



# Establishment of the transformants expressing human cytochrome P450 subtypes in HepG2, and their applications on drug metabolism and toxicology

S. Yoshitomi <sup>a,\*</sup>, K. Ikemoto <sup>a</sup>, J. Takahashi <sup>a</sup>, H. Miki <sup>b</sup>, M. Namba <sup>c</sup>, S. Asahi <sup>a</sup>

<sup>a</sup>*Drug Analysis and Pharmacokinetics Research Laboratories, Pharmaceutical Research Division, Takeda Chemical Industries Ltd, 2-17-85 Juso-Honmachi, Yodogawa-ku, Osaka 532-8686, Japan*

<sup>b</sup>*Discovery Research Laboratories IV, Discovery Research Division, Takeda Chemical Industries Ltd, 2-17-85 Juso-Honmachi, Yodogawa-ku, Osaka 532-8686, Japan*

<sup>c</sup>*Department of Cell Biology, Institute of Molecular and Cellular Biology, Okayama University Medical School, Shikata-Cho, Okayama 700-0914, Japan*

Accepted 24 January 2001

## Abstract

Transformants with stable expression of a series of human cytochrome P450 (CYP) subtypes in the human hepatic cell line, HepG2, were established. These transformants are designated 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 stably expressed human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, respectively. The expression of the CYP subtypes in the transformants was confirmed by both determination of enzyme activities and the reverse transcriptase polymerase chain reaction (RT-PCR) procedure. The apparent  $K_m$  values of the expressed CYP subtypes for their specific substrates were close to those of human liver microsomes. In addition to their CYP activities, these transformants retained glucuronide- and sulfate-conjugating activities. Furthermore, the activities of CYP2C9, CYP2D6 and CYP3A4 were inhibited by their specific inhibitors. The cytotoxicity of acetaminophen (APAP), cyclophosphamide (CPA) and benz[a]anthracene (BA) were analyzed by CYP-expressing transformants. The cytotoxicity depended on the expression of CYP subtypes and increased in a dose-dependent manner. These results show the metabolic activation of APAP, CPA and BA by the specific CYP subtypes expressed in the transformants and demonstrate the usefulness of these transformants for in vitro metabolic and toxicological studies in human liver. © 2001 Elsevier Science Ltd. All rights reserved.

**Keywords:** HepG2; Cytochrome P450; Stable expression; Drug metabolism; Toxicology

## 1. Introduction

The cytochrome P450 (CYP) gene superfamily encodes a number of CYP isoforms. They are widely distributed from bacteria to mammals and catalyze the

oxidative and reductive metabolism of a wide variety of chemicals, both endogenous as well as exogenous. Among the CYP subtypes, members CYP1 through CYP3, present in mammalian hepatic microsomes, are characteristic in their ability to metabolize a large number of chemicals. Similarly, CYPs catalyze to form toxic reactive intermediates from many chemicals (Thakker et al., 1985; Guengerich, 1991; Porter et al., 1991). As it is well known that there are significant quantitative and qualitative differences between laboratory animals and humans in their CYP subtypes, it is necessary to use human CYP isoforms to predict the metabolism and toxicity of chemicals in humans. In human liver, about 70% of the total CYP could be accounted for by CYP1A2, CYP2A6, CYP2B6, CYP2C, CYP2D6,

*Abbreviations:* APAP, acetaminophen; BA, benz[a]anthracene; BSO, L-buthionine sulfoximine; CPA, cyclophosphamide; CYP or P450, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; GSH, reduced glutathione; 7-HC, 7-hydroxycoumarin; LDH, lactate dehydrogenase; NAPQI, N-acetyl-p-benzoquinone imine; PAHs, polycyclic aromatic hydrocarbons; PNP, p-nitrophenol; RT-PCR, reverse transcriptase polymerase chain reaction; TBAP, tetra-n-butyl-ammonium phosphate.

\* Corresponding author. Tel.: +81-6-6300-6105; fax: +81-6-6300-6206.

E-mail address: Yoshitomi\_Sumie@takeda.co.jp (S. Yoshitomi).

CYP2E1 and CYP3A proteins (Rendic and Di Carlo, 1997). Most human drug metabolism is catalyzed by CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. CYP1A1 is present in the extrahepatic organs such as lungs and kidneys.

Genetically engineered mammalian cells expressing CYP subtypes have provided new tools for investigation of the metabolism and CYP-mediated metabolic activation of chemicals. The stable expression system of CYP in cells has made it possible to evaluate the relative risk of a chemical in *in vitro* toxicological testing (Sawada et al., 1998). The choice of recipient cells is an important factor in determining the usefulness of the genetically engineered cells (Sawada et al., 1998; Friedberg et al., 1999). Among the human hepatic cell lines, HepG2 was derived from a human liver tumor and has been characterized to retain many xenobiotic-metabolizing activities as compared to fibroblasts (Knowles et al., 1980; Rueff et al., 1996). Therefore, HepG2 is useful in the prediction of the metabolism and cytotoxicity of chemicals in human liver. Cederbaum and colleagues reported the establishment of stable expression of human CYP2E1 in HepG2 and they examined the cytotoxicity of ethanol, acetaminophen and ferric-nitrilotriacetate (Dai et al., 1993; Dai and Cederbaum, 1995; Wu et al., 1996; Chen et al., 1998; Sakurai et al., 1998). To assess the metabolic and toxicological characteristics of the xenobiotics in human liver, we established a series of transformants stably expressing 10 CYP subtypes, which make a large contribution to the metabolism of xenobiotics in humans. In this study, we examined the possibility of these transformants to be useful tools for the prediction of drug metabolism in, and toxicity to, the human liver.

## 2. Materials and methods

### 2.1. Materials

Resorufin, 7-ethoxyresorufin, 7-hydroxyresorufin and 7-ethoxycoumarin were obtained from Molecular Probes (Eugene, OR, USA); coumarin, testosterone and acetaminophen (APAP) were from Wako Pure Chemicals (Osaka, Japan); L-buthionine sulfoximine (BSO), benz[*a*]anthracene (BA), sulfaphenazole, quinidine, cyclophosphamide (CPA) and MTT were from Sigma (St Louis, MO, USA); 7-hydroxycoumarin was from Extrasynthèse (France); 6 $\beta$ -hydroxytestosterone, (S)-mephenytoin, 4'-hydroxymephenytoin, bufuralol and 1'-hydroxybufuralol were from Sumika Chemical Analysis Service (Osaka, Japan); taxol was from Ultrafine Chemicals (Manchester, UK); tolbutamide was from Research Biochemicals International (Natick, MA, USA); 4-hydroxytolbutamide, 4-nitrophenol and 6-hydroxypacritaxel were from Gentest Corp. (Woburn, MA, USA); ketoconazole was from Biomol Research Labs (Plymouth

Meeting, PA, USA). All other chemicals and solvents were of the highest grade commercially available.

### 2.2. Cells

HepG2 was obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Bio Whittaker, Walkersville, MD, USA), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Gibco BRL) in a humidified atmosphere in 5% CO<sub>2</sub> at 37°C. Transformants were cultured in DMEM supplemented with 10% FCS, 200  $\mu$ g/ml G418 (Gibco BRL), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin.

### 2.3. Establishment of HepG2 transformants stably expressing CYP subtypes

A series of human CYP cDNAs were cloned using RT-PCR from a human liver cDNA library. The amplified products were cloned into the appropriate vector and further ligated into pcDNA3.1(+ (Invitrogen, Carlsbad, CA, USA). HepG2 cells were seeded onto a 60-mm culture dish. When the culture reached a confluence of 50–60%, 2  $\mu$ g of plasmid DNA was transfected by LIPOFECTAMINE PLUS Reagent (Gibco BRL). After incubation for 2 days, cells were selected for G418 resistance in medium containing 500  $\mu$ g/ml G418. The G418-resistant colonies were pooled and further selected with medium containing 200  $\mu$ g/ml G418. Surviving single colonies were picked up and the catalytic activity of the introduced CYP subtype was determined. The homogeneity of the transformants was assured by repeated subcloning.

HepG2 and its transformants were cultured to confluence. Total RNAs were extracted from  $5 \times 10^7$  cells with RNeasy Mini Kit (QIAGEN, Hilden, Germany). The concentration and purity of RNA were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer. Complementary DNA was synthesized with the ThermoScript™ RT-PCR System (Gibco BRL) from 2  $\mu$ g total RNA.  $\beta$ -Actin, CYP1A1/2, CYP2A6, CYP2B6/7, CYP2C8/9/19, CYP2D6, CYP2E1 and CYP3A4/5/7 cDNA fragments were amplified by KOD DNA Polymerase (TOYOBO, Japan) using primers as summarized in Table 1. RT-PCR reaction products were electrophoresed on a 3% agarose gel and stained with SYBR Green I (Molecular Probes). Stained bands were analyzed by a Fluor-S MultiImager (Bio-Rad, Hercules, CA, USA).

### 2.4. Determination of catalytic activities of CYP isoforms and conjugating enzymes

Cells were seeded onto 12-well tissue culture plates and grown to confluence. After the cells were washed with

phosphate buffered saline, 500  $\mu$ l of the appropriate substrate solution diluted by DMEM supplemented with 2% FCS was added to the cells and incubated. After an aliquot of the medium was removed for analysis of enzyme activity, the cells were harvested and lysed by sonication. Protein concentrations were determined with a BCA Protein Assay Kit (PIERCE, Rockford, IL, USA) using bovine serum albumin as a standard.

CYP1A1/2 activity was measured by 7-ethoxyresorufin *O*-deethylase activity. After incubation at 37°C for 30 min in the dark, the concentration of resorufin in the reaction mixture was determined by the measurement of fluorescence at 550 nm as excitation and at 586 nm as emission.

CYP2A6 activity was measured by 7-hydroxylation of coumarin. After incubation at 37°C for 60 min, the concentration of 7-hydroxycoumarin in the reaction mixture was determined by measurement of fluorescence at 390 nm as excitation and at 440 nm as emission.

CYP2B6 activity was measured by 7-ethoxycoumarin *O*-deethylase activity. After incubation at 37°C for 60 min, the concentration of 7-hydroxycoumarin in the reaction mixture was determined by the measurement of fluorescence at 390 nm as excitation and at 440 nm as emission.

CYP2C8 activity was measured by 6-hydroxylation activity of taxol. After incubation at 37°C for 3 h, formation of 6-hydroxypacritaxel was analysed by HPLC. The column used was a Capcell Pak C18 AG120 (5  $\mu$ m, 4.6 $\times$ 250 mm; Shiseido, Tokyo, Japan). The column temperature and flow rate were 40°C and 1.0 ml/min, respectively. The mobile phase was 40% acetonitrile. Detection was carried out at UV 230 nm.

CYP2C9 activity was measured by tolbutamide 4-hydroxylation. After incubation at 37°C for 3 h, formation of 4-hydroxytolbutamide was analyzed by HPLC.

The column was an Inertsil ODS (5  $\mu$ m, 4.6 $\times$ 250 mm; GL Science, Tokyo, Japan). The mobile phase was a mixture of 10 mM acetate buffer (pH 4.3) and acetonitrile (72:28, v/v). The column temperature and flow rate were 40°C and 1.0 ml/min, respectively. Detection was carried out at UV 230 nm.

CYP2C19 activity was measured by (S)-mephenytoin 4'-hydroxylation. After incubation at 37°C for 2 h, 4'-hydroxymephenytoin was analysed by HPLC. The column was a Capcell Pak C18 column AG120 (5  $\mu$ m, 4.6 $\times$ 250 mm). The mobile phase was a mixture of 0.05 M  $\text{KH}_2\text{PO}_4$  (pH 4.0) and acetonitrile (74:26, v/v). The column temperature and flow rate were 40°C and 0.8 ml/min, respectively. Detection was carried out at UV 204 nm.

CYP2D6 activity was measured by bufuralol 1'-hydroxylation. After incubation at 37°C for 2 h, formation of 1'-hydroxybufuralol was analyzed by HPLC. The column was an Inertsil ODS (5  $\mu$ m, 4.6 $\times$ 250 mm). The mobile phase contained 1 mM perchloric acid and 30% acetonitrile. The column temperature and flow rate were 40°C and 1.0 ml/min, respectively. Detection was carried out at 252 nm as excitation and 302 nm as emission.

CYP2E1 activity was measured by 4-nitrophenol hydroxylation. After incubation at 37°C overnight, 2 N NaOH was added and the absorbance of 4-nitrocatechol in the reaction mixture was measured at 492 nm.

CYP3A4 activity was measured by testosterone 6 $\beta$ -hydroxylation. After incubation at 37°C for 60 min, formation of 6 $\beta$ -hydroxytestosterone was analysed by HPLC. The column was a Capcell Pak C18 AG120 (5  $\mu$ m, 4.6 $\times$ 250 mm). The mobile phase (A) was a mixture of methanol, water and acetonitrile (80:113:7, by vol.) and the mobile phase (B) was a mixture of the same components (4:4:2, by vol.). The column temperature and flow rate were 40°C and 1.0 ml/min, respectively.

Table 1  
The sequences of primers for RT-PCR

Name		Sequence
$\beta$ -Actin	Sense	5'-CAA-gAg-ATg-gCC-ACg-gCT-gCT-3'
	Antisense	5'-TCC-TTC-TgC-ATC-CTg-TCg-gCA-3'
CYP1A1/2	Sense	5'-gAg-CAT-gTg-AgC-AAg-gAg-3'
	Antisense	5'-AAg-gAA-gAg-TgT-Cgg-AAg-3'
CYP2A6	Sense	5'-CCC-AAC-ACg-gAg-TTC-TAC-TTg-AAA-3'
	Antisense	5'-gAA-gAA-ATC-CCg-AAA-CTT-ggT-gTC-3'
CYP2B6/7	Sense	5'-CCA-TAC-ACA-gAg-gCA-gTC-AT-3'
	Antisense	5'-ggT-gTC-AgA-TCg-ATg-TCT-TC-3'
CYP2C8/9/19	Sense	5'-CTT-gTg-gAg-gAg-TTg-AgA-3'
	Antisense	5'-TCC-TgC-TgA-gAA-Agg-CAT-3'
CYP2D6	Sense	5'-TgA-TgA-gAA-CCT-gCg-CAT-Ag-3'
	Antisense	5'-ACC-gAT-gAC-Agg-TTg-gTg-AT-3'
CYP2E1	Sense	5'-AgC-ACA-ACT-CTg-AgA-TAT-gg-3'
	Antisense	5'-ATA-gTC-ACT-gTA-CTT-gAA-CT-3'
CYP3A4/5/7	Sense	5'-ggA-TgA-AgA-ATg-gAA-gAg-3'
	Antisense	5'-Tgg-ACA-TCA-ggg-TgA-gTg-3'

The time program for the gradient elution was as follows: the concentration of mobile phase (B) was linearly increased from 0 to 100% over a period of 20 min, and then held at 100% for 10 min and cycled back to the initial condition. Detection was carried out at UV 254 nm.

Sulfate- and glucuronide-conjugating activities were measured using 7-hydroxycoumarin (7-HC) as a substrate. The substrate solution was added to the cells, incubated for the designated times, and sulfate- and glucuronide-conjugates were analysed by HPLC. The column was a Supelcosil LC-8 (5  $\mu$ m, 4.6 $\times$ 50 mm, Supelco, Bellefonte, PA, USA). The column temperature and flow rate were 40°C and 1.5 ml/min, respectively. Mobile phase (A) was a mixture of 0.25% acetic acid (pH 5.3), 0.5 M *tetra-n*-butyl-ammonium phosphate (TBAP) and acetonitrile (90:1:9, by vol.) and mobile phase (B) was the mixture of same components (50:1:49, by vol.). The time program for gradient elution was as follows: mobile phase (A) was held at 100% for 5 min and mobile phase (B) was linearly increased from 0 to 73% over a period of 7 min and then cycled back to the initial condition. Detection was carried out at UV 325 nm.

The apparent  $K_m$  and  $V_{max}$  values were calculated from Lineweaver–Burk plots.

### 2.5. Growth rate of cells

Cells ( $1 \times 10^5$  cells) were seeded on 12-well plates and cultured for 10 days. The number of cells was counted every 2 or 3 days by Trypan blue staining. Doubling time was calculated using the following formula:  $DT$  (hour) =  $A \times 24 / \log_2(\text{cell number})_{\text{dayA}} / (\text{cell number})_{\text{day0}}$ .  $DT$  refers to doubling time, and  $(\text{cell number})_{\text{dayA}}$  and  $(\text{cell number})_{\text{day0}}$  refer to cell number on day A and day 0, respectively.

### 2.6. Inhibition of enzyme activity

Hepc/2C9.1, Hepc/2D6.39 or Hepc/3A4.2-30 ( $7 \times 10^4$  cells/100  $\mu$ l/well) were seeded onto a 96-well plate and cultured for 3 days. The medium was changed for a fresh one containing 400  $\mu$ M tolbutamide and various concentrations of sulfaphenazole for Hepc/2C9.1; 200  $\mu$ M bufuralol and various concentrations of quinidine for Hepc/2D6.39; 100  $\mu$ M testosterone and various concentrations of ketoconazole for Hepc/3A4.2-30. After incubation at 37°C for the designated time, the activity of CYP2C9, CYP2D6 or CYP3A4 was analyzed as described above.

### 2.7. LDH release and MTT assay

Cells were seeded onto a 96-well plate in the presence of various concentrations of chemicals diluted with

DMEM containing 2% FCS. After cultivation for the designated time, LDH release and MTT assays were performed as follows.

#### 2.7.1. LDH assay

After incubation, LDH release in the culture supernatants was measured by a Cytotox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, MI, USA) (referred to as  $LDH_{out}$ ). On the other hand, 10  $\mu$ l of 10% Triton X100 (Wako Pure Chemicals) was added to the cells and incubated at 37°C for 45 min, and then LDH activity was measured (referred to as  $LDH_{total}$ ). LDH leakage was calculated as the ratio of  $LDH_{out} / LDH_{total}$ .

#### 2.7.2. MTT assay

MTT (1 mg/ml in PBS) was added to each well and incubated at 37°C for 4 h and then 100  $\mu$ l of 10% sodium dodecylsulfate and 0.01 N HCl was added. After overnight incubation at 37°C, absorbance of the reaction mixture at 590 nm and 620 nm were measured. The difference between  $A_{590}$  and  $A_{620}$  was taken as the index of cell viability.

### 2.8. Statistical analysis

Data were analyzed using a statistical analysis system to determine the significance of observed differences.

## 3. Results

### 3.1. Establishment of transformants expressing CYP subtypes

The CYP cDNAs encoding 10 subtypes of CYPs were cloned by PCR from a human liver cDNA library. The nucleotide sequences of the cDNAs were equal to those reported. The nucleotides around the initial ATG codon and stop codon were modified to make appropriate restriction sites by using PCR. Each cDNA coded CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 was inserted into pcDNA3.1(+) and designated as 1A1/pcDNA3.1(+), 1A2/pcDNA3.1(+), 2A6L/pcDNA3.1(+), 2B6/pcDNA3.1(+), 2C8/pcDNA3.1(+), 2C9/pcDNA3.1(+), 2C19/pcDNA3.1(+), 2D6/pcDNA3.1(+), 2E1/pcDNA3.1(+), and 3A4/pcDNA3.1(+), respectively. The expression of the cDNA in the construct was driven by human cytomegalovirus (CMV) promoter. These plasmids were introduced into HepG2 and cells were selected for G418 resistant phenotype. The homogeneity of the cell lines was assured by repeated subcloning. Each G418-resistant transformant was assayed for CYP expression by determination of specific

enzyme activity. 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, CYP 2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, respectively. As a mock control, pcDNA3.1(+) was introduced into HepG2 and named Hepc. Expression of mRNA in each transformant was confirmed by RT-PCR analysis (Plate 1). In our experimental conditions, no definite RT-PCR product corresponding to each CYP mRNA was found in HepG2 and Hepc cells. On the other hand, a significant increased amount of

each CYP mRNA was detected in the corresponding transformant. Next, to evaluate the CYP activities of the transformants, kinetic analysis was carried for these transformants and the apparent  $K_m$  and  $V_{max}$  values were calculated from Lineweaver–Burk plots (Table 2, Fig. 1). The apparent  $K_m$  values of these transformants were nearly the same as those from human liver microsomes. These CYP activities have been stably expressed more than 2 years (data not shown). The proliferation rates of the transformants, except for Hepc/3A4.2-30, were around 35 h. These are close to those of HepG2 or Hepc (Table 2), and Hepc/3A4.2-30 grew more slowly than the other transformants.

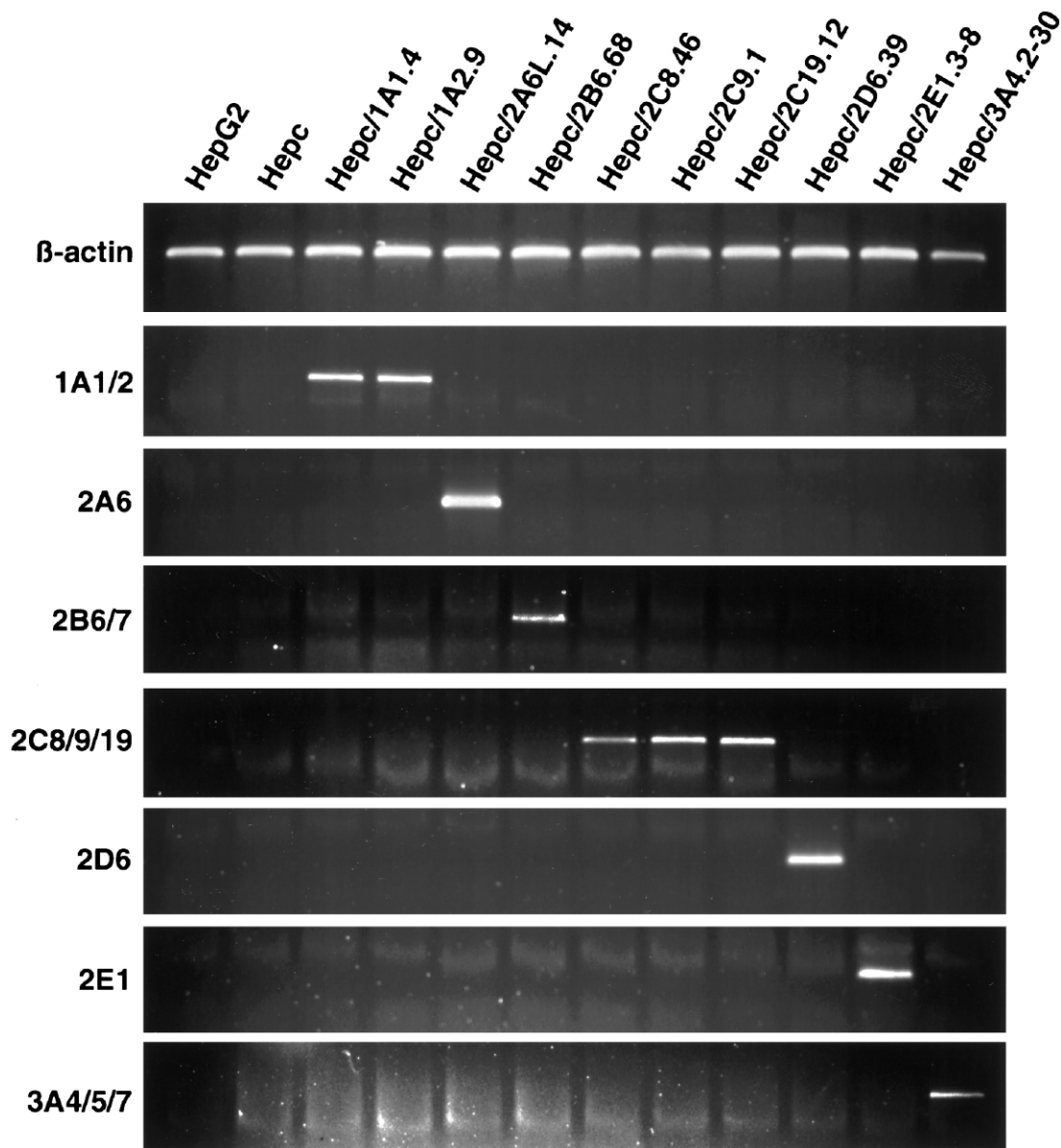


Plate 1. Expression of mRNA of each introduced CYP cDNA in the transformants. Total RNAs were extracted and cDNA was synthesized with the ThermoScript RT-PCR system. Fragments of CYP subtypes and  $\beta$ -actin were amplified by KOD DNA polymerase. Primers were summarized in Table 1. RT-PCR reaction products were electrophoresed on 3% agarose gel and stained with SYBR Green I.

### 3.2. Formation of sulfate and glucuronide conjugates from 7-hydroxycoumarin

Cells were cultivated with 50  $\mu\text{M}$  of 7-HC, and the sulfate and glucuronide conjugates of 7-HC were analysed. Both the conjugates formed in the culture supernatant of HepG2 in a time-dependent manner (Fig. 2). All transformants derived from HepG2 also showed similar conjugating activities (Table 3).

### 3.3. Cell-based CYP inhibition assay

The inhibitory effects of specific CYP inhibitors on the transformants were examined (Fig. 3). 4-Hydroxylation of tolbutamide by Hepc/2C9.1, 1'-hydroxylation of bufuralol by Hepc/2D6.39 and 6 $\beta$ -hydroxylation of testosterone by Hepc/3A4.2-30 were inhibited by sulfaphenazole ( $\text{IC}_{50}$  = 0.33  $\mu\text{M}$ ), quinidine ( $\text{IC}_{50}$  = 0.10  $\mu\text{M}$ ) and ketoconazole ( $\text{IC}_{50}$  = 0.18  $\mu\text{M}$ ), respectively.

### 3.4. Cytotoxicity of acetaminophen (APAP) to Hepc/1A2.9, Hepc/2E1.3-8 and Hepc/3A4.2-30

HepG2, Hepc, Hepc/1A2.9, Hepc/2E1.3-8 and Hepc/3A4.2-30 were treated with various concentrations of APAP. Cytotoxicity was determined by LDH assay and MTT assays (Fig. 4A–E). In HepG2, Hepc, Hepc/1A2.9, Hepc/2E1.3-8 and Hepc/3A4.2-30, LDH leakage was increased and the MTT level (cell viability) was decreased after treatment with APAP (2–10 mM) in a dose-dependent manner (Fig. 4A and D). Compared with Hepc, strains Hepc/1A2.9, Hepc/2E1.3-8 and

Hepc/3A4.2-30 were much more sensitive to APAP. In comparison with strain Hepc, strains Hepc/1A2.9, Hepc/2E1.3-8 and Hepc/3A4.2-30 caused significant LDH leakage (Fig. 4A) ( $P \leq 0.01$ ). Similarly, the decrement of MTT level was seen significantly in strains Hepc/1A2.9 ( $P \leq 0.01$ ), Hepc/2E1.3-8 ( $P \leq 0.01$ ) and Hepc/3A4.2-30 ( $P \leq 0.05$ ) (Fig. 4D). The effect of simultaneous treatment of BSO, which is a potent inhibitor of glutathione (GSH) biosynthesis, was also examined (Fig. 4B and E). The treatment with BSO (100  $\mu\text{M}$ ) alone hardly changed LDH leakage and MTT levels of the strains examined. In addition to this, the simultaneous addition of BSO to APAP hardly altered LDH leakage and MTT levels of Hepc and HepG2 (cf. Fig. 4A with B, and D with E). Compared with Hepc, strains Hepc/1A2.9, Hepc/2E1.3-8 and Hepc/3A4.2-30 were significantly much more sensitive to the simultaneous addition of BSO to APAP (Fig. 4B and D) ( $P \leq 0.01$ ). Furthermore, the simultaneous addition of BSO resulted in no cytotoxic effects on strains Hepc and HepG2; however, addition of BSO amplified the cytotoxicity of APAP to strains expressing CYP isoforms. Results from LDH or MTT assays in each strain exposed to 10 mM APAP or 10 mM APAP + 100  $\mu\text{M}$  BSO were expressed as the values relative to the results of the assays from Hepc exposed to 10 mM APAP. Strains Hepc/1A2.9 ( $P < 0.01$ ), Hepc/2E1.3-8 ( $P \leq 0.01$ ), and Hepc/3A4.2-30 ( $P \leq 0.05$ ) caused a significant increase in LDH leakage by the simultaneous addition of BSO (Fig. 4C). Similarly, the decrement of MTT level by the simultaneous addition of BSO was seen significantly in strains Hepc/1A2.9 and Hepc/2E1.3-8 ( $P \leq 0.01$ ) (Fig. 4F).

Table 2  
Characteristics of transformants

Name of transformant	Expressed CYP subtype	Catalytic reaction measured	Kinetic analysis <sup>a</sup>			
			Transformant $K_m$ ( $\mu\text{M}$ )	Human liver microsomes <sup>b</sup> $K_m$ ( $\mu\text{M}$ )	Transformant $V_{\text{max}}$ (pmol/min/mg)	Doubling time <sup>c</sup> (fold)
Hepc/1A1.4	CYP1A1	7-Ethoxyresorufin <i>O</i> -deethylation	0.25	0.19	56	1.03
Hepc/1A2.9	CYP1A2	7-Ethoxyresorufin <i>O</i> -deethylation	0.72	0.39	2	1.06
Hepc/2A6L.14	CYP2A6	Coumarin 7-hydroxylation	5.1	2.3	812 000	0.91
Hepc/2B6.68	CYP2B6	7-Ethoxycoumarin <i>O</i> -deethylation	81	–	80 000	0.94
Hepc/2C8.46	CYP2C8	Taxol 6-hydroxylation	7.4	24	9400	1.01
Hepc/2C9.1	CYP2C9	Tolbutamide 4-hydroxylation	45	120	25 000	1.02
Hepc/2C19.12	CYP2C19	(S)-Mephenytoin 4'-hydroxylation	8.3	16	140 000	0.91
Hepc/2D6.39	CYP2D6	Bufuralol 1'-hydroxylation	17	40	14	0.91
Hepc/2E1.3-8	CYP2E1	p-Nitrophenol hydroxylation	88	30	120	1.08
Hepc/3A4.2-30	CYP3A4	Testosterone 6 $\beta$ -hydroxylation	96	89	71	1.28

<sup>a</sup> Cells were seeded on 12-well tissue culture plates and grown to confluence. After cells were washed, 500  $\mu\text{l}$  of the appropriate substrate solution were added to the cells, which were incubated as described in the methods. The values are the means of duplicate determinations. The apparent  $K_m$  and  $V_{\text{max}}$  values were calculated from Lineweaver–Burk plots.

<sup>b</sup> Iwata et al., 1998.

<sup>c</sup> Cells were seeded on 12-well tissue culture plates and cultured for 10 days. The number of cells was counted every 2 or 3 days after Trypan blue staining. The values are the means of duplicate determinations. The values compared with HepG2 (doubling time = 35 h) were shown.

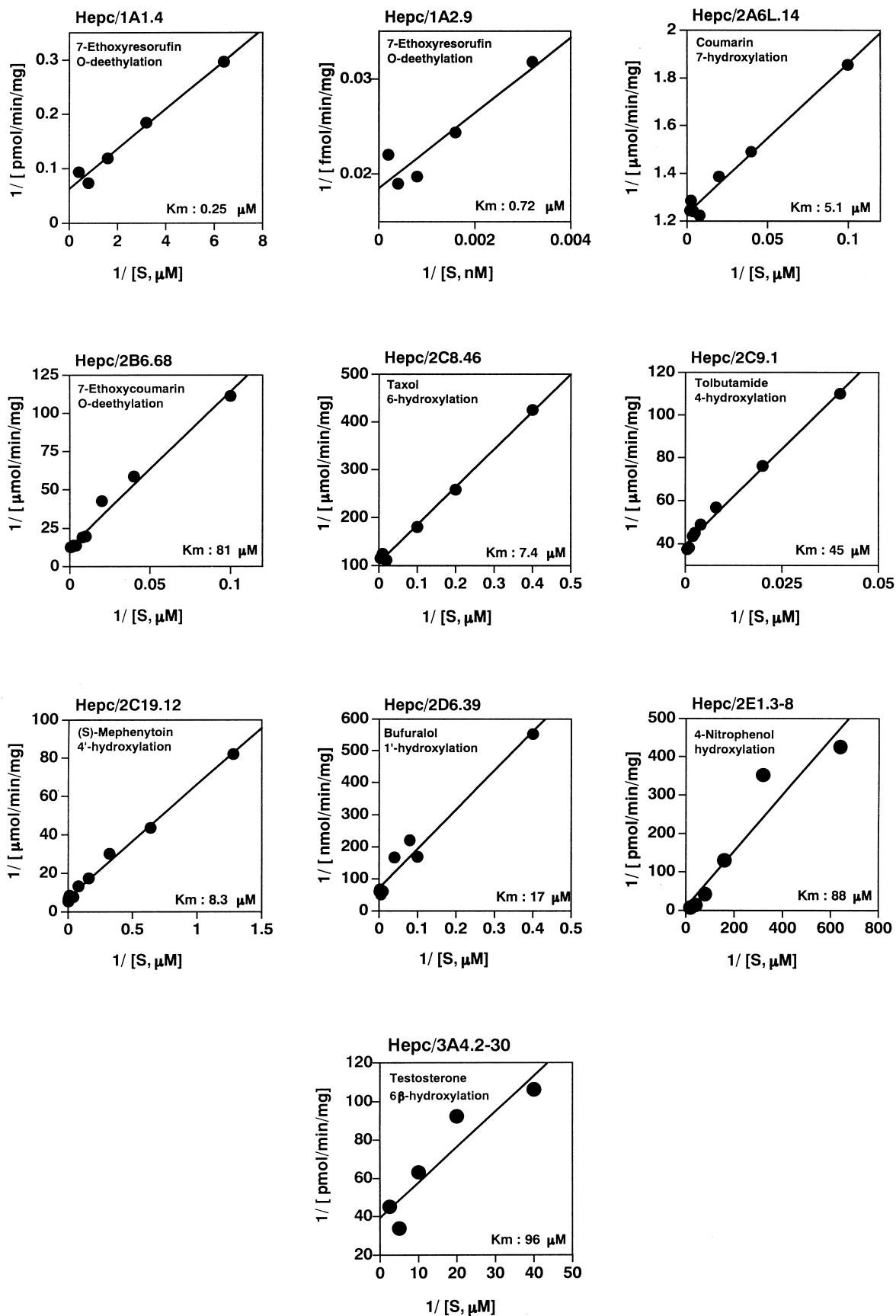


Fig. 1. Lineweaver–Burk plots for the metabolism of each specific substrate by a series of CYP-expressing transformants. The values are the means of duplicate determinations. The  $K_m$  values are expressed as  $\mu\text{M}$ .

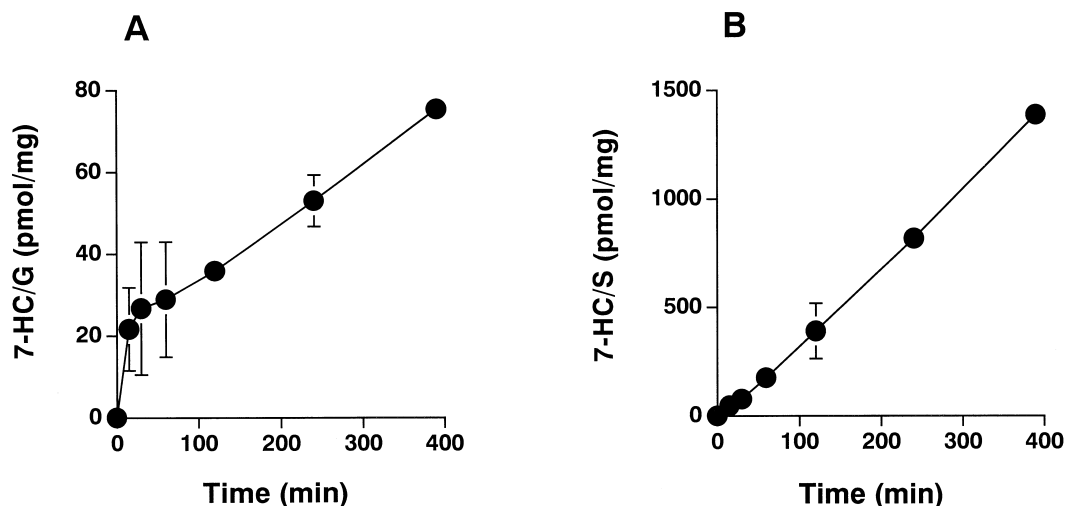


Fig. 2. Time course of formation of glucuronide and sulfate conjugates of 7-HC by HepG2. HepG2 ( $1 \times 10^5$  cells/well) were seeded on a 96-well tissue culture plate and cultured for 3 days. Medium was changed to a fresh one containing  $50 \mu\text{M}$  7-HC and incubated at  $37^\circ\text{C}$  for designated times. Each point and bar represents the mean  $\pm$  S.D. of four experiments. (A) Glucuronide conjugate (7-HC/G); (B) sulfate conjugate (7-HC/S).

### 3.5. Cytotoxicity of cyclophosphamide (CPA) to Hepc/2B6.68, Hepc/2A6L.14, Hepc/2C8.46, and Hepc/2C9.1

The role of CYP2B6, CYP2A6, CYP2C8 and CYP2C9 in the activation of CPA was examined. Cytotoxicity was determined by LDH and MTT assays (Fig. 5). In HepG2, Hepc, Hepc/2B6.68, Hepc/2A6L.14, Hepc/2C8.46 or Hepc/2C9.1, LDH leakage was increased (Fig. 5A) after treatment with CPA (8–16 mM) and the MTT level was decreased (Fig. 5B) with CPA (4–16 mM) in a dose-dependent manner. As compared with Hepc, strains Hepc/2B6.68, Hepc/2A6L.14, Hepc/2C8.46 or Hepc/2C9.1 caused a significant increase in LDH leakage after treatment with 16 mM CPA ( $P \leq 0.01$ ) and a decrease in the MTT level after treatment with 8 mM CPA ( $P \leq 0.01$ ).

### 3.6. Cytotoxicity of benz[a]anthracene (BA) to Hepc/1A1.4

We further studied the activation of BA by the expression of CYP1A1 and cytotoxicity of its reactive metabolite. The cytotoxicity was determined by LDH and MTT assays (Fig. 6). In HepG2, Hepc or Hepc/1A1.4, LDH leakage was increased (Fig. 6A) and the MTT level was decreased (Fig. 6B) after treatment with BA (2–7  $\mu\text{M}$ ) in a dose-dependent manner. As compared with Hepc, strain Hepc/1A1.4 caused a significant increase in LDH leakage ( $P \leq 0.05$ ) and a decrease in the MTT level ( $P \leq 0.01$ ) after treatment with BA (7  $\mu\text{M}$ ).

## 4. Discussion

The stable expression system of CYP isoforms has made it possible to evaluate the relative risk of chemicals

in vitro (Sawada and Kamataki, 1998; Doehmer et al., 1999). We have established a series of transformants stably expressing human CYP isoforms in the human hepatic cell line, HepG2. In this study, we selected 10 CYP isoforms that make a significant contribution to the metabolism of xenobiotics in human liver. This is the first report on the establishment of stable transformants expressing a series of CYP isoforms in HepG2. This cell system enables us to analyse the function of each CYP isoform in the metabolism and toxicology of chemicals.

In this study, HepG2 was selected as a recipient cell line because it is derived from human liver and retains

Table 3  
Formation of sulfate and glucuronide conjugates of 7-hydroxycoumarin (7-HC)<sup>a,b</sup>

Cell	7-HC/G <sup>c</sup> (pmol/mg)	7-HC/S <sup>d</sup> (pmol/mg)
HepG2	53.66 $\pm$ 7.77	929.67 $\pm$ 27.18
Hepc	78.97 $\pm$ 2.72	534.10 $\pm$ 18.23
Hepc/1A1.4	48.54 $\pm$ 2.89	723.23 $\pm$ 12.17
Hepc/1A2.9	45.91 $\pm$ 15.65	665.76 $\pm$ 34.83
Hepc/2A6L.14	139.46 $\pm$ 3.20	456.36 $\pm$ 13.57
Hepc/2B6.68	68.60 $\pm$ 2.04	865.38 $\pm$ 9.31
Hepc/2C8.46	228.62 $\pm$ 10.25	1400.56 $\pm$ 99.84
Hepc/2C9.1	57.95 $\pm$ 11.06	891.60 $\pm$ 30.11
Hepc/2C19.12	63.42 $\pm$ 16.93	707.96 $\pm$ 48.63
Hepc/2D6.39	45.41 $\pm$ 4.19	345.95 $\pm$ 5.42
Hepc/2E1.3-8	160.61 $\pm$ 14.79	601.74 $\pm$ 8.22
Hepc/3A4.2-30	162.58 $\pm$ 15.34	682.19 $\pm$ 21.13

<sup>a</sup> Cells were seeded on 96-well tissue culture plates and cultured for 3 days. After cells were washed, they were treated with  $50 \mu\text{M}$  7-HC at  $37^\circ\text{C}$  for 6 h. The production of 7-HC/G and 7-HC/S was detected by HPLC analysis as described in Section 2.

<sup>b</sup> Results showed mean  $\pm$  S.D. ( $n = 4$ ).

<sup>c</sup> 7-HC/G, 7-HC glucuronide.

<sup>d</sup> 7-HC/S, 7-HC sulfate.



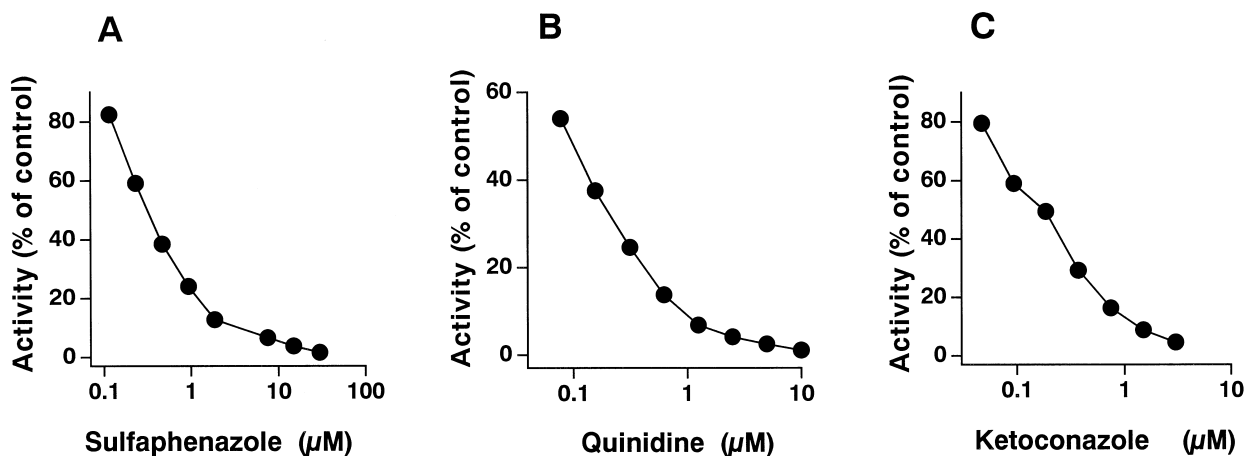


Fig. 3. Effects of CYP subtype specific inhibitors on the activity of CYP2C9, 2D6 and 3A4 expressed in HepG2. Hepc/2C9.1, Hepc/2D6.39 or Hepc/3A4.2-30 ( $7 \times 10^4$  cells/100  $\mu\text{l}$ /well) were seeded on a 96-well tissue culture plate and cultured for 3 days. Medium was changed to a fresh one containing 400  $\mu\text{M}$  tolbutamide and various concentrations of sulfaphenazole for Hepc/2C9.1 and incubated at 37°C for 3 h; 200  $\mu\text{M}$  bufuralol and various concentrations of quinidine for Hepc/2D6.39 and incubated at 37°C for 3 h; 100  $\mu\text{M}$  testosterone and various concentrations of ketoconazole for Hepc/3A4.2-30 and incubated at 37°C for 1 h. CYP2C9, CYP2D6 or CYP3A4 activity in each sample was determined as described in Materials and Methods. Results showed percent of control response. Each point and bar represents the mean  $\pm$  S.D. of three to four experiments. (A) Hepc/2C9.1; (B) Hepc/2D6.39; (C) Hepc/3A4.2-30.

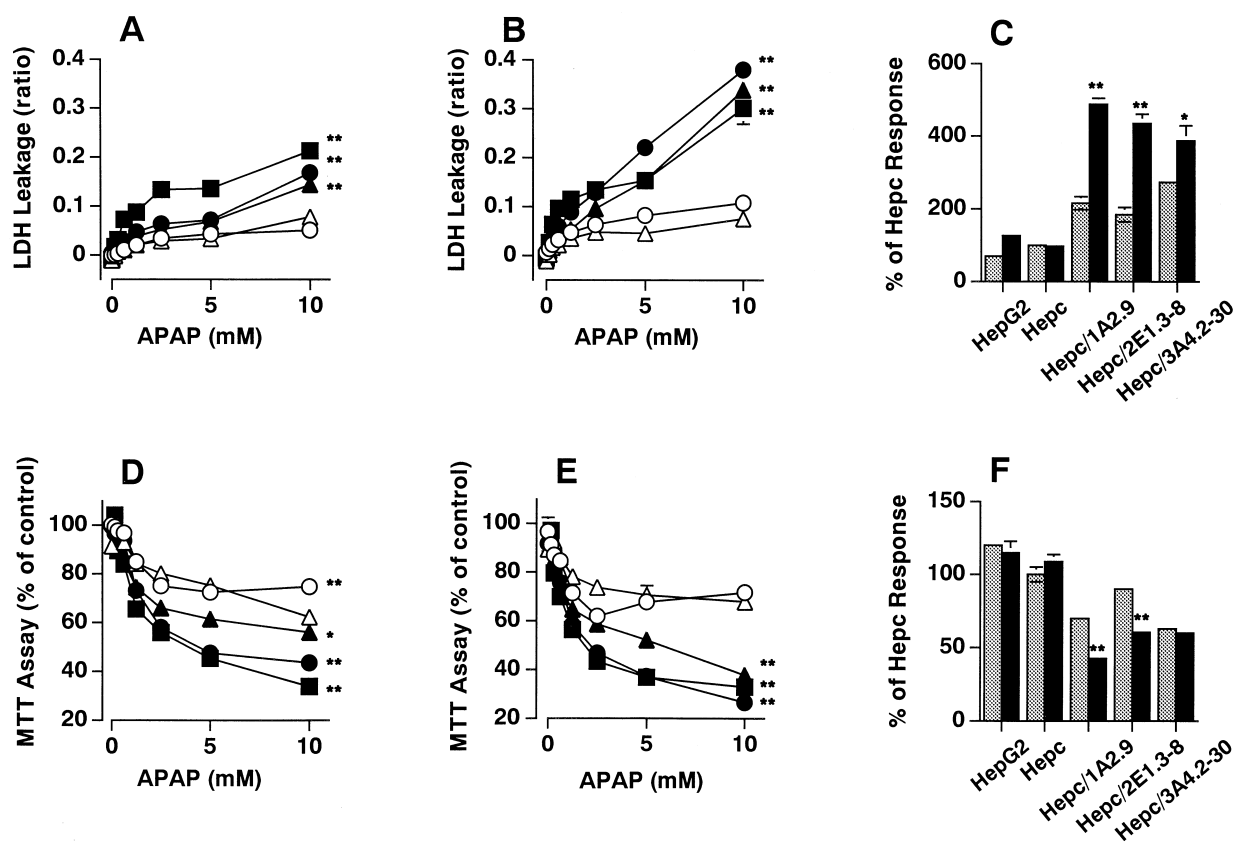


Fig. 4. Metabolic activation of APAP by expression of CYP1A2, CYP2E1 and CYP3A4. Cells ( $3 \times 10^4$  cells) were seeded onto a 96-well microplate with APAP (A,C) or both APAP and 100  $\mu\text{M}$  BSO (B,D), and cultured for 3 days. Each point and bar represents the mean  $\pm$  S.D. of three to four experiments. Data (10 mM APAP) were tested using Student's *t*-test (\*\* $P \leq 0.01$ ; \* $P \leq 0.05$ , compared with Hepc). (A) and (B): LDH assay. LDH activity was determined in culture medium and total cell extract. LDH leakage was expressed as the ratio of  $\text{LDH}_{\text{out}}/\text{LDH}_{\text{total}}$ . (D) and (E): MTT assay. Cell viability was determined by MTT assay. Results were expressed as percent of control response. HepG2 (○); Hepc (△); Hepc/1A2.9 (●); Hepc/2E1.3-8 (▲); Hepc/3A4.2-30 (■). (C) and (F): The percentage to Hepc at 10 mM APAP treatment was determined in the LDH (C) and MTT assay (F). The enhancement of LDH leakage or the decrement of MTT level by both of 10 mM APAP + 100  $\mu\text{M}$  BSO treatment (solid bars) were compared with 10 mM APAP treatment (dotted bars) in each strain. Each data was tested using Student's *t*-test (\*\* $P \leq 0.01$ ; \* $P \leq 0.05$ , compared with APAP treatment).

many xenobiotic metabolizing activities (Dierickx, 1989; Rueff et al., 1996). Therefore, HepG2 has been used as the test strain for the prediction of toxicity, carcinogenicity and cell mutagenicity in humans. Although HepG2 shows about 10% of the P450-dependent monooxygenase activity of freshly isolated human adult hepatocytes, the cell line retains many Phase II metabolic functions (Doostdar et al., 1988; Rueff et al., 1996). Generally, the reactions catalyzed by cytochrome P450 molecules require co-existence of NADPH-cytochrome P450 reductase and cytochrome  $b_5$  supports some CYP-mediated reactions. HepG2 has been shown to have NADPH-cytochrome P450 reductase activity

and cytochrome  $b_5$ , although the levels are lower than those of human liver (Aoyama et al., 1990; Waxman et al., 1991; Patten et al., 1992). Therefore, the cell system established here does not need co-expression of reductase and/or cytochrome  $b_5$  with CYP enzymes and the reaction continues for a few weeks (data not shown).

In this cell-based system, each transformant has predominantly only one CYP activity, and high linearity in Lineweaver–Burk plots was obtained from all transformants in this study. The apparent  $K_m$  values of these transformants were nearly equal to those of human liver microsomes; however, a small discrepancy between transformants and human liver microsomes existed for

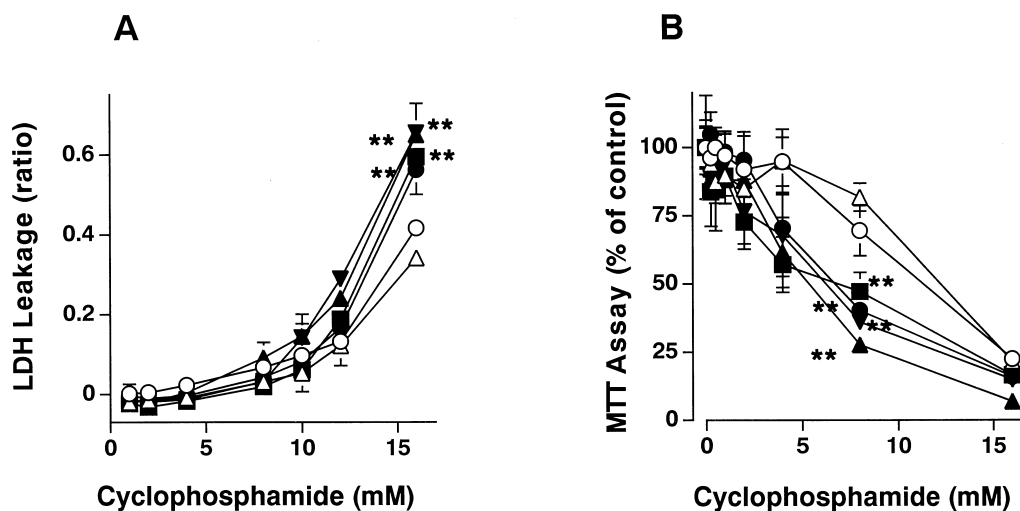


Fig. 5. Metabolic activation of CPA by expression of CYP2A6, CYP2B6, CYP2C8 and CYP2C9. Hep/2A6L.14 (■), Hepc/2B6.68 (▼); Hepc/2C8.46 (●), Hepc/2C9.1 (▲), Hepc (△) or HepG2 (○) ( $2 \times 10^4$  cells) were seeded onto a 96-well microplate with CPA and grown for 3 days. Each point and bar represents the mean  $\pm$  S.D. of three to four experiments. Data were tested using Student's *t*-test (\*\* $P \leq 0.01$  compared with Hepc). (A): *LDH assay*. LDH activity was determined in culture medium and total cell extract. LDH leakage was expressed as the ratio of  $LDH_{out}/LDH_{total}$ . (B) *MTT assay*. Cell viability was determined by MTT assay. Results were expressed as percent of control response.

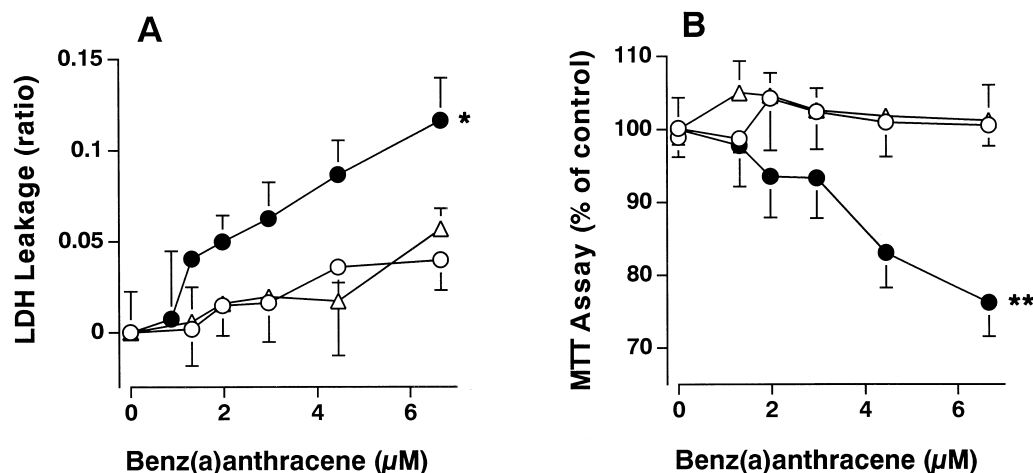


Fig. 6. Metabolic activation of BA by expression of CYP1A1. Hepc/1A1.4 (●), Hepc (△) or HepG2 (○) ( $2 \times 10^4$  cells) were seeded onto a 96-well microplate with BA and grown for 3 days. Each point and bar represents the mean  $\pm$  S.D. of three to four experiments. Data were tested using Student's *t*-test (\*\* $P \leq 0.01$ ; \* $P \leq 0.05$ , compared with Hepc). (A): *LDH assay*. LDH activity was determined in culture medium and in total cell extract. LDH leakage was expressed as the ratio of  $LDH_{out}/LDH_{total}$ . (B): *MTT assay*. Cell viability was determined by MTT assay. Results were expressed as percent of control response.

some CYP isoforms. These discrepancies may come from the existence of multiple CYP isoforms in the liver microsomes.

Among several types of drug–drug interactions, inhibition of a CYP isoform by a drug leads to an increase or decrease in the plasma concentration of a concomitantly administered drug, and in some cases the inhibition causes a serious adverse effect of the drug. Therefore, it is necessary to examine whether a drug would inhibit the activities of CYP isoforms. Sulfaphenazole, quinidine and ketoconazole are known to be potent inhibitors of CYP2C9, CYP2D6 and CYP3A4, respectively. The activities of CYP isoforms in Hepc/2C9.1, Hepc/2D6.39 and Hepc/3A4.2-30 were inhibited by their specific inhibitors in a dose-dependent manner, and  $IC_{50}$  values were nearly equal to those reported (Bourrié et al., 1996). In addition to the CYP isoforms, the co-existence of conjugating enzymes permits a more precise reconstruction of hepatic metabolism. Therefore, these transformants might become an aid for predicting those drug–drug interactions where CYP3A4, CYP 2C or CYP2D6 are involved.

To investigate the usefulness of the cell system in toxicological studies, the metabolic activation of some well-known chemicals, for example APAP, CPA and BA, was examined. APAP is bioactivated to form a reactive intermediate, *N*-acetyl-*p*-benzoquinone imine (NAPQI), by such CYP isoforms as CYP1A2, CYP2E1 and CYP3A4 (Raucy et al., 1989; Thummel et al., 1993). In normal conditions, conjugation with intercellular GSH reduces the toxicity of NAPQI. When intercellular GSH is depleted, over-produced NAPQI can initiate severe liver injury (Albano et al., 1985; Moore et al., 1985). Direct linkage between APAP cytotoxicity and APAP metabolism by CYP2E1 has also been demonstrated in culture systems with a human liver-derived cell line (Dai and Cederbaum, 1995). In the present study, we examined the mechanism of APAP toxicity using a series of CYP transformants. When intracellular GSH was depleted by the treatment with 100  $\mu$ M BSO, it significantly enhanced APAP cytotoxicity to Hepc/1A2.9, Hepc/2E1.3-8 and Hepc/3A4.2-30 ( $P \leq 0.01$ ). It has been reported that treatment of HepG2 with BSO (100  $\mu$ M) caused approximately 90% depletion of intracellular GSH (Dai and Cederbaum, 1995). These results suggest that Hepc/1A2.9, Hepc/2E1.3-8 and Hepc/3A4.2-30 exhibit both activation of APAP by the CYP molecule and detoxification of the metabolic intermediate by the GSH conjugating process. In addition to GSH conjugating activity, the cell system has glucuronide- and sulfate-conjugating activity (Fig. 2, Table 3). Simultaneous expression of Phase I CYP isoforms and Phase II conjugating enzymes in a cell is one of the remarkable advantages of this cell system. CPA is an anticancer alkylating agent, and it is metabolised to produce pharmacologically active but

cytotoxic species (Sladek, 1988). CYPs 2A6, 2B6, 2C8, 2C9 and 3A4 are reported to form the activated molecules (Chang et al., 1993). In this study, transformants expressing CYP2A6, 2B6, 2C8 and 2C9, namely Hepc/2A6L.14, Hepc/2B6.68, Hepc/2C8.46 and Hepc/2C9.1, were much more sensitive to CPA than HepG2 and Hepc. The results indicate that the cytotoxicity of CPA was enhanced by the reactive intermediate produced by the expressed CYP isoforms. CYP1A1 is known to be particularly active on polycyclic aromatic hydrocarbons (PAHs) such as BA. In this study, the metabolic activation of BA was examined using Hepc/1A1.4, and the results of the study supported the activation of the molecule by CYP1A1. As already observed with CYP1A1 transformed V79 strain (Schmalix et al., 1993), this transformant might also be an indicator for the mutagenic effects caused by PAHs.

In summary, we have demonstrated the practicality of the stable CYP expression cell systems in the metabolism and toxicology of chemicals, for example metabolism, CYP inhibition and metabolic activation. Furthermore, this cell system is useful for the investigation of metabolism and toxicology in human liver, because oxidizing reactions and conjugating reactions are progressed simultaneously in this cell system, similar to those in the human liver. Inhibition of a particular enzyme reaction and/or co-culture of a particular transformant enables the generation of metabolic imbalance in the hepatic cell lines. Therefore, this cell system may be useful to elucidate the mechanism of idiosyncratic hepatotoxicity by particular drugs concerned with metabolic imbalance. This system should be used at the preclinical stage as a complement to animal studies, and as a means of predicting and planning clinical pharmacokinetic and toxicological studies.

## Acknowledgements

We thank Mr Migifumi Ino and Ms Aki Suzuki-Yoneda for their excellent technical support.

## References

- Albano, E., Rundgren, M., Harvison, P.J., Nelson, S.D., Moldeus, P., 1985. Mechanism of *N*-acetyl-*p*-benzoquinone imine cytotoxicity. *Molecular Pharmacology* 28, 306–312.
- Aoyama, T., Korzekwa, K., Nagata, K., Gillette, J., Gelboin, H.V., Gonzalez, F.J., 1990. Estradiol metabolism by complementary deoxyribonucleic acid-expressed human cytochrome P450s. *Endocrinology* 126, 3101–3106.
- Bourrié, M., Meunier, V., Berger, Y., Fabre, G., 1996. Cytochrome P450 isoform inhibitors as a tool for the investigation of metabolic reactions catalyzed by human liver microsomes. *Journal of Pharmacology and Experimental Therapeutics* 277, 321–332.

- Chang, T.K.H., Weber, G.F., Crespi, C.L., Waxman, D.J., 1993. Differential activation of cyclophosphamide and isofosphamide by cytochromes P-450 2B and 3A in human liver microsomes. *Cancer Research* 53, 5629–5637.
- Chen, Q., Cederbaum, A.I., 1998. Cytotoxicity and apoptosis produced by cytochrome P450 2E1 in HepG2 cells. *Molecular Pharmacology* 53, 638–648.
- Dai, Y., Cederbaum, A.I., 1995. Cytotoxicity of acetaminophen in human cytochrome P4502E1-transfected HepG2 cells. *Journal of Pharmacology and Experimental Therapeutics* 273, 1497–1505.
- Dai, Y., Rashba-Step, J., Cederbaum, A.I., 1993. Stable expression of human cytochrome P4502E1 in HepG2 cells: characterization of catalytic activities and production of reactive oxygen intermediates. *Biochemistry* 32, 6928–6937.
- Dierickx, P.J., 1989. Cytotoxicity testing of 114 compounds by the determination of the protein content in HepG2 cell cultures. *Toxicology in Vitro* 3, 189–193.
- Doehmer, J., Buters, J.T.M., Luch, A., Soballa, V., Baird, W.M., Morisson, H., Stegeman, J.J., Townsend, A.J., Greenlee, W.F., Glatt, H.R., Seidel, A., Jacob, J., Greim, H., 1999. Molecular studies on the toxifying effects by genetically engineered cytochromes P450. *Drug Metabolism Reviews* 31, 423–435.
- Doodard, H., Duthie, S.J., Burke, M.D., Melvin, W.T., Grant, M.H., 1988. The influence of culture medium composition on drug metabolizing enzyme activities of the human liver derived HepG2 cell line. *FEBS Letters* 241, 15–18.
- Friedberg, T., Pritchard, M.P., Bandera, M., Hanlon, S.P., Yao, D., McLaughlin, L.A., Ding, S., Burchell, B., Wolf, C.R., 1999. Merits and limitations of recombinant models for the study of human P450-mediated drug metabolism and toxic: an intralaboratory comparison. *Drug Metabolism Reviews* 31, 523–544.
- Guengerich, F.P., 1991. Reactions and significance of cytochrome P-450 enzymes. *Journal of Biological Chemistry* 266, 10019–10022.
- Iwata, H., Fujita, K., Kushida, H., Susuki, A., Konno, Y., Nakamura, K., Fujino, A., Kamataki, T., 1988. High catalytic activity of human NADPH-cytochrome P450 reductase in *Escherichia coli*. *Biochemical Pharmacology* 55, 1315–1325.
- Knowles, B.B., Howe, C.C., Aden, D.P., 1980. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science* 209, 497–499.
- Moore, M., Thor, H., Moore, G., Nelson, S.D., Moldeus, P., Orrenius, S., 1985. The toxicity of acetaminophen and N-acetyl-p-benzoquinone imine in isolated hepatocytes is associated with thiol depletion and increased calcium. *Journal of Biological Chemistry* 260, 13035–13040.
- Patten, C.J., Ishizaki, H., Aoyama, T., Lee, M., Ning, S.M., Huang, W., Gonzalez, F.J., Yang, C.S., 1992. Catalytic properties of the human cytochrome P450 2E1 produced by cDNA expression in mammalian cells. *Archives of Biochemistry and Biophysics* 299, 163–171.
- Porter, T.D., Coon, M.L., 1991. Cytochrome P-450. Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *Journal of Biological Chemistry* 266, 13469–13472.
- Raucy, J.L., Lasker, J.M., Lieber, C.S., Black, M., 1989. Acetaminophen activation by human liver cytochromes P-450IIE1 and P-450IA2. *Archives of Biochemistry and Biophysics* 271, 270–283.
- Rendic, S., Di Carlo, F.J., 1997. Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metabolism Reviews* 29, 413–580.
- Rueff, J., Chiapella, C., Chipman, J.K., Darroudi, F., Silva, I.D., Bogaert, M.D., Fonti, E., Glatt, H.R., Isern, P., Laires, A., Léonard, A., Llagostera, M., Mossesso, P., Natarajan, A.T., Palitti, F., Rodrigues, A.S., Schinoppi, A., Turchi, G., W-Schneider, G., 1996. Development and validation of alternative metabolic systems for mutagenicity testing in short-term assays. *Mutation Research* 353, 151–176.
- Sakurai, K., Cederbaum, A.I., 1998. Oxidative stress and cytotoxicity induced by ferric-nitritotriacetate in HepG2 cells that express cytochrome P450 2E1. *Molecular Pharmacology* 54, 1024–1035.
- Sawada, M., Kamataki, T., 1998. Genetically engineered cells stably expressing cytochrome P450 and their applications to mutagen assays. *Mutation Research* 411, 19–43.
- Schmalix, W.A., Mäser, H., Kiefer, F., Reen, R., Wiebel, F.J., Gonzalez, F., Seidel, A., Glatt, H., Greim, H., Doehmaer, J., 1993. Stable expression of human cytochrome P450 1A1 cDNA in V79 Chinese hamster cells and metabolic activation of benzo[a]pyrene. *European Journal of Pharmacology* 248, 251–261.
- Sladek, N.E., 1988. Metabolism of oxazaphosphorines. *Pharmacology and Therapeutics* 37, 301–355.
- Thakker, D.R., Yagi, H., Levin, W., Wood, A.W., Conney, A.H., Jerina, D.M., 1985. Polycyclic aromatic hydrocarbons: Metabolic activation to ultimate carcinogens. In: *Anderd, M.W. (Ed.), Bioactivation of Foreign Compounds*. Academic Press, New York, pp. 177–242.
- Thummel, K.E., Lee, C.A., Kunze, K.L., Nelson, S.D., Slattery, J.T., 1993. Oxidation of acetaminophen to N-acetyl-p-aminobenzoquinone imine by human CYP3A4. *Biochemical Pharmacology* 45, 1563–1569.
- Waxman, D.J., Lapenson, D.P., Aoyama, T., Gelboin, H.V., Gonzalez, F.J., Korzekwa, K., 1991. Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochrome P450s. *Archives of Biochemistry and Biophysics* 290, 160–166.
- Wu, D., Cederbaum, A.I., 1996. Ethanol cytotoxicity to a transfected HepG2 cell line expressing human cytochrome P4502E1. *Journal of Biological Chemistry* 271, 23914–23919.