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Advantages of Human Hepatocyte-Derived Transformants Expressing a Series of Human Cytochrome P450 Isoforms for Genotoxicity Examination

Tsuneo Hashizume,* Sumie Yoshitomi,† Satoru Asahi,* Rieko Uematsu,* Shigeo Matsumura,* Fumio Chatani,* and Hiroaki Oda‡,[1]

*Development Research Center and †Discovery Research Center, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, 17-85, Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532-8686, Japan; and ‡Laboratory of Nutritional Biochemistry, Nagoya University Graduate School of Bioagricultural Sciences, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

[1]To whom correspondence should be addressed. Fax: 81-52-789-5050. E-mail: hirooda@agr.nagoya-u.ac.jp.

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Metabolites of chemicals can often be ultimate genotoxic species; thus, *in vitro* routine testing requires the use of rat liver S9. However, there is a question as to whether this represents an appropriate surrogate for human metabolism. We have previously demonstrated the usefulness of HepG2 transformants expressing major human cytochrome P450 (CYP) isoforms to assess the genotoxicity of metabolites. We further assessed the advantages of these transformants from the following three aspects. First, the sensitivity of these transformants was confirmed with micronucleus (MN) induction by 7,12-dimethylbenz[*a*]anthracene or ifosfamide in transformants expressing the corresponding CYP1A1 or CYP2B6 and CYP2C9, respectively. Second, by using these transformants, β-endosulfan, a chemical for which the CYP isoforms contributing to its genotoxicity are unknown, was found to induce MN through the CYP3A4-mediated pathway. This result was confirmed by the facts that the decreased CYP3A4 activity using a inhibitor or short interfering RNA (siRNA) repressed MN induction by β-endosulfan and that endosulfan sulfate, one of the metabolites produced by CYP3A4, induced MN in the transformants harboring an empty vector. Third, the interaction between phase I and II drug-metabolizing enzymes was demonstrated by MN induction with inhibitors of uridine diphosphate (UDP)-glucuronosyltransferases in tamoxifen-treated transformants harboring the corresponding CYP3A4 or with inhibitors of glutathione *S*-transferase in safrole-treated transformants harboring the corresponding CYP2D6, whereas neither tamoxifen nor safrole alone induced MN in any transformant. These advantages provide the benefits of newly established transformants for *in vitro* genotoxicity testing that reflects comprehensive metabolic pathways including not only human CYP isoforms but also the phase II enzymes.

Key Words: **HepG2 transformants; human cytochrome P450; phase II enzymes; genotoxic metabolite.**

It is well known that the metabolites of chemicals are often the ultimate species responsible for genotoxicity and carcinogenicity. However, most genotoxicity studies are carried out under *in vitro* conditions using bacteria (*Salmonella typhimurium*) or mammalian cells (Chinese hamster ovary, V79, Chinese hamster lung cells, and lymphocytes) that are devoid of enzymes involved in the activation of promutagens. Therefore, *in vitro* testing to identify the potential genotoxic hazard for human of new chemicals routinely utilizes the liver S9 fraction from rats treated with Aroclor 1254 or phenobarbital/5,6-benzoflavone as a metabolic activation system (Ames *et al.*, 1973; Paolini and Cantelli-Forti, 1997).

However, it can be questioned if the induced rat liver S9 fraction represents an appropriate surrogate for the metabolic capabilities of humans for the following reasons (Ku *et al.*, 2007; Obach and Dobo, 2008). First, it is now known that the rat and human cytochrome P450 (CYP) enzymes can differ in their substrate specificities and the reactions catalyzed (Guengerich, 1997). Second, with phenobarbital/5,6-benzoflavone induction, although the expression levels of CYP1A and 2B enzymes are markedly elevated, others such as CYP3A are affected only in a minor way, whereas others (e.g., CYP2C11) may decrease (Guengerich *et al.*, 1982). Third, the system is set up to favor CYP-mediated metabolism. Some phase II enzymes, such as UDP-glucuronosyltransferases (UGT), glutathione *S*-transferases (GST), sulfotransferase (SULT), or methyl transferases, are not active in the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)-supplemented S9 system because other cofactors and additives (e.g., uridine diphosphate glucuronic acid, glutathione, acetyl-coenzyme A, etc.) would be needed (Ku *et al.*, 2007; Obach and Dobo, 2008). This can be essential not only for reducing potential false positives (e.g., reactive electrophiles that would be rapidly quenched by conjugation *in vivo* before being able to cause mutation) but also for false negatives because some conjugation reactions can yield metabolites that are more reactive than their substrate (e.g., sulfation of N-hydroxy-2-acetylaminofluorene or acetylation of N-hydroxylated heterocyclic amines; Dashwood, 2002; Ku *et al.*, 2007). The NADPH-supplemented

S9 system may represent an incomplete picture of the metabolism that can occur *in vivo*.

Another important issue for using induced rat liver S9 to detect the potential hazard in *in vitro* genotoxicity testing is that some genotoxic metabolites have to be formed by enzymes within the target cell (Ku *et al*., 2007). In addition, the reactions catalyzed by CYP molecules require the presence of NADPH CYP reductase and cytochrome *b5* to support some CYP-mediated reactions appropriately; thus, the permanent cell lines with endogenous bioactivation capacity such as human hepatoma cell lines are preferable. Of these cell lines, the most promising is the HepG2 (Aden *et al*., 1979) as this cell line possesses the factors necessary for the function of CYP. Furthermore, HepG2 cells possess the different phase I and phase II drug-metabolizing enzymes involved in the activation/detoxification of genotoxic carcinogens (for review, see Knasmüller *et al*., 1998, 2004). On the other hand, use of HepG2 requires caution because of its low CYP activity when compared with that in human primary hepatocytes (Wilkening *et al*., 2003). Therefore, we previously established a series of HepG2 transformants expressing the cytochromes 1A1, 1A2, 2A6, 2B6, 2C8, 2C19, 2D6, 2E1, and 3A4 with the apparent *V*max values for their characteristic substrates (Yoshitomi *et al*., 2001). Our previous study demonstrated the applicability of this HepG2 transformant system for *in vitro* micronucleus (MN) tests with validation of the sensitivity using well-studied genotoxic chemicals requiring CYP activation and with exploratory testing to see if this system could identify the CYP isoforms responsible for the genotoxicity of a chemical whose CYP activation is unknown (Hashizume *et al*., 2009).

In the present study, the advantages of this system were further assessed through the following three trials. First, the sensitivity of this system to detect genotoxicity requiring CYP activation was confirmed in *in vitro* MN tests with two well-studied chemicals, 7,12-dimethylbenz[*a*]anthracene (DMBA) and ifosfamide. Second, this system allowed us to investigate the genotoxicity of a chemical, β-endosulfan, for which the contributing CYP isoforms, especially those mediated by CYP3A4 which is known to metabolize many drugs in humans, have not yet been identified. Third, the relevance of the interaction between phase I and phase II drug-metabolizing enzymes, e.g., UGT, GST, and SULT, in the test system was demonstrated in a MN test of tamoxifen or safrole, which has been reported to be metabolized by enzymes of both phases.

In conclusion, we have demonstrated the benefits of a newly established transformant system for *in vitro* genotoxicity testing that reflects comprehensive metabolic pathways including not only human CYP isoforms but also the phase II drug-metabolizing enzymes.

MATERIALS AND METHODS

Chemicals and solvents used. DMBA, dimethyl sulfoxide, β-endosulfan, ifosfamide, safrole, and endosulfan sulfate were obtained from Wako Pure Chemicals (Osaka, Japan); mitomycin C (MMC) was from Kyowa Hakko Kogyo (Tokyo, Japan); ketoconazole was from BD Gentest (MA); physiological saline solution (saline) was from Otsuka Pharmaceutical Factory (Tokushima, Japan); and tamoxifen and diclofenac were from MP Biomedicals (OH). Ethacrynic acid was supplied from Tokyo Chemical Industry (Tokyo, Japan).

The In Vitro MicroFlow Kit was purchased from Litron Laboratories (Rochester, NY). This kit included Nuclei Acid Dye A Solution (contains ethidium monoazide dye), Nuclei Acid Dye B Solution (contains SYTOX Green dye), Lysis Solutions 1 and 2, and RNase Solution. Six-micron fluorescent beads were used as counting beads and were obtained from Invitrogen (Grand Island, NY; cat. no. P14828).

Cell culture. HepG2 cells and its transformants expressing human CYP isoforms were used (Hashizume *et al*., 2009; Yoshitomi *et al*., 2001). The selected transformants were designated as Hepc/1A1.4, Hepc/1A2.9, Hepc/2A6L.14, Hepc/2B6.68, Hepc/2C8.46, Hepc/2C9.1, Hepc/2C19.12, Hepc/2D6.39, Hepc/2E1.3-8, and Hepc/3A4.2-30, which expressed CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, respectively. As a mock control, pcDNA3.1(+) was introduced into HepG2 and named Hepc-Mock. These transformants have already been confirmed to have nearly the same K_m values as those from human liver microsomes (Table 1). In addition, we have found that the parent HepG2 and mock control Hepc-Mock cells still did not have any detectable reverse transcription-PCR products corresponding to each CYP messenger RNA (Yoshitomi *et al*., 2001). Therefore, each transformant is considered to have predominantly only one CYP activity. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 100μM nonessential amino acids (Invitrogen) in a humidified atmosphere in 5% CO_2 at 37°C. The transformants were cultured in the above-mentioned DMEM-supplemented 200 μg/ml G418 (Invitrogen).

Chemical treatments. Approximately 24 h before the treatment, the cells were seeded at 1×10^5 cells per well in 24-well tissue culture plates. The cells were incubated with various concentrations of the chemicals in a single culture for 48 h at 37°C. For DMBA, tamoxifen or β-endosulfan, a stock solution was prepared and frozen, and immediately prior to treatment, the stock solutions were diluted with dimethyl sulfoxide (DMSO) as 200× concentrations. Safrole was diluted with DMSO immediately prior to treatment. Ifosfamide was dissolved directly with the culture medium to make the intended concentrations. A 0.5% (vol/vol) solvent was used for a negative control. As a positive control, 25 ng/ml of MMC was administered in every experiment to ensure that the assays worked properly. Experiments for each chemical were repeated at least three times independently.

Flow cytometric scoring of MNs. After 48-h treatment, the cells were collected by trypsinization. Cells from each culture were sampled for flow cytometric staining, according to the In Vitro MicroFlow Kit procedure, and 10,000 events were automatically collected from a Becton Dickinson FACS Calibur machine using CellQuest (Version 3.3) software (Avlasevich *et al*., 2006; Bryce *et al*., 2007, 2008). Cells were collected via centrifugation at approximately 300 × g for 2 min. Supernatants were removed, cells were resuspended, and 300 μl of Nucleic Acid Dye A Solution was added. These tubes were placed in aluminum blocks on crushed ice. A fluorescent light bulb was positioned approximately 15 cm above the specimens for 30 min.

After the photoactivation period, 1 ml of cold kit-supplied buffer solution with counting beads (1 drop per 10 ml buffer solution) was added to each sample. The cells were collected via centrifugation, and the supernatants were removed so that approximately 100 μl of supernatant remained per tube. The cells were gently resuspended, and then 500 μl of kit-supplied Lysis Solution 1 was added to each tube, which was immediately vortex mixed. These samples were kept at 37°C for 1 h, and then 500 μl of kit-supplied Lysis Solution 2 was injected forcefully into each tube. After vortex mixing, these samples were stored at room temperature until flow cytometric analysis.

Procedures to obtain flow cytometric data, including configuration of regions and gating logic, were as described in the In Vitro MicroFlow Kit

TABLE 1
Characteristics of the Transformants[a]

Name of transformant	Expressed CYP isoform	Catalytic reaction measured	Kinetic analysis: Transformant K_m (μM)	Kinetic analysis: Human liver microsomes[b] K_m (μM)	Kinetic analysis: Transformant *V*max (ρmol/min/mg)
Hepc/1A1.4	CYP1A1	7-Ethoxyresorufin *O*-deethylation	0.25	0.19	56
Hepc/1A2.9	CYP1A2	7-Ethoxyresorufin *O*-deethylation	0.72	0.39	2
Hepc/2A6L.14	CYP2A6	Coumarin 7-hydroxylation	5.1	2.3	812,000
Hepc/2B6.68	CYP2B6	7-Ethoxycoumarin *O*-deethylation	81	—	80,000
Hepc/2C8.46	CYP2C8	Taxol 6-hydroxylation	7.4	24	9400
Hepc/2C9.1	CYP2C9	Tolbutamide 4-hydroxylation	45	120	25,000
Hepc/2C19.12	CYP2C19	(*S*)-mephenytoin 4′-hydroxylation	8.3	16	140,000
Hepc/2D6.39	CYP2D6	Bufuralol 1′-hydroxylation	17	40	14
Hepc/2E1.3-8	CYP2E1	*p*-Nitrophenol hydroxylation	88	30	120
Hepc/3A4.2-30	CYP3A4	Testosterone 6β-hydroxylation	96	89	71

[a]Yoshitomi *et al*. (2001).
[b]Iwata *et al*. (1988).

manual and in the report by Bryce *et al*. (2007). Briefly, data acquisition was accomplished with a flow cytometer providing 488 nm excitation. SYTOX-associated fluorescence emission was collected in the FL1 channel (530/30 band-pass filter), and EMA-associated fluorescence was collected in the FL3 channel (670 long-pass filter). Events were triggered on FL1 fluorescence. The incidence of MN was determined through the acquisition of at least 10,000 gated nuclei per culture.

Inhibitory treatment using chemical inhibitor or siRNA specific to each CYP isoform. To assess the involvement of CYP3A4 enzymatic activity in the MN induction by β-endosulfan, ifosfamide or MMC, and ketoconazole, a specific inhibitor of CYP3A4 (Andrew and Brian, 2007) was coadministered with β-endosulfan to HepG2 transformants expressing CYP3A4. Likewise, diclofenac and ethacrynic acid were selected as UGT (Kiang *et al*., 2005; Uchaipichat *et al*., 2004) and GST inhibitors (Ahokas *et al*., 1984), respectively. These chemical inhibitors were dissolved fresh in DMSO and diluted to a 1000× concentration to give a final concentration of 0.1% in DMSO. The cells were cotreated with the chemicals and the corresponding inhibitor for 48 h.

To examine the inhibition of CYP3A4 activity by ketoconazole, the cells were separately plated onto 24-well plates at 1×10^5 cells per well, and on the following day, the cells were exposed to ketoconazole for 48 h. Using CYP3A4-specific substrate Luciferin-isopropyl acetal (IPA), the activities in cells were determined both at the beginning and at the end of the ketoconazole treatment as described below.

siRNA to CYP3A4 (Stealth select HSS102565) and negative control siRNA (Stealth RNAi negative control low GC duplex) were obtained from Invitrogen. Transfection complexes were prepared in 100 μl of Opti-MEM I reduced serum medium (Invitrogen) by mixing 1.5 μl of Lipofectamine RNAiMAX (Invitrogen) and 50nM of siRNA in the wells of a 24-well tissue culture plate. These complexes were incubated for 20 min at room temperature. The cell suspension (1×10^5 cells per 0.5 ml per well) was transferred into the wells and mixed gently with these complexes. After incubation for 8 h at 37°C, the cells were re-covered for 16 h with fresh medium. Then the cells were treated with the various chemicals for 48 h. To examine the inhibition of CYP3A4 activity by siRNA, the cells were separately plated in the absence or presence of siRNA in another 24-well plate, and on the following day, the cells were cultured with fresh medium for additional 48 h. Using Luciferin-IPA, CYP3A4 activities in cells were determined immediately both after the 16-h recovery and after the additional 48-h incubation as described below.

Determination of CYP3A4 activity. CYP3A4 activity was measured using a P450-Glo CYP3A4 Assay (Luciferin-IPA) (Promega, WI). The assay was performed using culture cells according to the instruction manual with some modifications. The cells were incubated at 37°C for 1 h with fresh medium containing Luciferin-IPA at 3μM. For the ketoconazole treatment, the medium also contained 1μM ketoconazole. After the incubation, 150 μl of the supernatant in each well were transferred into the wells of a 96-well white plate. The reaction was terminated by mixing with 150 μl of luciferase detection reagent. The luminescent was determined with a plate-reading luminometer (Perkins Elmer). After transferring the supernatants from the wells, the cells were collected, and the number of cells per well was counted by a Coulter counter (Beckman Coulter). For each measurement, wells without substrate were assayed, and values obtained were subtracted from the wells with substrate. Furthermore, these values were normalized to the cell number. Mean light units per 10^5 cells in duplicate wells were calculated, and the results were expressed as relative values compared with the units in the wells treated with neither inhibitor nor siRNA.

Statistical analysis. The experiments were repeated at least three times independently. All data are given as means ± SDs.

For the contribution of each CYP isoform between HepG2 and each transformant, the *F*-test, Student's *t*-test, and Aspin and Welch *t*-test were used according to the literature (Snedecor and Cochran, 1980). First, the data were tested by the *F*-test for homogeneity of variance between the groups. When the variance was homogeneous, Student's *t*-test was used, and when the variance was heterogeneous, the Aspin and Welch *t*-test was performed to compare the mean in the HepG2 with that in the each transformant. The same procedures were applied for the comparison between Hepc-Mock and each transformant.

The assessment of MN induction was analyzed statistically as follows. First, the data were tested by Bartlett's test (Snedecor and Cochran, 1980) for homogeneity of variance. When the variance was homogeneous, Williams' test (Williams, 1972), which assumes a dose-related trend, was performed to determine the (lowest) dosage level in which the statistical difference was observed. When the variance was heterogeneous, the Shirley-Williams' test (Shirley, 1977), which assumes a dose-related trend, was performed to determine the (lowest) dosage level in which the statistical difference was observed.

The *F*-test was conducted at the significance level of 0.20; Bartlett's test was conducted at the significance level of 0.05; and the other tests were conducted at the two-tailed significance level of 0.05.

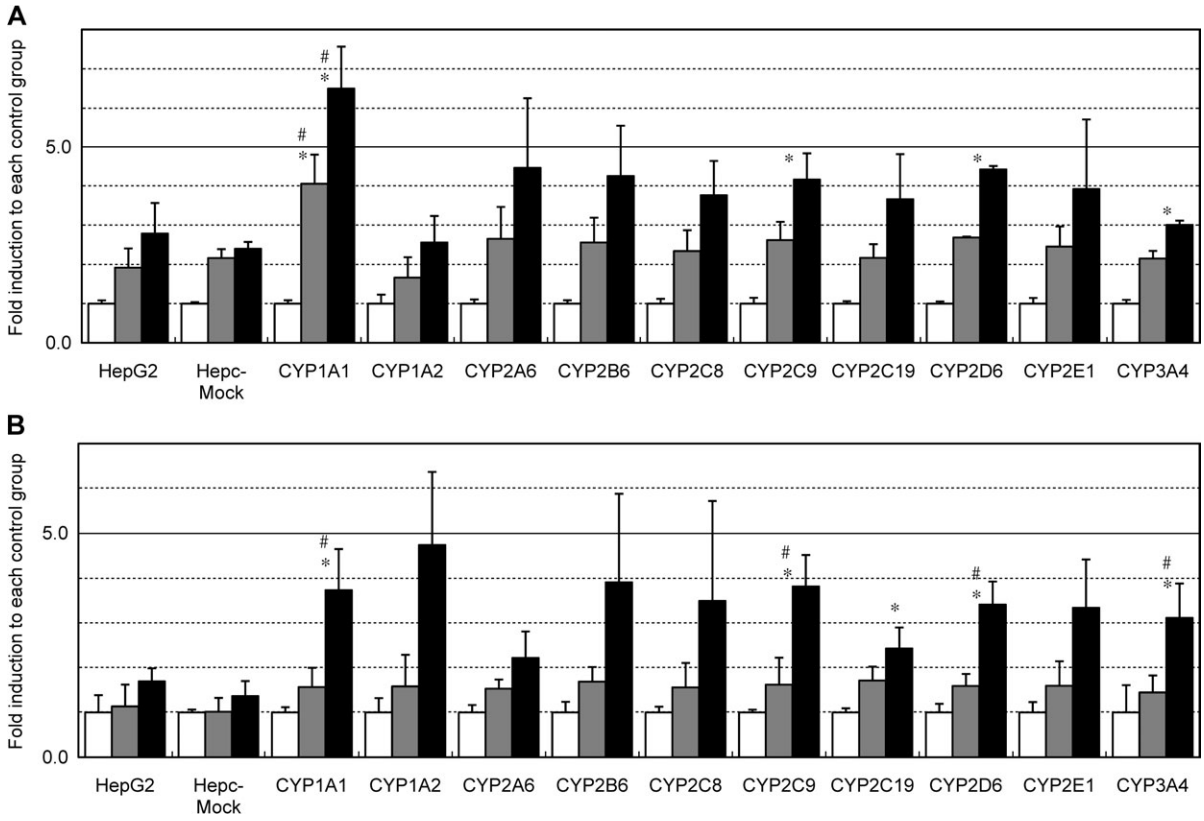

FIG. 1. Micronucleus induction of DMBA (A) or of ifosfamide (B) by expression of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. (A) The cells (1×10^5 cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% DMSO (open bars), 78 ng/ml (gray bars), or 156 ng/ml (solid bars) DMBA. (B) The cells (1×10^5 cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% saline (open bars), 500 μg/ml (gray bars), or 1000 μg/ml (solid bars) ifosfamide. Values were normalized with the mean DMSO- or saline-treated control value of three experiments for each transformant. Each bar represents the mean ± SD. Data were tested using Student's *t*-test when the variance was homogeneous or Aspin and Welch *t*-test when the variance was heterogeneous (*$p < 0.05$, compared with Hepc-Mock; #$p < 0.05$, compared with HepG2).

RESULTS

Confirmation of Sensitivity of the Transformants Using Model Chemicals Requiring CYP Activation

We selected DMBA and ifosfamide as model chemicals exhibiting CYP-mediated MN induction. In the metabolism of DMBA, it had been shown that CYP1A1 had clearly the highest activity among the hepatic CYP isoforms (Shimada and Fujii-Kuriyama, 2004, Shou *et al.*, 1996) and that significant formation of some metabolites was also observed with CYP1A2, CYP2B6, and CYP2C9 (Shou *et al.*, 1996). Ifosfamide had been demonstrated to be efficiently metabolized by CYP2B6, CYP2C9, and CYP3A4 (Chang *et al.*, 1993, Jing *et al.*, 2006). HepG2 and its transformants were treated with two concentrations of these model chemicals in order to validate the sensitivity of this series of transformants for *in vitro* genotoxic assessment.

DMBA treatment significantly increased the frequency of MN regardless of the expressed CYP isoforms (Fig. 1A). For example, the frequencies of MN were approximately 1.9- and 2.8-fold in HepG2 or 2.2- and 2.4-fold in Hepc-Mock, the transformant harboring an empty vector only, higher than that with DMSO in the DMBA treatment at 78 and 156 ng/ml DMBA, respectively. Among the transformants, the fold induction of the percent MN in the transformant expressing CYP1A1 was significantly higher than those in HepG2 and Hepc-Mock cells. When compared with Hepc-Mock cells, significant differences were also seen in the fold induction of the percent MN at 156 ng/ml DMBA in the transformants expressing CYP2C9, CYP2D6, and CYP3A4.

Ifosfamide did not increase the MN frequency in HepG2 as well as Hepc-Mock cells (Fig. 1B); however, significant MN induction was seen in all transformants at 1000 μg/ml. When compared with Hepc-Mock cells, significant increases were found in the transformants expressing CYP1A1, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 at 1000 μg/ml, whereas ifosfamide did not increase the MN frequency in Hepc-Mock or in HepG2. Similarly, when compared with HepG2 cells, statistically significant increases were obtained in the transformants expressing CYP1A1, CYP2C9, CYP2D6, and CYP3A4 at 2000 μg/ml. No significant differences were observed in the transformants such as

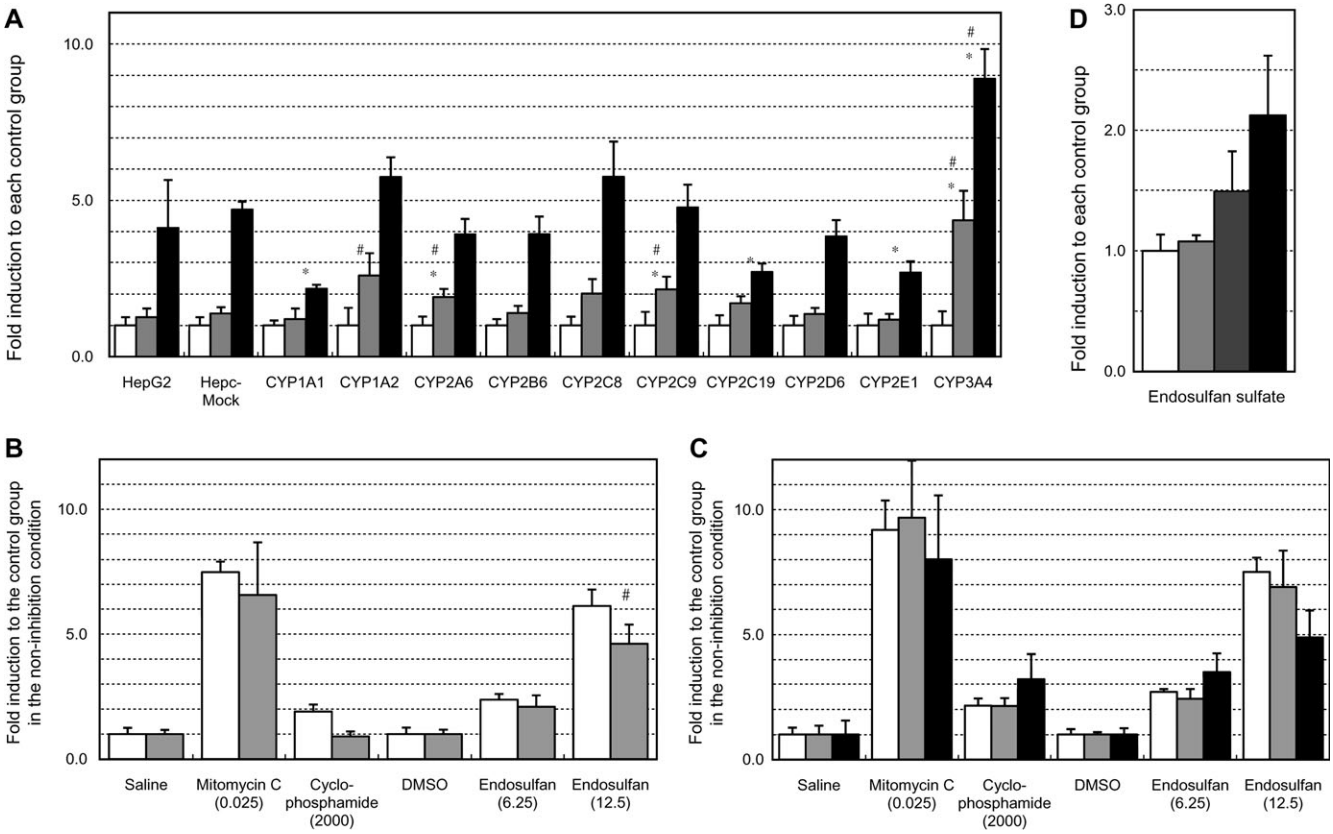

FIG. 2. MN induction of β-endosulfan by CYP3A4-mediated activation. (A) A MN induction of β-endosulfan by expression of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. The cells (1×10^5 cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% DMSO (open bars), 6.25 μg/ml (gray bars), or 12.5 μg/ml (solid bars) β-endosulfan. (B) Effects of ketoconazole, a CYP3A4-specific inhibitor for MN induction by various chemicals in the transformant expressing CYP3A4. The cells (1×10^5 cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% saline, 100 ng/ml MMC, 1000 μg/ml cyclophosphamide, 0.5% DMSO, 6.25, or 12.5 μg/ml β-endosulfan in the absence (open bars) or presence (gray bars) of 1μM ketoconazole. (C) Effects of siRNA to CYP3A4 on MN induction by various chemicals in the transformant expressing CYP3A4. The cells (1×10^5 cells) were seeded onto a 24-well plate for 8 h in absence (open bars) or the presence of 50nM siRNA for negative control (gray bars) or CYP3A4 (solid bars). (D) MN induction of endosulfan sulfate in Hepc-Mock, the transformant expressing an empty vector only. The cells (1×10^5 cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% DMSO (open bars), 8 μg/ml (light gray bars), 10 μg/ml (heavy gray bars), or 12.5 μg/ml (solid bars) endosulfan sulfate. Statistical analysis was done in the same procedure as Figure 1, except for the Student's *t*-test (#$p < 0.05$, compared with the DMSO-treated control group) in Figure 2D.

CYP1A2, CYP2A6, CYP2B6, CYP2C8, and CYP2E1 due to the large deviations.

Because MN was significantly induced in the transformant expressing CYP1A1 by DMBA and in the transformants expressing CYP2C9 and CYP3A4 by ifosfamide, it was confirmed that this series of transformants has sufficient sensitivity to detect model chemicals requiring CYP activation other than the chemicals previously reported by us such as benzo(*a*)pyrene and cyclophosphamide (Hashizume *et al.*, 2009).

Identification of CYP Contributing to MN Induction by a Novel Chemical

In order to elucidate the possibility that the series of transformants can be used to identify the CYP isoforms contributing to MN induction in genotoxic assessment of newly developed drugs, we chose β-endosulfan, because Lu *et al.*, (2000) reported that MNs were increased by β-endosulfan in HepG2 cells; CYP isoforms involved in the metabolic activation of β-endosulfan to exert its genotoxicity have not been identified to the best of our knowledge. Treatment with β-endosulfan induced a fold increase of the percent MN in almost all the HepG2 transformants (Fig. 2A); however, the fold induction to the DMSO control group was 4.4 at 6.25 μg/ml and 8.9 at 12.5 μg/ml β-endosulfan in the transformant expressing CYP3A4. When compared with HepG2 and Hepc-Mock cells, a significant increase was observed only in this transformant. This result suggested that CYP3A4 was involved in the metabolic activation of β-endosulfan and that this series of transformants could be used to identify the CYP-mediated genotoxicity of novel chemicals.

To confirm the involvement of CYP3A4 activity, we examined the effect of ketoconazole, a specific inhibitor of CYP3A4, on MN induction by β-endosulfan in the

TABLE 2
CYP3A4 Activity in Hepc/3A4.2-30 Treated with a Specific Inhibitor or Pretreated with siRNA

Inhibition	Relative values[a]	
	24 h after plating	At the end of 48-h incubation
Nontreated	100	100
Ketoconazole[b]	6.0 ± 2.0	8.2 ± 4.5
Negative control siRNA[c]	101.9 ± 26.0	108.7 ± 10.9
siRNA to CYP3A4[c]	22.6 ± 8.3	16.3 ± 2.7

[a]Values are the mean ± SD in the independent four experiments for ketoconazole treatment and three experiments for siRNA pretreatment.

[b]At the beginning and the end of 48-h treatment by ketoconazole, catalytic activity was determined using 3μM Luciferin-IPA as described in Determination of CYP3A4 activity section.

[c]Twenty-four hours after plating and at the end of 48-h incubation with fresh medium, catalytic activity was determined as described in Materials and Methods section.

transformant expressing CYP3A4 (Fig. 2B). Whereas ketoconazole did not affect MN formation in the DMSO-treated group, MN formation by β-endosulfan at 12.5 μg/ml was significantly reduced by cotreatment with 1μM ketoconazole. In addition, a significant decrease in the frequency of MN was caused by cyclophosphamide as a positive control chemical requiring CYP3A4 activation (Fig. 2B). However, the fold induction of the percent MN by MMC, which does not require activation by CYPs, was not affected by cotreatment with ketoconazole (Fig. 2B), indicating that ketoconazole itself did not have any reducing action of the MN formation by MMC. To confirm the decrease in the enzymatic activity, the activity of CYP3A4 in the transformant was assayed using Luciferin-IPA, the substrate of CYP3A4. Ketoconazole at 1μM decreased approximately 5–10% the activity of the no-inhibitor control within 1 h, and this repression continued during the 48-h treatment period (Table 2).

The involvement of CYP3A4 activity in MN induction by β-endosulfan was further examined using an siRNA specific to CYP3A4. Negative control siRNA did not affect the relative value of MN induction, whereas small but significant decreases were observed in cells exposed with siRNA to CYP3A4 (Fig. 2C). The MN formation did not seem to be affected by siRNA with CYP3A4 either in the negative (DMSO) or positive (MMC) control group, demonstrating that the repressing effect on β-endosulfan–induced MN correlated with the knockdown condition of the CYP3A4 (Fig. 2C). However, pretreatment with siRNA to CYP3A4 did not affect MN induction by cyclophosphamide yet as a positive control chemical requiring CYP3A4 activation, contrary to the cotreatment with ketoconazole. Pretreatment with siRNA to CYP3A4 resulted in a nearly 90% reduction in CYP3A4 activity in the corresponding transformant when compared with the non-pretreated control (Table 2). Pretreatment with the negative control siRNA did not alter the CYP3A4 activity (Table 2).

Lee *et al.* (2006) reported that β-endosulfan was mainly metabolized to endosulfan sulfate in an incubation mixture with human liver microsomes. They also reported that the formation of endosulfan sulfate was inhibited by ketoconazole and that the microsomes expressing human CYP3A4 complementary DNA (cDNA) produced endosulfan sulfate (Lee *et al.*, 2006). Based on these reports, we examined the genotoxicity of endosulfan sulfate in the Hepc-Mock cells harboring an empty vector only in order to investigate whether this sulfate is the metabolite that induces MN in the β-endosulfan–treated transformant expressing CYP3A4. As shown in Figure 2D, endosulfan sulfate induced MN with statistical significance at 12.5 μg/ml. This result demonstrated that endosulfan sulfate was the genotoxic metabolite and that this metabolite was formed by CYP3A4 in the transformant treated with β-endosulfan.

These results of the exploratory experiment with β-endosulfan clearly demonstrated that this system was capable of determining the genotoxicity of a chemical for which the contributing CYP isoforms have not yet been identified.

Genotoxic Assessment of Chemicals Metabolized by Phase I and Phase II Drug-Metabolizing Enzymes

In order to evaluate the relevance of the interaction between phase I and phase II drug-metabolizing enzymes in the test system, the transformants were treated with tamoxifen and safrole. Tamoxifen is reported to be metabolized by CYP3A4 to α-hydroxytamoxifen and further metabolized by SULT to α-hydroxytamoxifen sulfate ester as the putative reactive intermediate (Brown, 2009; White, 2003; Zhao *et al.*, 2009). This intermediate reacts with the exocyclic amino group of guanines (the major reaction) and adenines (a minor reaction) in DNA (Osborne *et al.*, 1996). UGT plays a detoxification role through the glucuronidation of α-hydroxytamoxifen (Brown, 2009; White, 2003; Zhao *et al.*, 2009). Safrole is also reported to be hydroxylated predominantly by CYP2A6, 2C9, 2D6, or 2E1 and further metabolized by SULT to 1′-sulfooxysafrole (Andrew and Brian, 2007; Rietjens *et al.*, 2005). This intermediate forms the electrophilic carbocation of safrole, suggesting the production of DNA adduct (Rietjens *et al.*, 2005). On the other hand, the safrole-2′, 3′-oxide formed from the parent safrole by epoxide hydrolases or 1′-hydroxysafrole-2′, 3′-oxide from 1′-hydroxysafrole are reported to be detoxified by GST (Rietjens *et al.*, 2005). Because these major phase I and II enzymes are functional in HepG2 cells, we examined whether or not tamoxifen and safrole could induce MNs in a series of HepG2 transformants expressing CYP isoforms.

As shown in Figures 3A and 3B, both tamoxifen and safrole did not significantly induce MN at any concentration tested in any transformant. At much higher concentrations of each chemical,

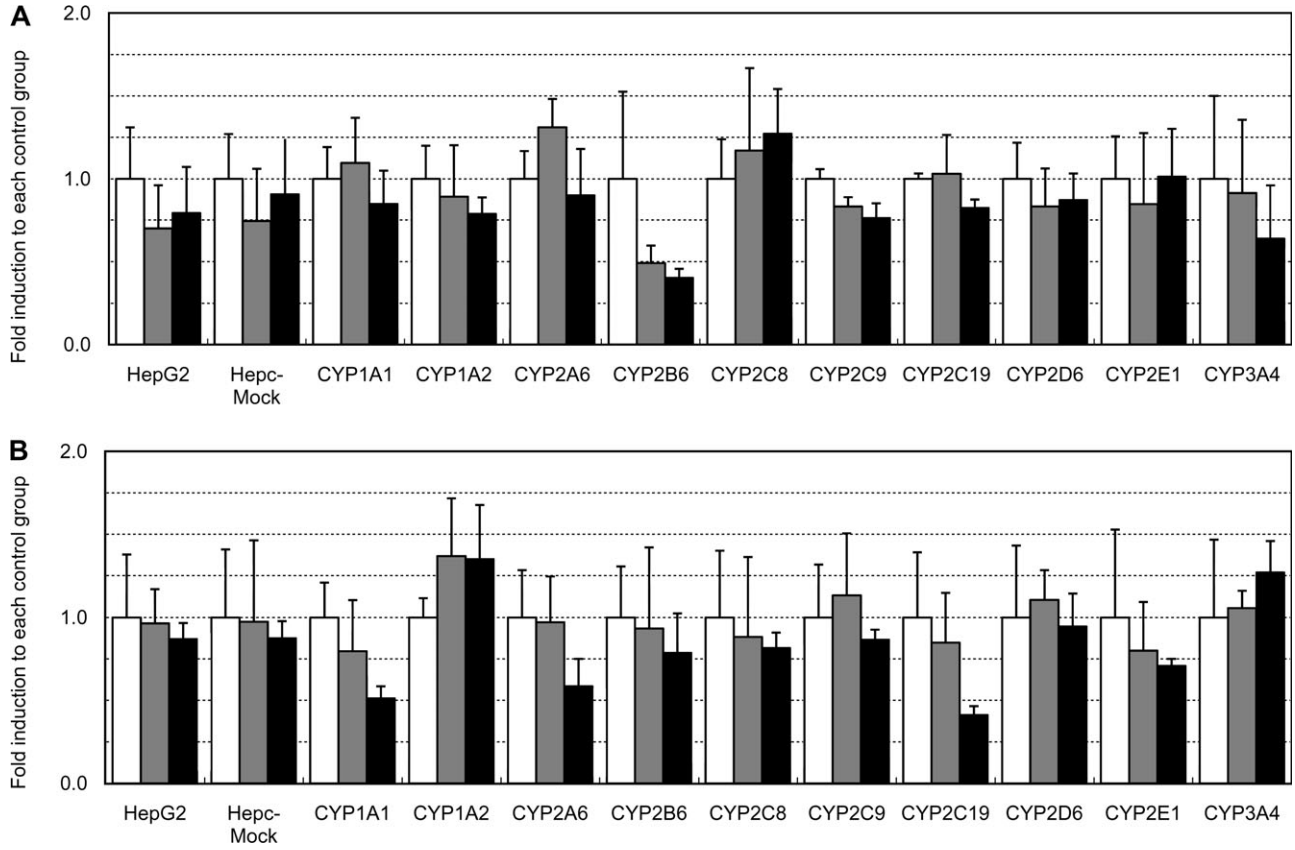

FIG. 3. MN induction of tamoxifen (A) or of safrole (B) by expression of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. (A) The cells (1×10^5 cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% DMSO (open bars), 1 μg/ml (gray bars), or 2 μg/ml (solid bars) tamoxifen. (B) The cells (1×10^5 cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% DMSO (open bars), 83.3 μg/ml (gray bars), or 125 μg/ml (solid bars) safrole. No statistically significant increase was observed when compared with both Hepc-Mock and HepG2.

the frequencies of the MNs were decreased (data not shown), suggesting that the tested concentrations were appropriate to evaluate MN induction. Then to investigate the involvement of the detoxification pathway by UGT in the metabolism of tamoxifen and by GST in the metabolism of safrole, we tested the effect of UGT and GST inhibitors on the MN induction by tamoxifen and safrole, respectively. A small but significant increase in MN by tamoxifen was observed in the presence of the UGT inhibitor, diclofenac, in the transformants expressing CYP3A4, which contribute to the metabolic activation of tamoxifen to α-hydroxytamoxifen (Fig. 4A). This result indicated that the CYP3A4-mediated metabolite, α-hydroxytamoxifen, was further metabolized by UGT to a genotoxically inactive substance. In a similar way, significant increases were also seen in the presence of the GST inhibitor, ethacrynic acid, in the transformants expressing CYP2D6 responsible for the genotoxic activation of safrole to 1′-hydroxysafrole (Fig. 4B). This result suggested that CYP2D6-mediated metabolite, 1′-hydroxysafrole, was further metabolized by GST not exerting its genotoxicity in the metabolic pathway.

These results demonstrated that phase I and phase II drug-metabolizing enzymes were functional in the metabolic activation by CYP or SULT and inactivation by UGT or GST in the HepG2 transformant. The interaction between phase I and II enzymes could not be investigated in the routine *in vitro* genotoxicity systems due to lack of cofactors necessary for several phase II enzymes. Thus, this system is considered to be more suitable for elucidating the mechanism of genotoxicity of chemicals through metabolic activation and inactivation.

DISCUSSION

In the current genotoxicity testing guidelines (ICH S2B, 1997), induced rat liver S9 fraction has been adopted for *in vitro* genotoxicity tests as an exogenous activation system for detecting promutagens, but the relevance of the rat liver S9 has been called into question when considering the risk to humans (Ku *et al.*, 2007; Obach and Dobo, 2008, Paolini and Cantelli-Forti, 1997). Ideally, the permanent cell lines with endogenous bioactivation capacity such as human hepatoma cell lines have been preferred to detect some genotoxic metabolite to be formed in the target cells. We have previously reported the useful HepG2 transformant system expressing

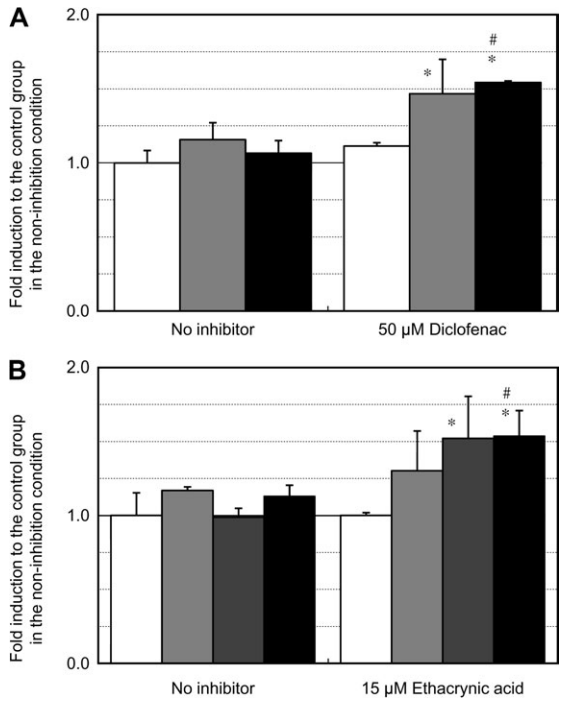

FIG. 4. Effects of a phase II enzyme inhibitor on MN induction by tamoxifen and safrole. (A) The transformant cells expressing CYP3A4 (1×10^5 cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% DMSO (open bars), 2 μg/ml (gray bars), or 3 μg/ml (solid bars) tamoxifen in the absence or presence of 50μM diclofenac, a UGT inhibitor. (B) The transformant cells expressing CYP2D6 (1×10^5 cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% DMSO (open bars), 41.7 μg/ml (light gray bars), 62.5 μg/ml (heavy gray bars), or 83.3 μg/ml (solid bars) safrole in the absence or presence of 15μM ethacrynic acid, a GST inhibitor. The incidence of MN was determined, and statistical analysis was done in the same procedure as Figure 1.

a series of human CYP isoforms for an *in vitro* genotoxicity assessment of chemicals requiring CYP activation (Hashizume *et al*., 2009). In the present study, we have shown the following advantages of this system to elucidate the genotoxicity of metabolites: (1) the detection of genotoxicity mediated by other CYP isoforms than the most active one in the validation with well-known chemicals, (2) the identification of CYP isoforms related to the generation of the genotoxic metabolites from a novel chemical, and (3) the importance of the interaction between the phase I and II drug-metabolizing enzymes to reveal a more comprehensive picture of the *in vivo* metabolism of chemicals.

(1) The Validation with Well-Known Chemicals Requiring CYP Activation

We have previously confirmed that the HepG2 transformant expressing a series of human CYP isoforms has an adequate sensitivity to detect the genotoxicity of model chemicals requiring CYP activation such as benzo(*a*)pyrene and cyclophosphamide (Hashizume *et al*. 2009). In addition to these chemicals, in the present study, we validated that HepG2 transformants could detect other chemicals, which have been extensively studied for CYP activation. We selected DMBA and ifosfamide as model chemicals and examined MN induction as a genotoxic endpoint in the treatment with these chemicals.

As shown in Figure 1A, MN induction in a HepG2 transformant expressing CYP1A1, which is known to be the most active among the CYP isoforms to metabolize DMBA, was significantly higher than those in HepG2 and Hepc-Mock cells. Increases in the percent MN above the value in Hepc-Mock were also seen unexpectedly with other transformants, although some increases were significant (e.g., CYP2C9, 2D6, and 3A4) while others were not (CYP2A6, 2B6, 2C8, 2C19, and 2E1). Similar results were obtained with treatment with ifosfamide (Fig. 1B). A significant increase in the percent MN was observed in the transformants expressing the most related CYP isoforms, CYP2C9 and 3A4. The transformant expressing CYP2B6 also showed an increase compared with the HepG2 and Hepc-Mock cells without statistical significance, whereas other transformants expressing CYP1A1, 2C9, 2D6, or 3A4 also showed a statistically significant increase of percent MN by ifosfamide (Fig. 1B).

Shou *et al*. (1996) reported that CYP1A1 was most active to metabolize DMBA into its ultimate electrophilic metabolite, 3,4-diol-1,2-epoxide. On the other hand, they reported that CYP2B6, 2C9, and 1A2 were also capable of metabolizing DMBA, whereas CYP2C8, 2E1, and 3A4 exhibited relatively low activity (Shou *et al*., 1996). Likewise, although the most active CYP isoforms to form final alkylating metabolites are CYP2B6 and 3A4 (Jing *et al*., 2006), other CYP isoforms such as CYP1A1, 2A6, 2B6, and 2C19 were reported to be able to produce a proximate metabolite, 4-hydroxyifosfamide, in experiments using microsomes prepared from baculovirus-infected insect cells expressing single human CYP and human NADPH CYP reductase (White, 2003). Therefore, the present validation study showed that genotoxic metabolites could be produced by not only the most active CYP isoform but also by other less active CYPs and that this transformant system could detect the genotoxic potential of chemicals requiring CYP activation not tested routinely in the early stage of drug development.

(2) Identification of CYP Isoforms Related to Genotoxicity

β-Endosulfan is reported to induce MN in HepG2 cells, suggesting that CYP activation might be involved in the MN induction (Lu *et al*., 2000); however, the contributing CYP isoform to induce MN has not yet been investigated to the best of our knowledge. Therefore, we examined whether a series of HepG2 transformants could identify the CYP isoform contributing to the MN induction by β-endosulfan as a model chemical. β-Endosulfan significantly increased the fold induction of MN in the transformant expressing CYP3A4 compared with that obtained with the transformant expressing

an empty vector (Fig. 2A). Furthermore, inhibitory effects of a specific inhibitor of CYP3A4 and of siRNA to CYP3A4 on MN induction by β-endosulfan were shown (Figs. 2B and 2C, respectively). The activity of CYP3A4 in the transformant using the luminogenic substrate demonstrated that these inhibitory conditions decreased the activity to approximately 10% compared with the control level (Table 2). These results indicated that MN induction by β-endosulfan could be associated with the presence of CYP3A4 activity and suggested that CYP3A4 is involved in producing the genotoxic metabolites of the chemical.

Lee *et al*. (2006) reported that β-endosulfan is metabolized by CYP3A4 based on the results in the study with CYP isoform–selective inhibitor in human liver microsomes and with the incubation study of cDNA-expressed enzymes. They have also reported that human liver microsome incubation of endosulfan in the presence of NADPH resulted in the formation of endosulfan sulfate (Lee *et al*., 2006). Based on their and our results that endosulfan sulfate was the genotoxic metabolite to induce MN (Fig. 2D), this metabolite was considered to be produced in the HepG2 transformant expressing human CYP3A4 by the treatment with β-endosulfan.

These results clearly demonstrated that the HepG2 transformant system was able to identify the CYP isoform related to the genotoxicity of chemical metabolites and was useful to elucidate the genotoxicity of a new chemical or a drug candidate in the presence of the metabolic activation system.

(3) Importance of Interaction between the Phase I and II Enzymes in the Genotoxicity of Chemicals

The biotransformation of chemicals is catalyzed mainly by four enzyme systems: hydrolysis, reduction, oxidation, and conjugation (Andrew and Brian, 2007). A liver S9 fraction from rats treated with Aroclor 1254 or phenobarbital/5,6-benzoflavone is widely used in *in vitro* genotoxicity studies. However, the rat liver S9 fraction system lacks the conjugating reactions due to an absence of the appropriate cofactors for phase II enzymes, which are known to be important not only for reducing potential genotoxicity but also for increasing genotoxicity (Ku *et al*., 2007).

In our experiment, tamoxifen did not induce MN in any transformants including CYP3A4 (Fig. 3A). Furthermore, cotreatment of tamoxifen with a specific UGT inhibitor, diclofenac, slightly but significantly increased MN in the transformants expressing CYP3A4 (Fig. 4A), suggesting that a metabolic activation resulting in the genotoxicity could occur in the transformant but detoxification by glucuronidation might be predominant in the overall biotransformation. Similarly, we observed that cotreatment of safrole with a GST inhibitor, ethacrynic acid, produced MN in the transformants expressing CYP2D6 (Fig. 4B), whereas safrole alone did not (Fig. 3B), indicating that safrole was detoxified by GST. The results for tamoxifen and safrole clearly demonstrated that interaction between the phase I and II drug-metabolizing enzymes was crucial to assess the genotoxicity of chemicals in the presence of a metabolic activation system. The interplay between the phase I and II enzymes is lacking in the NADPH-supplemented rat liver S9 system due to an absence of cofactor necessary for several phase II enzymes such as UGT or GST. Furthermore, the reactive intermediates have to be formed in the target cell because some conjugates have poor membrane permeability. These results raise the possibility that the induced rat liver S9 system may generate mutagenic metabolites of no relevance or worse even may not generate a mutagenic metabolite that would be generated by human enzymes. Therefore, a set of HepG2 transformants is a superior test system for mimicking the metabolism occurring in the human liver, and the use of this system can potentially provide more relevant data than current genotoxicity tests.

In summary, we have demonstrated the following advantages of a HepG2 transformant system expressing a series of CYP isoforms in the assessment of the genotoxicity of chemicals: First, the sensitivity of these transformants was confirmed by chemicals extensively studied for CYP activation. Second, these transformants indicated which CYP isoforms contribute to the genotoxicity of a novel chemical whose genotoxicity with metabolic activation is unknown. Third, the relevance of the interaction between the phase I and phase II drug-metabolizing enzymes was demonstrated by the genotoxicity of chemicals requiring CYP activation in the presence of inhibitors of the phase II enzymes. Even though we tested only a limited number of chemicals, these advantages provide the benefits of a newly established transformant system in *in vitro* genotoxicity testing that reflects comprehensive metabolic pathways including not only human CYP isoforms but also the phase II drug-metabolizing enzymes.

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