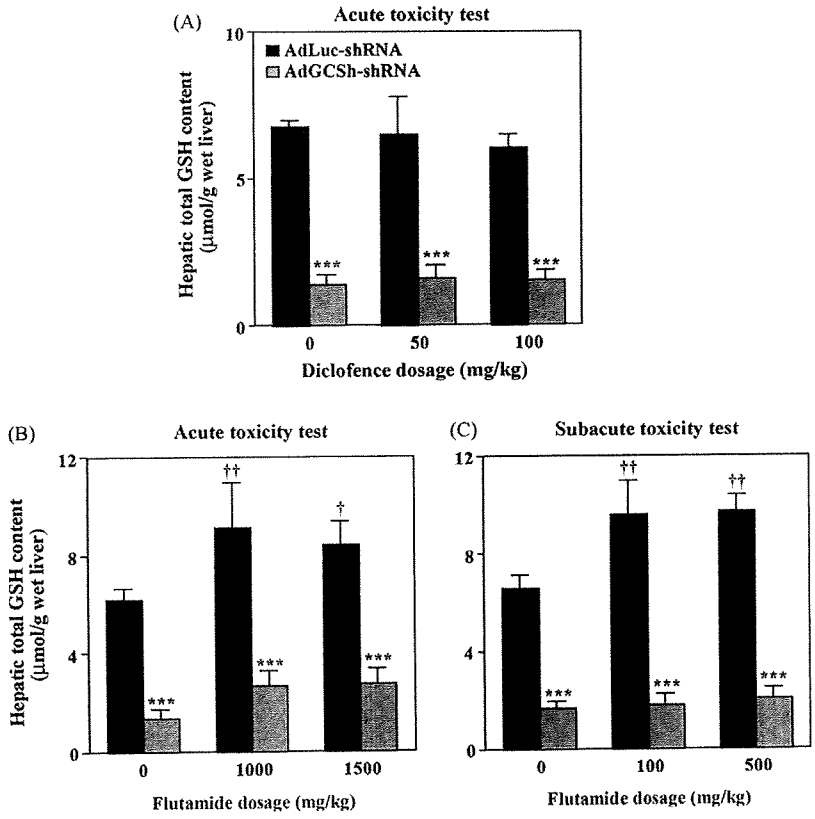

Fig. 4. Total GSH content in diclofenac or flutamide administered γ-GCS-knockdown rats. Total hepatic GSH content was measured 6 h after the administration in the acute toxicity test of diclofenac (A), 24 h after administration in the acute toxicity test of flutamide (B) and 24 h after the last administration in the subacute toxicity test of flutamide (C). Data are mean ± SD ($n = 3–5$). ***$P < 0.001$ compared with each AdLuc-shRNA-infected group (Student's t-test). †$P < 0.05$ and ††$P < 0.01$ compared with AdLuc-shRNA-infected and no drug administered group (Dunnett's post hoc test).

rat or human microsomes and the formation of GSH or mercapturic acid conjugates in rats treated with the drug (Tang et al., 1999; Poon et al., 2001). In the present study, supporting the latter hypothesis, the involvement of GSH in the diclofenac-induced acute hepatotoxicity was demonstrated. However, it is unclear that the involvement of acylglucuronide in diclofenac-induced hepatotoxicity in the present study.

In the flutamide-induced 24 h hepatotoxicity study, the γ-GCS-knockdown rats showed similar hepatotoxicity when administered 1000 and 1500 mg/kg (Fig. 2). This could be caused by saturated absorption. We also investigated of 500 mg/kg flutamide in 24 h study, and no hepatotoxicity was observed (data not shown). In addition, the bilirubin level was slightly increased in γ-GCS-knockdown rats administered flutamide at 1000 and 1500 mg/kg, however, cholestasis was not observed by histology. This is the first report of flutamide-induced acute hepatotoxicity in rat.

Flutamide-induced subacute hepatotoxicity (100 mg/kg for 15 days) was reported by Mannaa et al. (2005). In our study, rats were administered flutamide at 100 or 500 mg/kg for 7 days and ALT was significantly elevated. Histological hepatotoxicity was also observed. This condition is different from that described by Mannaa et al. (2005). Nevertheless, the γ-GCS-knockdown rat is the first successful model sensitive for flutamide-induced acute and subacute hepatotoxicity.

Although the mechanisms of flutamide-induced hepatotoxicity have not been precisely elucidated, CYP-mediated oxidative bioactivation to reactive metabolites is thought to be a cause of the toxicity (Berson et al., 1993; Fau et al., 1994). Recently, GSH or mercapturic acid conjugates of flutamide were detected in human liver microsomes or urine of prostate cancer patients (Kang et al., 2007; Soglia et al., 2006; Tevell et al., 2006). Conjugates of flutamide were also detected in mouse liver microsomes (Ohbuchi et al., 2009). In the present study, the involvement of GSH in the flutamide-induced acute and subacute hepatotoxicity was clearly demonstrated. In addition, GSH depleted mice showed hepatotoxicity (ALT; 200 IU/L) when administered 400 mg/kg of flutamide (Matsuzaki et al., 2006). GSH content was reduced to 27% of normal mice with an amino acid-deficient diet for 2 weeks. This hepatotoxicity was not observed without amino acid-deficient diet (Matsuzaki et al., 2006). These data supported the involvement of GSH in the flutamide-induced hepatotoxicity.

Diclofenac- or flutamide-induced subacute hepatotoxicity was not detected or lower than acute hepatotoxicity in the present rat model. Responsiveness to toxicity of a drug occasionally decreases resulting from prior exposure to that drug, which is generically called tolerance. The major mechanisms for tolerance are as follows, one is due to decreased amount toxicant reaching the site where the toxicity is produced. The other is due to reduced responsiveness of a tissue to the drug (Eaton and Klaassen, 2001). In the present study, subacute hepatotoxicity would be lowered by tolerance, however the mechanism is awaited to be clarified.

Hepatic GSH content was successfully knockdown by AdGCSh-shRNA in diclofenac administered groups. On the other hand, hepatic GSH was increased by the administration of flutamide (Fig. 4). Hepatic γ-GCS is regulated by nuclear factor-erythroid 2-related factor 2 (Nrf2) (Copple et al., 2008). Nrf2 is partly regulated by protein kinase C (PKC) (Huang et al., 2002; Numazawa et al., 2003) and flutamide increases PKC expression (Montalvo

et al., 2002). Thus, flutamide could increase oxidative stress and PKC expression, and then hepatic GSH could be increased due to the induction of γ-GCS. In 7 days flutamide hepatotoxicity study, hepatic GSH content was induced in the control rats, but not in the γ-GCS knockdown rats, demonstrating the continuous knockdown of γ-GCS. However, GSH would be used to detoxify flutamide, and intrahepatic level should be decrease instead of increase. Further investigation would be needed to clarify this mechanism.

The maximum depletion of GSH was obtained 14 days after AdGCSh-shRNA infection in rats and continued about 2 weeks (Akai et al., 2007). Therefore, to maintain the GSH depletion level, we conducted the subacute toxicity study for a short time (7 days). In particular, we started drug administration 10 days after the injection and continued for a week. This would be enough time to determine the onset of hepatotoxicity.

The activity of hepatic glutathione *S*-transferase (GST) and hepatic GSH content is 10–20 times and about 2 times higher in rodent than that of humans, respectively (Grover and Sims, 1964; Woodhouse et al., 1983; Higashi et al., 1985). GSH conjugation plays an important role in protecting tissues, it is hard to predict DILI in preclinical study using rat. In addition, although *in vitro* test to detect reactive intermediates by GSH adduct is widely used, these data could not always correlate with *in vivo* (Soglia et al., 2006; Evans et al., 2004). It is partly because some GSH adducts are not stable and are subject to chemical degradation/rearrangement or enzymatic degradation, thereby escaping detection (Evans et al., 2004). In the present study, we could show the γ-GCS-knockdown rat model sensitively detect diclofenac- and flutamide-induced hepatotoxicity. Thus, decreasing glutathione levels in a rat model would be relevant and predictive of DILI in humans.

The present rat model is sensitive for hepatotoxic drugs compared to the normal rat. However, it is difficult to determine the absolute dosage of drugs for the purpose of extrapolating the hepatotoxicity in human. The present rat model has an advantage to compare the hepatotoxicity in structurally related compounds in drug development process.

In summary, in the present study, we demonstrated that the γ-GCS-knockdown rat model can sensitively detect diclofenac- and flutamide-induced hepatotoxicity, indicating the involvement of GSH in the hepatotoxic effect of these drugs. Thus, this rat model could be useful for highly sensitive tests to evaluating acute and subacute DILI in preclinical drug development.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Human Arylacetamide Deacetylase Is a Principal Enzyme in Flutamide Hydrolysis

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ABSTRACT:

Flutamide, an antiandrogen drug, is widely used for the treatment of prostate cancer. The initial metabolic pathways of flutamide are hydroxylation and hydrolysis. It was recently reported that the hydrolyzed product, 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1), is further metabolized to *N*-hydroxy FLU-1, an assumed hepatotoxicant. However, the esterase responsible for the flutamide hydrolysis has not been characterized. In the present study, we found that human arylacetamide deacetylase (AADAC) efficiently hydrolyzed flutamide using recombinant AADAC expressed in COS7 cells. In contrast, carboxylesterase1 (CES1) and CES2, which are responsible for the hydrolysis of many drugs, could not hydrolyze flutamide. AADAC is specifically expressed in the endoplasmic reticulum. Flutamide hydrolase activity was highly detected in human liver microsomes (K_m, 794 ± 83 μM; V_{max}, 1.1 ± 0.0 nmol/min/mg protein), whereas the activity was extremely low in human liver cytosol. The flutamide hydrolase activity in human liver microsomes was strongly inhibited by bis-(nonylphenyl)-phenylphosphate, diisopropylphosphorofluoride, and physostigmine sulfate (eserine) but moderately inhibited by sodium fluoride, phenylmethylsulfonyl fluoride, and disulfiram. The same inhibition pattern was obtained with the recombinant AADAC. Moreover, human liver and jejunum microsomes showing AADAC expression could hydrolyze flutamide, but human pulmonary and renal microsomes, which do not express AADAC, showed slight activity. In human liver microsomal samples (n = 50), the flutamide hydrolase activities were significantly correlated with the expression levels of AADAC protein (r = 0.66, p < 0.001). In conclusion, these results clearly showed that flutamide is exclusively hydrolyzed by AADAC. AADAC would be an important enzyme responsible for flutamide-induced hepatotoxicity.

Flutamide (3′-trifluoro-2-methyl-4′-nitro-2-methyl-propinoylanilide) is a nonsteroidal antiandrogen drug used for the treatment of prostate cancer. The combination of luteinizing hormone-releasing hormone agonist results in prolonged survival in prostate cancer patients (Crawford et al., 1989). However, flutamide occasionally causes severe hepatotoxicity (Thole et al., 2004). Flutamide itself is not toxic when used at the appropriate clinical dose, but bioactivation of flutamide has been considered to be the cause of flutamide-induced hepatotoxicity (Fau et al., 1994).

Flutamide is mainly metabolized to 2-hydroxyflutamide by human CYP1A2. It has been suggested that 2-hydroxyflutamide is associated with the therapeutic effect of flutamide (Katchen and Buxbaum, 1975). Flutamide is also hydrolyzed to 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1) by esterase (Katchen and Buxbaum, 1975; Schulz et al., 1988). FLU-1 is considered to have no therapeutic effect (Aizawa et al., 2003). Goda et al. (2006) recently reported that FLU-1 is further metabolized to *N*-hydroxyl FLU-1 by human CYP3A4. Many researchers have reported on the relationship between the toxicity and metabolism of flutamide. It was shown in CYP1A2 knockout SV129 mice but not in wild-type mice that the urinary concentration of FLU-1 was increased, and an abnormal elevation of alanine aminotransferase was shown after the knockout mice were fed an amino acid-deficient diet (Matsuzaki et al., 2006). It was also shown in humans that the urinary caffeine metabolite ratio, an indicator of the CYP1A2 activity, was significantly lower in patients with hepatic injury compared with patients with normal hepatic function after the same flutamide therapy (Ozono et al., 2002). In addition, a study on patients of prostate cancer taking flutamide showed that the incidence of hepatotoxicity was correlated with the plasma concentration of FLU-1 (Aizawa et al., 2003). More recently, Ohbuchi et al. (2009) reported that coadministration of FLU-1 and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, an inducer of CYP3A and CYP1A, to mice significantly increased serum alanine aminotransferase, and sev-

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ABBREVIATIONS: FLU-1, 4-nitro-3-(trifluoromethyl)phenylamine; CES, carboxylesterase; HLM, human liver microsome; BNPP, bis-(nonylphenyl)-phenylphosphate; AADAC, arylacetamide deacetylase; DFP, diisopropylphosphorofluoride; eserine, physostigmine sulfate; PMSF, phenylmethylsulfonyl fluoride; $AgNO_3$, silver nitrate; $CdCl_2$, cadmium chloride; $CoCl_2$, cobaltous chloride; $CuCl_2$, cupric chloride; PNPA, *p*-nitrophenyl acetate; NaF, sodium fluoride; HLC, human liver cytosol; HJM, human jejunum microsome; HPM, human pulmonary microsome; HRM, human renal microsome; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SNP, single nucleotide polymorphism; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography.

eral protein adducts were detected after incubation of the microsomal protein with *N*-hydroxy FLU-1. Therefore, the hepatotoxicity of flutamide might be related to *N*-hydroxy FLU-1. Considering these reports, the hydrolysis pathway may contribute to the hepatotoxicity of flutamide.

Carboxylesterase (CES) is the major serine esterase contributing to the hydrolysis of various drugs and xenobiotics. In human, CES isoforms are classified into three families: CES1, CES2, and CES3. CES1 and CES2 have been reported to be responsible for the biotransformation of a number of clinically used drugs and prodrugs such as imidapril, capecitabine, and irinotecan (Imai et al., 2006). CES3 appears to show extremely low activity compared with CES1 and CES2 (Sanghani et al., 2004). The flutamide hydrolysis by human liver microsomes (HLM) is inhibited by bis-(nonylphenyl)-phenylphosphate (BNPP), a general CES inhibitor (Heymann and Krisch, 1967; Block and Arndt, 1978; Mentlein et al., 1988). Thus, it is plausible that flutamide is hydrolyzed by CES. However, the flutamide hydrolase activity was not detected by using purified CES1 (pI 4.5) and CES2 (pI 5.3) (Takai et al., 1997). Therefore, we considered that another esterase expressed in HLM plays a role in flutamide hydrolysis. As a candidate enzyme, arylacetamide deacetylase (AADAC) could be considered.

AADAC, as well as CES1 and CES2, is a major serine hydrolase expressed in HLM (Ross and Crow, 2007). AADAC was first identified as the enzyme that catalyzes the deacetylation of 2-acetylaminofluorene (Probst et al., 1991). The active site domain of AADAC shares high homology with that of hormone-sensitive lipase (Probst et al., 1994). Therefore, AADAC has been classified as a lipase. In fact, Tiwari et al. (2007) proved that human AADAC was capable of hydrolyzing cholesterol ester when expressed in yeast. However, it is unknown whether AADAC is involved in the hydrolysis of clinical therapeutic drugs. In the present study, the involvement of human AADAC in flutamide hydrolysis was investigated.

Materials and Methods

Chemicals and Reagents. Flutamide, 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1), *p*-nitrophenol, diisopropylphosphorofluoride (DFP), physostigmine sulfate (eserine), phenylmethylsulfonyl fluoride (PMSF), disulfiram, silver nitrate ($AgNO_3$), cadmium chloride ($CdCl_2$), cobaltous chloride ($CoCl_2$), and cupric chloride ($CuCl_2$) were purchased from Wako Pure Chemical Industries (Osaka, Japan). *p*-Nitrophenyl acetate (PNPA), BNPP, and sodium fluoride (NaF) were purchased from Sigma-Aldrich (St. Louis, MO). Primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). The random hexamer and SYBR Premix Ex Taq were from Takara (Shiga, Japan). RevaTra Ace (Moloney murine leukemia virus reverse transcriptase RNase H−) was obtained from Toyobo (Tokyo, Japan). All the other chemicals used in this study were of analytical or the highest quality commercially available.

Human Tissues. The microsomes or cytosol from human liver, jejunum, lung, and kidney were used in this study. Pooled HLM (n = 50), individual HLM (24 donors), and pooled human liver cytosol (HLC, n = 22) were purchased from BD Gentest (Woburn, MA). Individual HLM from 15 donors were also obtained from Human and Animal Bridging Research Organization (Chiba, Japan), and those from 11 donors were obtained from autopsy materials that were discarded after pathological investigation. Human jejunum microsomes (HJM, pooled, n = 10), human pulmonary microsomes (HPM, single donor), and human renal microsomes (HRM, single donor) were purchased from Tissue Transformation Technologies (Edison, NJ). The pooled HLM, HJM, HPM, and HRM were used for the immunoblotting analysis and the assay of flutamide hydrolase activity. The HLC was used for the comparison of flutamide hydrolase activity with HLM. The individual HLM samples were used for the correlation analysis. The use of the human tissues was approved by the Ethics Committees of Kanazawa University (Kanazawa, Japan) and Iwate Medical University (Morioka, Japan).

TABLE 1

Primers used in the present study

Primer	Sequence
For detection of real-time RT-PCR	
AADAC-RT-S	5′-TTGTGGAGCTCCTGGGACTT-3′
AADAC-RT-AS	5′-TCTGTCTGCTGTCCATCTTG-3′
For construction of expression plasmids	
AADAC-S	5′-TAGAGACCAAGAAGCGGGAC-3′
AADAC-AS	5′-GCTACATGTTTTACTATAGATTTTCC-3′
CES1A1-S	5′-AGAGACCTCGCAGGCCCCGA-3′
CES1A2-S	5′-GAGACCTCGCAGGCCCCCG-3′
CES1A-AS	5′-CCATGGTAAGATGCCTTCTG-3′
CES2A1-S	5′-CCTGCCTACCACTAGATCCC-3′
CES2A1-AS	5′-CTCGCCTGTCAGCGAACCCAC-3′

Total RNA from Human Tissues and Reverse Transcription-Polymerase Chain Reaction Analyses. Total RNA samples from normal human liver (single donor), colon (pooled, n = 2), kidney (single donor), bladder (pooled, n = 2), breast (pooled, n = 2), ovary (single donor), and uterus (pooled, n = 3) were obtained from Stratagene (La Jolla, CA). Total RNA samples from normal human lung (single donor) and testis (single donor) were from Cell Applications (San Diego, CA). Total RNA samples from normal human stomach (single donor), adrenal gland (pooled, n = 62), and small intestine (pooled, n = 5) were from Clontech (Palo Alto, CA). The reverse transcription procedure was described previously (Nakajima et al., 2006).

For quantitative analysis, real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed for AADAC mRNA using an MX3000P real-time PCR system (Stratagene). The forward and reverse primers used for PCR were AADAC-RT-S and AADAC-RT-AS primers (Table 1). A 1-μl portion of the reverse-transcribed mixture was added to a PCR mixture containing 10 pmol of each primer and 12.5 μl of SYBR Premix Ex Taq solution in a final volume of 25 μl. After an initial denaturation at 95°C for 30 s, the amplification was performed by denaturation at 94°C for 4 s, annealing at 58°C for 7 s, and extension at 72°C for 20 s for 45 cycles. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was also quantified according to a method described previously (Tsuchiya et al., 2004). The copy numbers were calculated using standard amplification curves.

Construction of Plasmids Expressing Human AADAC, CES1A, and CES2. The full-length human AADAC, CES1A1, CES1A2, and CES2A1 cDNAs were obtained by RT-PCR using a human liver RNA sample as the initial template. The primers used are shown in Table 1. In this study, two clones of AADAC cDNA were obtained [c.931guanine (G) and adenine (A)]. In the reference sequence of NM 001086.2, the nucleotide at the c.931 position is G. Therefore, the clones with c.931G and c.931A were defined as the AADAC wild-type and AADAC variant, respectively. This single nucleotide polymorphism (SNP, c.931G>A) leads to an amino acid change of valine to isoleucine. The allele frequency of this SNP (ID: rs1803155) has been reported to be approximately 55 to 80% in each population, including European, Asian, and sub-Saharan African in the dbSNP database in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1803155). The PCR products were subcloned into pTARGET mammalian expression vector (Promega, Madison, WI). The nucleotide sequences were confirmed by DNA sequence analysis (Long-Read Tower DNA sequencer; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Transfection of Plasmids Expressing Human AADAC, CES1A, and CES2. African green monkey kidney cells, COS7 cells, were obtained from American Type Culture Collection (Manassas, VA). The COS7 cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose and 10% fetal bovine serum with 5% CO_2 at 37°C. The cells were transfected in 10-cm dishes with 7.5 μg of each expression plasmid using Lipofectamine (Invitrogen, Carlsbad, CA). After incubation for 48 h, the cells were harvested and suspended in a small amount of TGE buffer (10 mM Tris-HCl, 20% glycerol, 1 mM EDTA, pH 7.4) and disrupted by freeze-thawing three times. Each protein expression level was determined by immunoblot analysis as described below.

Immunoblot Analysis. SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to Laemmli (1970). Enzyme sources (30 μg) were separated on 10% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membrane, Immobilon-P (Millipore Corporation, Billerica, MA). The membranes were probed with monoclonal mouse anti-human AADAC (Abnova, Neihu District, Taipei City, Taiwan), polyclonal rabbit anti-human CES1 (Abcam, Cambridge, MA), and polyclonal rabbit anti-human CES2 antibodies (Antagene, San Francisco, CA), and the corresponding fluorescent dye-conjugated second antibody and an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) were used for the detection. The relative expression level was quantified using ImageQuant TL Image Analysis software (GE Healthcare).

Flutamide Hydrolase Activity. The flutamide hydrolase activity was determined as follows: a typical incubation mixture (final volume of 0.2 ml) contained 100 mM potassium phosphate buffer, pH 7.4, and various enzyme sources (human microsomal protein and COS7 cell homogenate expressing esterases, 0.4 mg/ml; human cytosolic protein, 1.0 mg/ml). In the preliminary study, we confirmed that the rate of formation of FLU-1 was linear with respect to the protein concentrations (<1.0 mg/ml human microsomal protein and COS7 cells homogenate expressing esterases and <1.5 mg/ml human cytosolic protein) and incubation time (<60 min). Flutamide was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the incubation mixture was 1.0%. The reaction was initiated by the addition of 25 to 750 μM flutamide after 2-min preincubation at 37°C. After the 30-min incubation at 37°C, the reaction was terminated by the addition of 0.1 ml of ice-cold acetonitrile. After removal of the protein by centrifugation at 9500*g* for 5 min, a 60-μl portion of the supernatant was subjected to high-performance liquid chromatography (HPLC). The HPLC analysis was performed using an L-7100 pump (Hitachi, Tokyo, Japan), an L-7200 autosampler (Hitachi), an L-7405 UV detector (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 376 nm with a noise-base clean Uni-3 (Union, Gunma, Japan), which can reduce the noise by integrating the output and increase the signal 3-fold by differentiating the output, and 5-fold by further amplification with an internal amplifier, resulting in a maximum 15-fold amplification of the signal. The mobile phase was 45% acetonitrile containing 25 mM ammonium acetate, pH 5.0. The flow rate was 1.0 ml/min. The column temperature was 35°C. The quantification of FLU-1 was performed by comparing the HPLC peak height with that of an authentic standard. Because FLU-1 contaminants exist in the commercially available flutamide to the extent of ~0.5%, the content of FLU-1 in the mixture incubated without the enzyme was subtracted from that with the enzyme to correct the activity. The activity in each concentration was determined as the mean value in triplicate. For kinetic analyses of flutamide hydrolase activity, the parameters were estimated from the fitted curves using a computer program (KaleidaGraph, Synergy Software, Reading, PA) designed for nonlinear regression analysis.

To clarify the involvement of various esterases, inhibition analysis of flutamide hydrolysis was performed by using representative esterase inhibitors. Organophosphates such as BNPP and DFP are known as general CES inhibitors (Heymann and Krisch, 1967; Yamaori et al., 2006). Eserine and NaF are cholinesterase inhibitors (Iwatsubo, 1965; Preuss and Svensson, 1996). EDTA is a paraoxonase inhibitor (Gonzalvo et al., 1997). PMSF is a serine hydrolase inhibitor (Johnson and Moore, 2000). Because heavy metals are frequently used for esterase inhibition studies, $AgNO_3$, $CdCl_2$, $CoCl_2$, and $CuCl_2$ were also used as inhibitors. Disulfiram was reported as a monoacylglycerol lipase inhibitor (Labar et al., 2007). The concentration of BNPP and DFP ranged from 0.001 to 1 mM, and that of the others was 0.1 and 1 mM. PMSF and disulfiram were dissolved in DMSO such that the final concentration of DMSO in the incubation mixture was 1.5%. Other inhibitors were dissolved in distilled water. The experimental procedure and condition were the same as above except that 500 μM flutamide was added. It was confirmed that 1.5% DMSO did not inhibit the flutamide hydrolase activity, and the control activity was determined in the presence of 1.5% DMSO.

***p*-Nitrophenyl Acetate Hydrolase Activity.** To confirm whether the recombinant AADAC, CES1, and CES2 are enzymatically active, the PNPA, a general esterase substrate, hydrolase activity was measured. The PNPA hydrolase activity was determined as follows: a typical incubation mixture was the same as above. PNPA was dissolved in DMSO, and the final concentration of DMSO in the incubation mixture was 1.0%. The reaction was initiated by the addition of 500 μM PNPA after 2-min preincubation at 37°C. After 5-min incubation at 37°C, the reaction was terminated by the addition of 0.1 ml of ice-cold methanol. The PNPA hydrolase activity was measured by the absorbance at 405 nm using Biotrak II plate reader (GE Healthcare). The quantification of *p*-nitrophenol, a metabolite of PNPA hydrolysis, was performed by comparing the absorbance with that of an authentic standard. Because *p*-nitrophenol contaminants exist in the commercially available PNPA to the extent of ~5%, the content of *p*-nitrophenol in the mixture incubated without the enzyme was subtracted from that with the enzyme to correct the activity.

Statistical Analysis. Comparison of two groups was made with unpaired, two-tailed Student's *t* test. Correlation analyses were performed by Spearman rank method. A value of $p < 0.05$ was considered statistically significant.

Results

PNPA and Flutamide Hydrolase Activities by Recombinant Human AADAC Wild-Type, AADAC Variant, CES1A1, CES1A2, and CES2. To compare the flutamide hydrolase activity between human AADAC, CES1A1, CES1A2, and CES2, they were transiently expressed in COS7 cells. The AADAC variant was also transiently expressed in COS7 cells. The protein expression levels were determined by immunoblot analysis (Fig. 1A). AADAC protein was specifically expressed in COS7 cells transfected with the expression plasmids of AADAC wild-type and variant. CES1A and CES2 proteins were specifically expressed in COS7 cells transfected with the expression plasmids of CES1A1 or CES1A2, and CES2, respectively. To confirm that these enzymes were enzymatically active, PNPA hydrolase activity was measured at a concentration of 500 μM PNPA (Fig. 1B). The recombinant CES2A1 showed the highest PNPA hydrolase activity (621 ± 61 nmol/min/mg protein), and the recombinant AADAC wild-type, variant, CES1A1, and CES1A2 showed similar activities (243 ± 11, 247 ± 24, 293 ± 19, and 245 ± 18 nmol/min/mg protein, respectively). The flutamide hydrolase activities were determined at a concentration of 500 μM flutamide (Fig. 1C). Among them, the AADAC wild-type and variant showed flutamide hydrolase activity (0.28 ± 0.03 and 0.30 ± 0.01 nmol/min/mg protein, respectively). In contrast, CES1A1, CES1A2, and CES2 showed almost no activity (0.003 ± 0.003, 0.008 ± 0.008, and 0.012 ± 0.007 nmol/min/mg protein, respectively), similar to mock COS7 cells (0.012 ± 0.007 nmol/min/mg protein). These results suggested that AADAC, but not the CES enzymes, contributed to the flutamide hydrolysis.

Kinetic Analyses of Flutamide Hydrolase Activity by HLM, HLC, and Recombinant Human AADAC. It was previously suggested that AADAC is localized to the endoplasmic reticulum lumen (Frick et al., 2004). Therefore, it is assumed that flutamide can be hydrolyzed in HLM rather than in HLC. In this study, the flutamide hydrolase activity in HLM and HLC was measured (Fig. 2A). The maximum concentration was 750 μM because of the limited solubility of flutamide in the incubation mixture. For HLM, the K_m and V_{max} values were 794 ± 83 μM and 1.1 ± 0.0 nmol/min/mg protein, respectively, resulting in an intrinsic clearance of 1.4 ± 0.1 μl/min/mg protein. For HLC, because the flutamide hydrolase activity ranging from 25 to 750 μM was linear, the K_m and V_{max} values could not be calculated by the Michaelis-Menten equation. The K_m value of the flutamide hydrolase activity by HLC appeared to be substantially higher than that by HLM. These results suggested that the contribution to flutamide hydrolysis by HLM was much higher than that by HLC. In addition, kinetic analyses of the flutamide hydrolase activity by the recombinant AADAC wild-type and variant were also performed (Fig. 2B). The K_m and V_{max} values of the AADAC wild-type were 778 ± 122 μM and 0.6 ± 0.1 nmol/min/mg

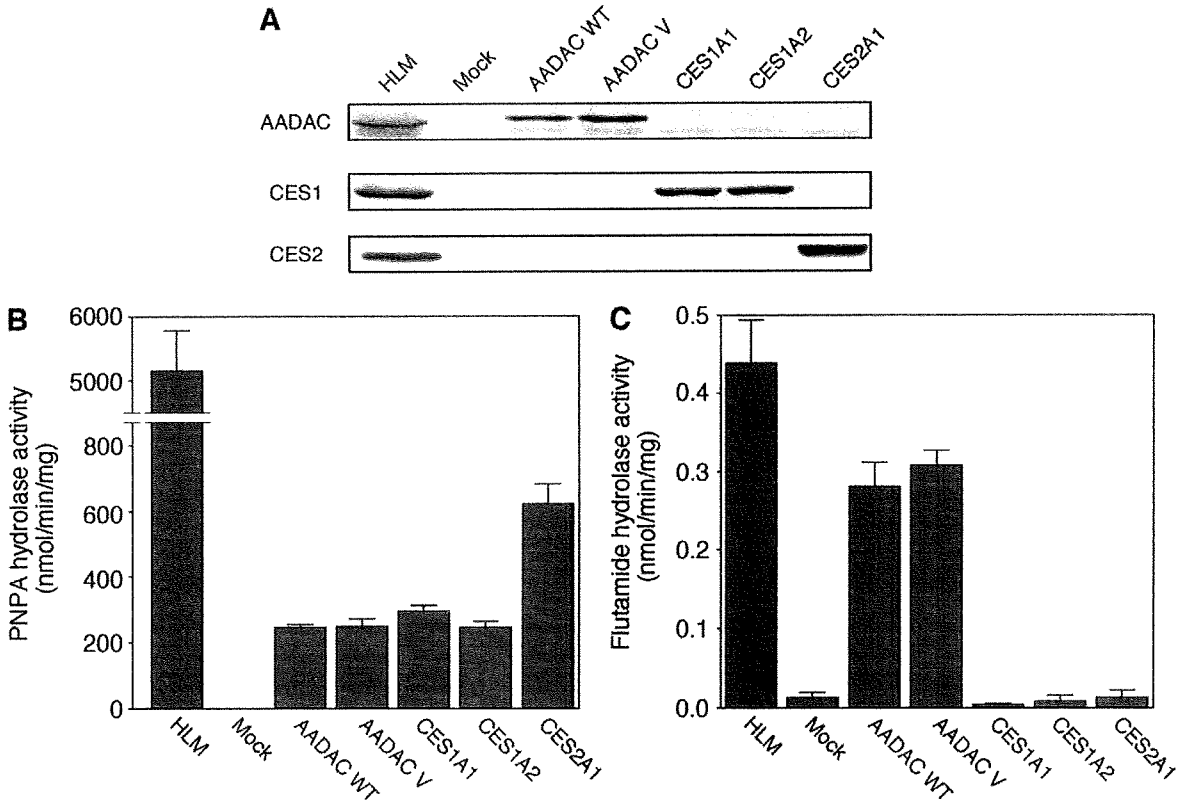

FIG. 1. A, immunoblot analysis of recombinant human AADAC, CES1, and CES2 expressed in COS7 cells. Total cell homogenates from COS7 cells (30 μg) were separated by electrophoresis using 10% SDS-polyacrylamide gel. PNPA hydrolase activities (B) and flutamide hydrolase activities (C) by recombinant AADAC, CES1, and CES2. The homogenates of COS7 cells expressing these enzymes were incubated with 100 μM PNPA or 500 μM flutamide. Each column represents the mean ± S.D. of triplicate determinations. WT, wild type; V, variant.

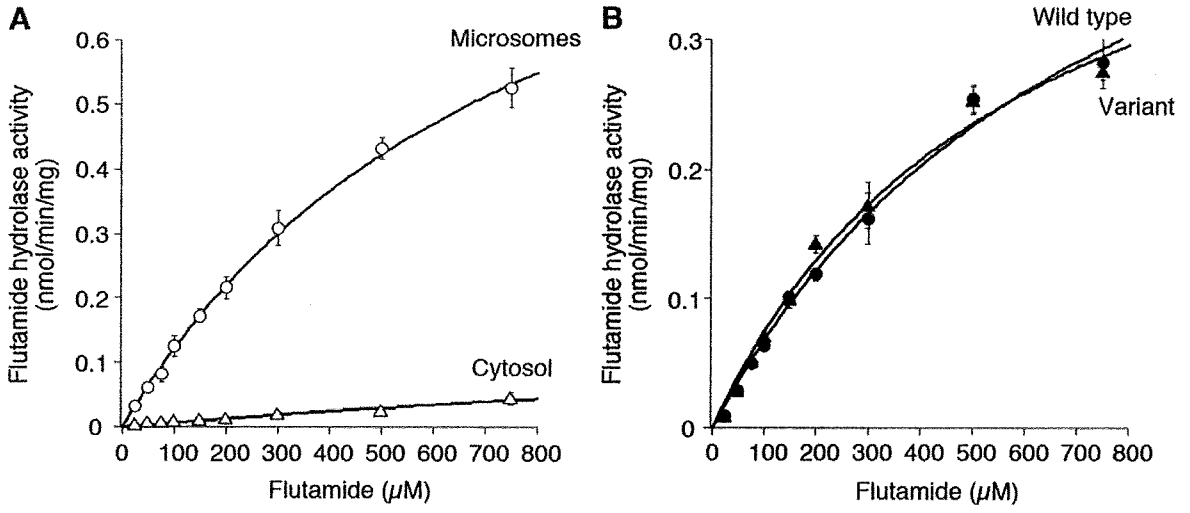

FIG. 2. Kinetic analyses of flutamide hydrolase activities in HLM and HLC (A) and by the recombinant AADAC wild-type and variant (B). The kinetic parameters were estimated from the fitted curve using the computer program KaleidaGraph designed for nonlinear regression analysis. Each data point represents the mean ± S.D. of triplicate determination.

protein, respectively, resulting in an intrinsic clearance of 0.8 ± 0.0 μl/min/mg protein. The K_m and V_{max} values of the AADAC variant were 591 ± 75 μM and 0.5 ± 0.0 nmol/min/mg protein, respectively, resulting in an intrinsic clearance of 0.9 ± 0.1 μl/min/mg protein. Thus, the AADAC variant did not alter the flutamide hydrolase activity. In addition, the K_m values of HLM and AADAC wild-type were not significantly different. These results suggested that AADAC was involved in flutamide hydrolysis in human liver.

Effects of Chemical Inhibitors on Flutamide Hydrolase Activity in HLM and Recombinant AADAC. To prove that AADAC is a principal enzyme for flutamide hydrolysis in human liver, the effects of inhibitors on the flutamide hydrolase activities by HLM and the

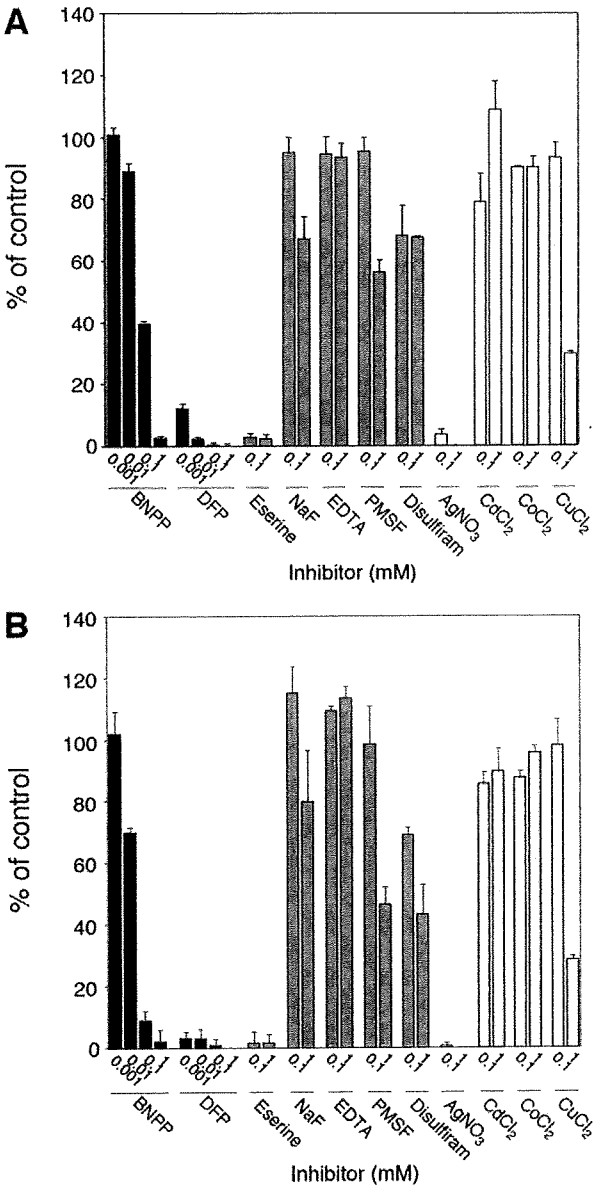

FIG. 3. Effects of chemical inhibitors on flutamide hydrolase activity. Flutamide hydrolase activities in HLM (A) and the recombinant AADAC (B) wild-type were determined at a substrate concentration of 500 μM. The control activity values were 0.438 (A) and 0.283 (B) nmol/min/mg protein, respectively. Each column represents the mean ± S.D. of triplicate determinations.

recombinant AADAC were analyzed. In this study, representative inhibitors of various esterases were used to clarify the involvement of various esterases and examine the inhibitory characteristics of AADAC. The flutamide hydrolase activity by HLM was inhibited in a BNPP concentration-dependent manner and was potently inhibited by 0.01 to 1 mM DFP, 0.1 to 1 mM eserine, and $AgNO_3$ (Fig. 3A). In addition, the flutamide hydrolase activity by HLM was moderately inhibited by 1 mM NaF, PMSF, disulfiram, and $CuCl_2$. However, no inhibition occurred by EDTA, $CaCl_2$, and $CoCl_2$. A similar inhibition pattern was obtained by the recombinant AADAC wild-type (Fig. 3B) and variant (data not shown). These results imply that AADAC is a major esterase responsible for the flutamide hydrolysis in human liver.

Expression of AADAC mRNA in Human Normal Tissues. The expression level of AADAC mRNA in human tissues was determined by

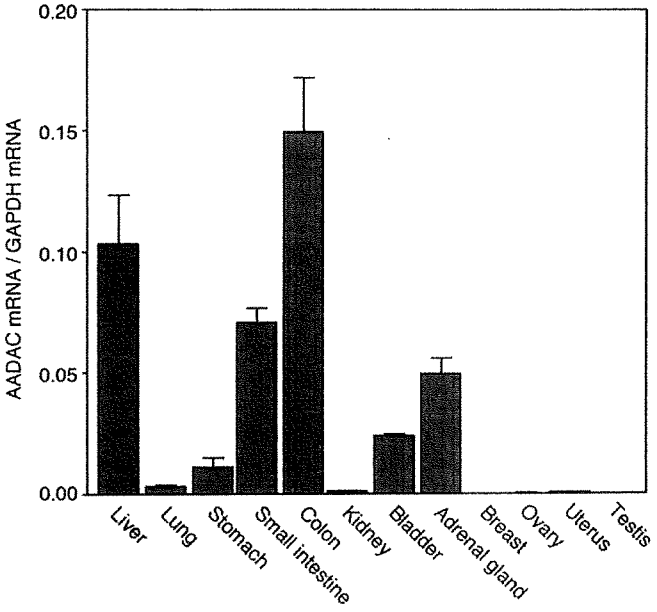

FIG. 4. Expression levels of AADAC mRNA in various human normal tissues. Relative copy numbers of AADAC to GAPDH in human tissues were determined by real-time RT-PCR analysis. Each column represents the mean ± S.D. of triplicate determinations.

real-time RT-PCR analysis (Fig. 4). The expression level of AADAC mRNA was normalized with GAPDH mRNA level. AADAC mRNA is highly expressed in colon, liver, and small intestine and moderately expressed in adrenal gland, bladder, and stomach. Other tissues investigated in this study showed low expression levels. We previously examined the expressions of CES1A1, CES1A2, and CES2 mRNA in human normal tissues (T. Maruichi, M. Katoh, S. Takahashi, M. Nakajima, and T. Yokoi, unpublished data). The expression levels of AADAC mRNA in all the tissues except colon and adrenal gland were lower than those of these CES mRNA. The expression level of AADAC in human liver was 116-, 23-, and 8-fold lower than those of CES1A1, CES1A2, and CES2, respectively.

Expression of AADAC Protein and Flutamide Hydrolase Activities in Human Tissues. To further analyze whether AADAC is responsible for the flutamide hydrolysis in humans, the expression of AADAC and the flutamide hydrolase activity in various human tissues were measured. The expression levels of AADAC, CES1A, and CES2 proteins in HLM, HJM, HPM, and HRM were determined by immunoblot analysis (Fig. 5A). AADAC protein was expressed in HLM and HJM but not in HPM and HRM. This result corresponded with the expression of AADAC mRNA (Fig. 4). CES1A protein was expressed in HLM and HPM, whereas CES2 protein was expressed in HLM, HJM, and HRM. High flutamide hydrolase activity at a concentration of 500 μM flutamide was detected in HJM (0.72 ± 0.02 nmol/min/mg protein) and HLM (0.43 ± 0.03 nmol/min/mg protein). The fact that the hydrolase activity in HJM was higher than that in HLM was supported by the results of the immunoblot analysis. On the other hand, HPM, in which CES1A is expressed, and HRM, in which CES2 is expressed, showed slight hydrolase activity (0.02 ± 0.00 and 0.01 ± 0.01 nmol/min/mg protein, respectively). These results suggested that AADAC is responsible for the flutamide hydrolysis in humans.

Correlation Analysis between Flutamide Hydrolase Activity and AADAC Protein Expression Level. The flutamide hydrolase activities in microsomes from 50 human livers were determined at a concentration of 500 μM. The flutamide hydrolase activities ranged

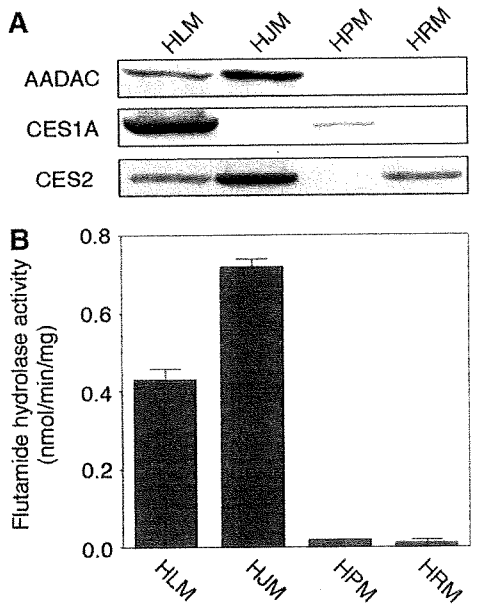

FIG. 5. A, immunoblot analysis of AADAC, CES1A, and CES2 in HLM, HJM, HPM, and HRM. Total cell homogenates from COS7 cells (30 μg) were separated by electrophoresis using 10% SDS-polyacrylamide gel. B, flutamide hydrolase activities in microsomes. The microsomes were incubated with 500 μM flutamide. Each column represents the mean ± S.D. of triplicate determinations.

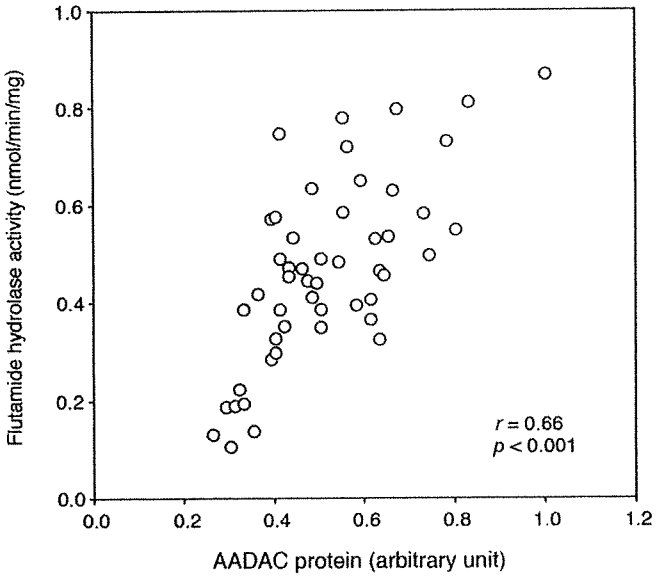

FIG. 6. Correlation between the expression levels of AADAC protein and flutamide hydrolase activities in 50 HLM. The expression level of AADAC protein was determined by immunoblot analysis. The flutamide hydrolase activity was determined by HPLC.

from 0.11 to 0.87 nmol/min/mg protein (mean ± S.D., 0.47 ± 0.19 nmol/min/mg protein), resulting in 7.9-fold interindividual variability. In addition, the expression levels of AADAC protein in microsomes from 50 human livers were determined by immunoblot analysis. The AADAC protein expression levels are represented as relative levels to the sample, which is the highest expression level. The relative expression levels of AADAC protein in HLM ranged from 0.26 to 1.00, resulting in 3.8-fold interindividual variability. As shown in Fig. 6, the expression level of AADAC protein and the flutamide hydrolase activity were significantly correlated ($r = 0.66$, $p < 0.001$). These results also supported that AADAC is a principal enzyme for the flutamide hydrolysis.

Discussion

Human AADAC was first identified as an enzyme that catalyzes the deacetylation of 2-acetylaminofluorene (Probst et al., 1991). It has been believed that AADAC might function as a lipase because of the high homology of the active site of AADAC with that of hormone-sensitive lipase (Probst et al., 1994). On the other hand, human CES enzymes are major serine esterases involved in the hydrolysis of various drugs and xenobiotics. AADAC is one of the major serine esterases expressed in HLM and CES enzymes (Ross and Crow, 2007), but it was unknown whether AADAC is involved in the hydrolysis of therapeutic drugs.

An antiandrogen drug, flutamide, has been widely used for prostate cancer, but severe hepatotoxicity sometimes occurred. Several studies suggested that the flutamide-induced hepatotoxicity was caused by FLU-1, a metabolite of hydrolyzed flutamide, or *N*-hydroxyl FLU-1, a metabolite of FLU-1 by CYP3A or CYP1A (Aizawa et al., 2003; Matsuzaki et al., 2006; Ohbuchi et al., 2009). Therefore, it was considered that flutamide hydrolysis was important in the occurrence of hepatotoxicity, but the flutamide hydrolase enzyme had never been identified. It was conceivable that human CES enzymes CES1A and CES2 are responsible for the hydrolysis of flutamide because they contribute largely to the hydrolysis of various drugs and xenobiotics. However, it was reported that the flutamide hydrolase activity was not detected by using purified CES1A and CES2 (Takai et al., 1997), which is consistent with our finding that the recombinant CES1A1, CES1A2, and CES2A1 did not show the flutamide hydrolase activity (Fig. 1C). Moreover, the activity was scarcely detected in HPM and HRM, in which CES1A and CES2 enzymes were expressed, respectively. On the other hand, the recombinant AADAC showed the flutamide hydrolase activity, and the activity was highly detected in HLM and HJM, in which AADAC is expressed. Furthermore, the similar activities of flutamide hydrolysis in HLM and the recombinant AADAC were consistent with the similar blot densities of AADAC (Fig. 1, A and C). Thus, we found that human AADAC is a major contributor to the flutamide hydrolysis. PNPA hydrolase activity, a general esterase activity, was detected in recombinant AADAC and CES1A1, CES1A2, and CES2A1 (Fig. 1B). However, the activity in HLM was substantially higher than the sum of those by recombinant AADAC and CES enzymes (Fig. 1B). Other esterases such as paraoxonase and butyrylcholinesterase may participate in the PNPA hydrolysis in HLM.

It has been reported that AADAC is located on the lumen side of endoplasmic reticulum (Frick et al., 2004). This corresponded to the present result that the flutamide hydrolase activity in HLM was substantially higher than in HLC (Fig. 2A). In addition, there was no significant difference between the K_m values of HLM (794 ± 83 μM) and recombinant AADAC wild-type (778 ± 122 μM) (Fig. 2, A and B). This finding also supported the involvement of AADAC in flutamide hydrolysis in human liver. However, the activity was also detected in HLC. It was already known that CES1A and CES2 enzymes are present in HLC (Xu et al., 2002; Tabata et al., 2004), but recombinant CES1A1, CES1A2, and CES2 could not hydrolyze flutamide (Fig. 1C). In addition, it was shown that sialic acid 9-*O*-acetylesterase, an alacepril hydrolase enzyme, purified from rat liver cytosol also could not hydrolyze flutamide (Usui et al., 2003). Therefore, other enzymes that can hydrolyze flutamide may be present in HLC, but the contribution to flutamide hydrolysis would be limited.

To confirm that flutamide hydrolysis in HLM is specifically cata-

lyzed by AADAC, we performed inhibition analyses by using various chemical inhibitors and heavy metals (Fig. 3). The flutamide hydrolase activity in HLM was inhibited in a BNPP concentration-dependent manner but was not completely inhibited by 1 mM PMSF (residual activity, 56% of control). It is known that the CES1A and CES2 enzyme activities are inhibited in a BNPP concentration-dependent manner and were substantially inhibited by 0.1 mM PMSF (Xie et al., 2002). Therefore, these enzymes would not contribute to the flutamide hydrolysis in HLM. Moreover, the activity was potently inhibited by DFP, eserine, and silver nitrate and moderately inhibited by NaF, disulfiram, and $CuCl_2$. Other inhibitors used in the present study did not cause inhibition. Because DFP and BNPP are organophosphates, general CES inhibitors, it is plausible that DFP also inhibits AADAC. Eserine and NaF are cholinesterase inhibitors (Iwatsubo, 1965; Ciliv and Ozand, 1972; Preuss and Svensson, 1996). It is of interest that flutamide hydrolysis was effectively inhibited by eserine but not by NaF. EDTA and disulfiram are known to be inhibitors of paraoxonase and monoacylglycerol lipase, respectively (Gonzalvo et al., 1997; Labar et al., 2007). Therefore, it was conceivable that these enzymes did not contribute to the flutamide hydrolysis in HLM. The inhibitory specificity of esterases by heavy metals has not been obvious, but in this study we found that AADAC was potently inhibited by $AgNO_3$ and moderately inhibited by $CuCl_2$. Probst et al. (1994) previously reported that the 2-acetylaminofluorene deacetylation catalyzed by AADAC in HLM was inhibited in a BNPP concentration-dependent manner but was not inhibited by increasing the concentrations of PMSF. The inhibition pattern of flutamide hydrolysis by BNPP and PMSF was similar to that of 2-acetylaminofluorene deacetylation.

To investigate the tissue distribution of AADAC mRNA in human normal tissues, real-time RT-PCR was performed (Fig. 4). AADAC mRNA was mainly expressed in human normal liver, small intestine, and colon. In addition, we confirmed that AADAC protein was expressed in human liver and jejunum microsomes but not in human pulmonary and renal microsomes (Fig. 5A). The tissue distribution of AADAC protein corresponded to that of AADAC mRNA. It is of interest that the expression of AADAC protein in jejunum was higher than in liver, although the mRNA expression was opposite. As shown in Fig. 6, there was interindividual variability in AADAC protein level and flutamide hydrolase activity in human liver. The discrepancy may be because of use of liver RNA derived from single donor. In general, the small intestine plays an important role in the first-pass metabolism of therapeutic drugs given orally (Lin et al., 1999). AADAC would play a certain role in the first-pass metabolism of flutamide in small intestine and in liver.

Correlation analysis was performed between the expression level of AADAC protein and the flutamide hydrolase activity using individual HLM samples (Fig. 6). The correlation between the expression level of AADAC and the flutamide hydrolase activity was strongly significant. Although the point appears to show an x-axis intercept greater than 0, several individual HLM samples may partially include inactive AADAC protein. The expression level of AADAC protein and the flutamide hydrolase activity were moderately variable. It is feasible that the induction by xenobiotics from the environment or diet and endobiotics, and genetic polymorphism of the *AADAC* gene affect the interindividual variability of the flutamide hydrolase activity and AADAC expression level. However, the regulation mechanisms of human AADAC expression are not fully understood. Saito et al. (2003) previously found 23 SNPs in the *AADAC* gene using DNA samples of 48 Japanese. Among them, only an SNP that was also found in this study (g.13651G > A, c.931G > A) leads to an amino acid change (V281I). However, AADAC variant (V281I) appeared not to alter the enzyme activity (Fig. 3B). It is considered that flutamide hydrolysis is important in the occurrence of hepatotoxicity (Aizawa et al., 2003; Matsuzaki et al., 2006; Ohbuchi et al., 2009). Therefore, the interindividual variability of AADAC might affect the incidence of flutamide-induced hepatotoxicity. Further study on the regulation mechanisms and genetic polymorphisms of human AADAC will be necessary.

In conclusion, we found that human AADAC is a principal enzyme in the flutamide hydrolysis. The present study is the first report of the contribution of human AADAC to the metabolism of a therapeutic drug.

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