Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J. Biol. Chem.*, **279**: 23847–23850 (2004).

27) Daujat, M., Peryt, B., Lesca, P., Fourtanier, G., Domergue, J. and Maurel, P.: Omeprazole, an inducer of human CYP1A1 and 1A2, is not a ligand for the Ah receptor. *Biochem. Biophys. Res. Commun.*, **188**: 820–825 (1992).

28) Quattrochi, L. C. and Tukey, R. H.: Nuclear uptake of the Ah (dioxin) receptor in response to omeprazole: transcriptional activation of the human CYP1A1 gene. *Mol. Pharmacol.*, **43**: 504–508 (1993).

29) Lesca, P., Peryt, B., Larrieu, G., Alvinerie, M., Galtier, P., Daujat, M., Maurel, P. and Hoogenboom, L.: Evidence for the ligand-independent activation of the AH receptor. *Biochem. Biophys. Res. Commun.*, **209**: 474–482 (1995).

30) Daujat, M., Charrasse, S., Fabre, I., Lesca, P., Jounaïdi, Y., Larroque, C., Poellinger, L. and Maurel, P.: Induction of CYP1A1 gene by benzimidazole derivatives during Caco-2 cell differentiation. Evidence for an aryl-hydrocarbon receptor-mediated mechanism. *Eur. J. Biochem.*, **237**: 642–652 (1996).

31) Kalthoff, S., Ehmer, U., Freiberg, N., Manns, M. P. and Strassburg, C. P.: Coffee induces expression of glucuronosyltransferases by the aryl hydrocarbon receptor and Nrf2 in liver and stomach. *Gastroenterology*, **139**: 1699–1710 (2010).

32) Ueda, R., Iketaki, H., Nagata, K., Kimura, S., Gonzalez, F. J., Kusano, K., Yoshimura, T. and Yamazoe, Y.: A common regulatory region functions bidirectionally in transcriptional activation of the human CYP1A1 and CYP1A2 genes. *Mol. Pharmacol.*, **69**: 1924–1930 (2006).

33) Matsunaga, T., Maruyama, M., Harada, E., Katsuyama, Y., Sugihara, N., Ise, H., Negishi, N., Ikeda, U. and Ohmori, S.: Expression and induction of CYP3As in human fetal hepatocytes. *Biochem. Biophys. Res. Commun.*, **318**: 428–434 (2004).

34) Takezawa, T., Matsunaga, T., Aikawa, K., Nakamura, K. and Ohmori, S.: Lower expression of HNF4$\alpha$ and PGC1$\alpha$ might impair rifampicin-mediated CYP3A4 induction under conditions where PXR overexpressed in human fetal liver cells. *Drug Metab. Pharmacokinet.*, **27**: 430–438 (2012).

35) Duan, Y., Catana, A., Meng, Y., Yamamoto, N., He, S., Gupta, S., Gambhir, S. S. and Zern, M. A.: Differentiation and enrichment of hepatocyte-like cells from human embryonic stem cells in vitro and in vivo. *Stem Cells*, **25**: 3058–3068 (2007).

Drug Metab. Pharmacokinet. 27 (4): 430–438 (2012). 

## Regular Article

# Lower Expression of HNF4α and PGC1α Might Impair Rifampicin-mediated CYP3A4 Induction under Conditions Where PXR Is Overexpressed in Human Fetal Liver Cells

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**Summary:** Pregnane X receptor (PXR) mRNA was detected in HepG2 cells by RT-PCR, but not in human fetal liver (HFL) cells. CYP3A4 was induced by rifampicin (RIF), mifepristone (RU486), clotrimazole (CTZ), and dexamethasone (DEX) in HepG2 cells, while these PXR ligands with the exception of DEX did not induce CYP3A4 mRNA expression in HFL cells. Ad-PXR infection increased mRNA levels of PXR and CYP3A4 in both cells despite the absence of PXR ligands. Similar results were observed in reporter gene assays. However, in HFL cells, RIF-mediated CYP3A4 induction was insufficient compared with HepG2 cells, despite PXR overexpression. The expression levels of five coactivators (HNF4α, PGC1α, SRC1, CBP, and P300) related to CYP3A4 expression in HepG2, HFL cells, and human adult liver were analyzed by RT-PCR. Expression levels of HNF4α and PGC1α in HFL cells were downregulated to 20% of those in the human adult liver. On the other hand, the expression level of HNF4α in HepG2 cells was higher than that in HFL cells, although PGC1α expression level was almost the same as that in HFL cells. HNF4α mRNA expression level in HepG2 cells was 57% of that in human adult liver, and the level in HFL cells was 30% of that in HepG2 cells. These results suggested that lower expression of HNF4α and PGC1α may impair RIF-mediated CYP3A4 induction under conditions of PXR overexpression in HFL cells.

**Keywords: PXR; drug-mediated induction; CYP3As; fetal liver; HNF4α; PGC1α**

## Introduction

Cytochrome P450 (CYP) is a superfamily of heme proteins that play a critical role in the oxidative metabolism of endogenous and xenobiotic substances.[1)] The human CYP3A subfamily includes four functional members, CYP3A4, CYP3A5, CYP3A7, and CYP3A43, which are expressed at different levels in the liver, kidney, and gastrointestinal tract.[2,3)] CYP3A4 is the predominant isoform expressed in the adult human liver and was reported to be responsible for the metabolism of more than 60% of therapeutic drugs.[4,5)] In contrast, CYP3A7 is the major CYP isoform in the human fetal liver.[6)]

CYP expression is modulated by a large variety of endogenous and exogenous substances, including drugs, and induction of CYP expression has been analyzed extensively in experimental animals. Toxicological and pharmacological studies designed to evaluate the safety and efficacy of candidate drugs and chemicals in the human fetus are largely dependent on extrapolation from animal models. However, it is difficult to estimate alterations of drug metabolism by inducers of drug-metabolizing enzymes in the human fetus due to the problems of differences among species.[7,8)] Species differences in the inducibility of drug-metabolizing enzymes result in important differences in the metabolism of drugs and potential carcinogens. Therefore, primary cultures of hepatocytes and human hepatoma HepG2 cells have been widely used to study drug metabolism.[7,9)] On the other hand, there have been few reports regarding the expression and induction of drug-metabolizing enzymes in human fetal hepatocytes in culture.

*Received October 20, 2011; Accepted January 23, 2012*
*J-STAGE Advance Published Date: February 14, 2012, doi:10.2133/dmpk.DMPK-11-RG-126*
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This work was partly supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Nos. 20590142 and 18590138).

Previous studies of the induction of CYP3As by rifampicin (RIF) and glucocorticoids have elucidated a mechanism of regulation in which orphan nuclear receptors, such as the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) and steroid receptors, such as the glucocorticoid receptor (GR), play central roles in regulation of gene expression.[10–15] Upon ligand binding, PXR forms a heterodimer with retinoid X receptor α (RXRα) and transactivates ER-6 (everted repeat separated by six nucleotides) and DR-3 (direct repeat separated by three nucleotides) elements upstream of CYP3A4.[16–20] We reported previously that CYP3A4 and CY3A7 mRNA expression levels were markedly upregulated by dexamethasone (DEX), but not by RIF, in human fetal liver (HFL) cells. We considered that CYP3A4 and CYP3A7 mRNA expression were not induced by RIF because PXR expression level was very low or absent in HFL cells.[11,21]

Recently, some transcription factors (coactivators and corepressors) that mediate CYP3As expression have been reported. Hepatocyte nuclear factor 4α (HNF4α) is one of the most important liver-enriched transcription factors for hepatocyte differentiation.[22] HNF4α plays critical roles not only in specification of the hepatic phenotype during liver development but also in the transcriptional regulation of genes involved in glucose, cholesterol, fatty acid, and xenobiotic metabolism and in the synthesis of blood coagulation factors.[23,24] Peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) is a key metabolic regulator and was originally identified as a peroxisome proliferator-activated receptor γ interacting coactivator in brown adipose tissue.[25] Recent studies showed that PGC1α is a versatile coactivator for numerous nuclear receptors and has been implicated in diverse biological activities, including lipid and carbohydrate metabolism.[26,27] PGC1α has been shown to increase the HNF4α-mediated transactivation of CYP7A1, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase genes.[27–29] PXR promotes CYP3A4 gene transcription by interacting with HNF4α, steroid receptor coactivator 1 (SRC1), and PGC1α. Moreover, RIF strongly stimulates PXR and HNF4α interaction and CYP3A4 promoter activity, which are further enhanced by PGC1α and SRC1 but inhibited by the small heterodimer partner (SHP).[13,30]

In the present study, we investigated the effects of PXR overexpression on drug-mediated induction of CYP3As mRNA expression in HFL cells and the expression levels of other transcription factors that were reported to be related to CYP3As expression.

## Materials and Methods

**Materials:** Dulbecco's modified Eagle's medium and Williams' medium E were purchased from Sigma Chemical Co. (St. Louis, MO). Penicillin–streptomycin–neomycin antibiotic mixture and SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) were from Invitrogen Corp. (Carlsbad, CA). Cellbanker, clotrimazole (CTZ), DEX, dimethyl sulfoxide (DMSO), mifepristone (RU486), RIF, and human normal adult liver total RNA (male, 64 years old) were from Wako Pure Chemicals (Osaka, Japan). An illustra RNAspin Mini RNA Isolation Kit was obtained from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire, UK). The Luciferase Assay System was from Promega Co. (Madison, WI). ExTaq and PrimeSTAR HS DNA Polymerases were from Takara Bio Inc. (Otsu, Japan). Collagen (type I) was from Koken (Tokyo, Japan). PCR primers were purchased from Sigma Genosys (Ishikari, Japan). All other reagents used were of the highest quality available.

**Cells:** HFL cells were obtained from Applied Cell Biology Research Institute (Kirkland, WA). Cell culture was initiated from a pool of six normal human liver tissues (average gestation 13 weeks) by elutriation following dispase digestion of tissue. The cells were subcultured when the culture reached 90–100% confluence, and suspended in Cellbanker after proliferation. Aliquots of the suspensions were cryopreserved at −150°C until use. HepG2 cells were obtained from Cell Resource Center for Biomedical Research, Tohoku University, Japan.

**Cell culture and adenovirus infection:** HepG2 and HFL cells were seeded onto dishes coated with collagen (type I) and cultured in Dulbecco's modified Eagle's medium and Williams' medium E, respectively, supplemented with 10% FBS, 2 mM glutamine, and antibiotics (50 μg/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml neomycin) under an atmosphere of 5% $CO_2$ in air at 37°C. The medium was replaced every 24 h. The recombinant adenoviruses, Ad-*lacZ*, Ad-PXR, and Ad-CYP3A4-362-7.7k-Luc, were provided by Yamazoe and Nagata. HepG2 and HFL cells were subcultured for 3 or 7 days after seeding of $4 \times 10^4$ cells onto 6- or 24-well culture plates. When subculture was performed, the cells were infected with Ad-*lacZ* (50 MOI), Ad-PXR (50 MOI), and Ad-CYP3A4-362-7.7k-Luc (25 MOI) solution for 1 h. In the drug treatment groups, HepG2 and HFL cells were treated with DEX (100 μM), RIF (40 μM), RU486 (10 μM), and CTZ (10 μM) for 48 h. The above compounds were dissolved in DMSO (vehicle), which was added to the culture medium at a final concentration of 0.1%. The medium was replaced daily with fresh medium containing either the test compounds dissolved in vehicle or vehicle alone. After treatment with the test compounds for 48 h, the cells were harvested using the cell lysis solution for RT-PCR and reporter gene assays.

**RNA extraction and reverse transcription reaction:** Total RNA was extracted using an illustra RNAspin Mini RNA Isolation Kit according to the manufacturer's instructions. First-strand cDNA was generated from 1–2 μg of total RNA. Reverse transcription reaction was performed using a SuperScript III First-Strand Synthesis System for RT-PCR in accordance with the manufacturer's instructions.

**Table 1. Sequences and semiquantitative RT-PCR conditions**

| Primer name | Forward primer sequence (5′ to 3′) | Reverse primer sequence (5′ to 3′) | Product size (bp) | Annealing temp. | Number of cycles | GenBank accession number |
|---|---|---|---|---|---|---|
| PXR | CAAGCGGAAGAAAAGTGAACG | CTGGTCCTCGATGGGCAAGTC | 442 | 68 | 32 | NM_022002 |
| RXRα | CAAACAGCTTTTCACCCTG | AGTAGGCCTCCAAGGAC | 392 | 56 | 32 | NM_002957 |
| HNF4α | GCCCTACCTCAAAGCCATCAT | GACCCTCCCAGCAGCATCTC | 273 | 60 | 35 | NM_000457 |
| PGC1α | TCAGTCCTCACTGGTGGACA | CTGCTTCGTCGTCAAAAACAGC | 352 | 64 | 33 | NM_013261 |
| SRC1 | ATGGTGAGCAGAGGCATGACA | AAACGGTGATGCTCATGTTG | 349 | 64 | 33 | Linja *et al.*[54] |
| P300 | CCTGAGTAGGGGCAACAAGA | GTGTCTCCACATGGTGCTTG | 353 | 64 | 32 | Linja *et al.*[54] |
| CBP | GATCGCCACGTCCCTTAGTA | CCCCAAGTGTCCCTGATCTA | 202 | 64 | 36 | U47741 |
| SMRT | AAGTCCATCCTCACGTCCAC | TGAAGCACACTGGGTCTCTG | 203 | 60 | 34 | U37146 |
| SHP | GCTGTCTGGAGTCCTTCTGG | GAAAGAAGAGGTCCCCAAGCAG | 261 | 60 | 34 | AB058644 |
| NcoR1 | CAAGTTTCCTCGCAGACTCC | CTCCTCTCTGGGGATTTTCC | 221 | 64 | 34 | NM_006311 |
| ApoCIII | ATGAAGCACGCCACCAAGAC | TGGGGTAGGAGAGCACTGAGA | 317 | 58 | 32 | AY422951 |
| PEPCK | CTGGGCGATGGGGAGTTTGTCA | GATTGTGTTCTTCTGGATGGTCTTG | 519 | 58 | 32 | BC023978 |
| GAPDH | CATCACCATCTTCCAGGAGC | CATGAGTCCTTCCACGATACC | 307 | 59 | 22 | BC013310 |

**Table 2. Sequences and SYBR Green real-time RT-PCR condition**

| Primer name | Forward primer sequence (5′ to 3′) | Reverse primer sequence (5′ to 3′) | Product size (bp) | Annealing temp. | Number of cycles | GenBank accession number |
|---|---|---|---|---|---|---|
| CYP3A4 | CTGTGTGTTTCCAAGAGAAGTTAC | TGCATCAATTTCCTCCTGCAG | 298 | 60 | 45 | AF182273 |
| CYP3A5 | CTCTCTGTTTCCAAAAGATACC | TGAAGATTATTGACTGGGCTG | 194 | 60 | 45 | NM_000777 |
| CYP3A7 | AGATTTAATCCATTAGATCCATTCG | AGGCGACCTTCTTTTATCTG | 148 | 60 | 45 | AF315325 |
| GAPDH | GAGTCAACGGATTTGGTCGT | GACAAGCTTCCCGTTCTCAG | 185 | 60 | 45 | BC013310 |

**Semiquantitative RT-PCR:** The mRNA expression levels of nuclear receptors, transcription factors, and target genes of HNF4α and PGC1α were analyzed by semiquantitative RT-PCR. PCR was carried out with the cDNAs thus obtained using a MyCycler thermal cycler (Bio-Rad, Hercules, CA) and PCR Express thermal cycler (Hybaid Ltd., Teddington, Middlesex, UK). The primers and amplification conditions used are summarized in **Table 1**. The levels of these mRNAs were quantified from their band densities on agarose gels using Printgraph AE-6914 and Scion Image Software (by W. Rasband), and were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

**Real-time RT-PCR analysis:** CYP3As mRNA expression levels were analyzed by SYBR Green real-time RT-PCR. PCR was carried out with the cDNAs thus obtained using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The primers and amplification conditions used are summarized in **Table 2**. The levels of these mRNAs were normalized relative to that of GAPDH mRNA.

**Reporter gene assay:** Reporter gene assay was performed according to the manufacturer's instructions using a Sirius Luminometer (Berthold Japan, Tokyo, Japan). The activity was expressed as relative light units (RLU)/mg of protein. The effects of drugs are presented as the ratio of treatment to no treatment.

**Statistical analysis:** Statistical comparisons were performed with Student's *t*-test.

## Results

**PXR and RXRα mRNA expression levels in HepG2 and HFL cells:** PXR and RXRα are nuclear receptors that play important roles in RIF-mediated CYP3As induction in the human adult liver. First, RT-PCR was used to analyze mRNA expression levels of PXR and RXRα in HepG2 and HFL cells. RXRα mRNA was detected in HepG2 and HFL cells. RXRα mRNA expression level in HFL cells was similar to that in HepG2 cells. PXR mRNA was detected in HepG2 cells, but not in HFL cells. Therefore, for overexpression of PXR, both cell lines were infected with Ad-PXR. Adenovirus-mediated transfection of PXR caused significant increases in PXR mRNA expression level in HepG2 and HFL cells (**Fig. 1**).

**CYP3As mRNA expression levels in HepG2 and HFL cells:** RIF is a highly effective inducer of CYP3A enzymes in adult human liver cells in primary culture.[1] Here, we evaluated the effects of PXR ligands including RIF on CYP3A4, CYP3A5, and CYP3A7 mRNA expression in HepG2 and HFL cells by real-time RT-PCR. CYP3A4,

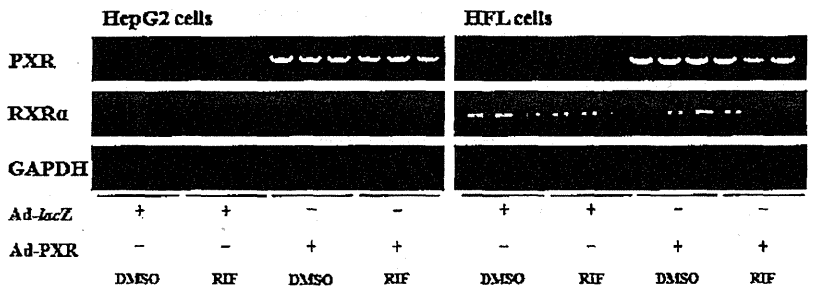


**Fig. 1. Nuclear receptor mRNA expression in HepG2 and HFL cells**
Total RNA was extracted from HepG2 and HFL cells infected with Ad-*lacZ* or Ad-PXR at 50 MOI for 1 h. mRNA was analyzed by semiquantitative RT-PCR as described in Materials and Methods. Images of ethidium bromide-stained agarose gels are shown.

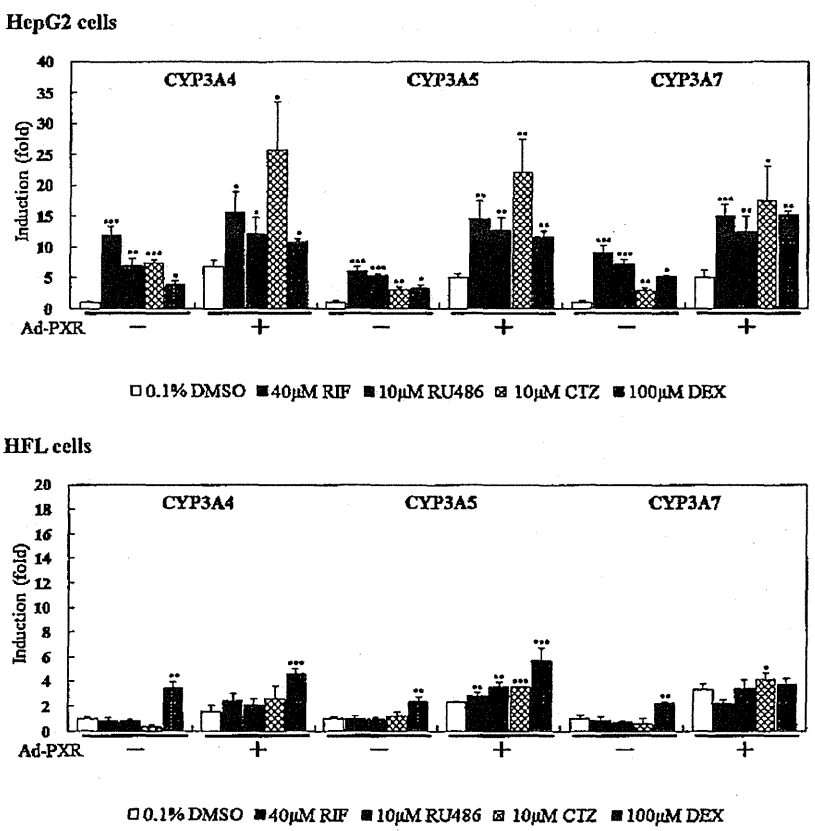


**Fig. 2. Effects of inducers and PXR infection on CYP3As mRNA expression in HepG2 and HFL cells**
HepG2 and HFL cells were infected with Ad-*lacZ* or Ad-PXR at 50 MOI and treated with vehicle (DMSO), 40 μM RIF, 10 μM RU486, 5 μM CTZ, or 100 μM DEX for 48 h. After treatment, mRNA was analyzed by SYBR Green real-time RT-PCR as described in Materials and Methods. Data are presented as the ratios of CYP3A4, CYP3A5, or CYP3A7 to GAPDH and normalized at 1.0 for Ad-*lacZ* infection and DMSO treatment alone. Columns and vertical bars represent means and SD ($n = 4$). Significantly different from DMSO group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

CYP3A5, and CYP3A7 mRNA expression levels in HepG2 cells were increased from 6- to 11-fold by RIF (Fig. 2). The other PXR ligands, RU486, CTZ, and DEX, induced an increase in CYP3A4, CYP3A5, and CYP3A7 mRNA expression by 3- to 7.4-fold in HepG2 cells. In contrast, RIF, RU486, and CTZ did not appreciably induce CYP3A4, CYP3A5, or CYP3A7 mRNA expression in HFL cells, although the expression levels of CYP3A4, CYP3A5, and CYP3A7 were increased to 3.4-, 2.3-, and 2.2-fold, respectively, by DEX.

Next, we examined the effects of PXR overexpression on PXR ligand-mediated CYP3As mRNA expression. Adenovirus-mediated transfection of PXR caused significant increases in CYP3A4, CYP3A5, and CYP3A7 mRNA expres-

sion in HepG2 and HFL cells without PXR ligands (**Fig. 2**). Under conditions of PXR overexpression, PXR ligand-mediated CYP3A4, CYP3A5, and CYP3A7 mRNA expression levels were increased to a greater extent than in HepG2 cells uninfected with Ad-PXR. Regardless of PXR overexpression in HFL cells, PXR ligands except DEX did not appreciably induce expression of CYP3A4, CYP3A5, or CYP3A7 mRNAs compared to HepG2 cells (**Fig. 2**).

**CYP3A4 promoter activity in HepG2 and HFL cells:** We used a luciferase promoter construct (Ad-CYP3A4-362-7.7k-Luc) to investigate the effects of PXR-mediated transactivation and PXR ligands on CYP3A4 promoter activity in HFL cells. Ad-CYP3A4-362-7.7k-Luc construct contained the core promoter (−362/+11) and the enhancer region (−7.2k/−7.8k) including ER-6 and DR-3 motifs.

CYP3A4 transactivation was detected without overexpression of PXR in HepG2 cells by PXR ligands, while no transactivation was detected in HFL cells (**Fig. 3**). Overexpression of PXR enhanced the transactivation in HepG2 and HFL cells. The activity was also increased to a greater extent by PXR ligands (RIF, RU486, and CTZ) in HepG2 cells. However, appreciable RIF-induced transactivation was not observed in HFL cells, even after overexpression of PXR. Similarly, other PXR ligands except CTZ did not show sufficient transactivation in HFL cells. However, CTZ significantly increased CYP3A4 transactivation after overexpression of PXR in HFL cells (7.2-fold, $p < 0.01$) (**Fig. 3**).

**Transcription factor expression levels in HFL cells:** We measured the expression levels of five coactivators in HepG2 and HFL cells and in human adult liver by RT-PCR. The expression levels of HNF4$\alpha$ and PGC1$\alpha$ mRNAs in HFL cells were about 20% of those in human adult liver. The expression level of PGC1$\alpha$ mRNA in HepG2 cells was similar to that in HFL cells. On the other hand, HNF4$\alpha$ mRNA level in HepG2 cells was 57% of that in human adult liver. The expression levels of CBP and P300 were 60–70% of those in the human adult liver, while SRC1 mRNA expression level in HFL cells was higher than those in HepG2 cells and human adult liver (1.3-fold) (**Fig. 4**). We also examined the expression of three co-repressors, SMRT, NCoR1, and SHP. The expression levels of SMRT and NCoR1 in HFL cells were similar to those in HepG2 cells and 70–90% of those in human adult liver. SHP mRNA expression was not detected in HFL cells (**Fig. 4**). Ad-PXR infection did not affect mRNA levels of the above coactivators or corepressors in HepG2 or HFL cells (data not shown).

**HNF4$\alpha$ and PGC1$\alpha$ target gene expression in HFL cells:** To confirm the functions of HNF4$\alpha$ and PGC1$\alpha$ in HFL cells, we examined mRNA expression levels of ApoCIII and PEPCK, which are target genes of HNF4$\alpha$ and PGC1$\alpha$. ApoCIII mRNA was detected in HepG2 cells, although the expression level was about 10% of that in human adult liver. On the other hand, ApoCIII mRNA was not detected in HFL cells under the present analytical conditions. PEPCK mRNA was not detected in HFL or HepG2 cells (**Fig. 5**).

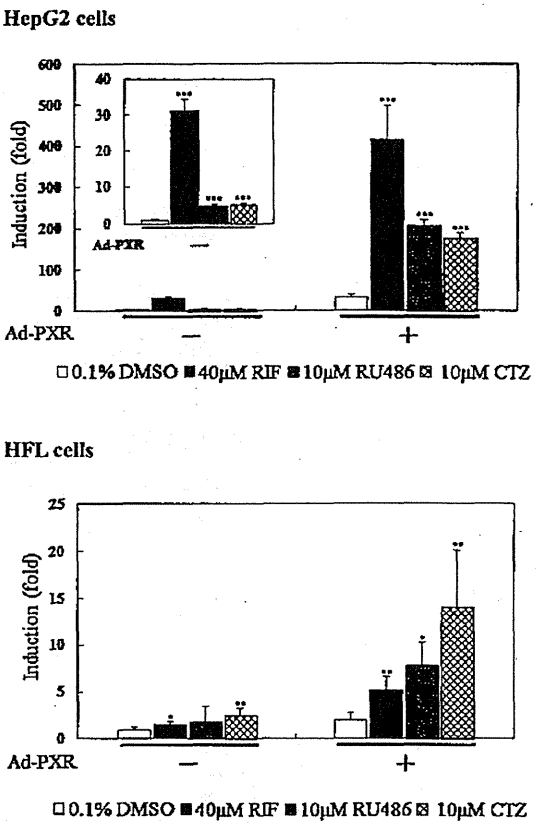


**Fig. 3. Effects of inducers and PXR infection on CYP3A4 promoter activity in HepG2 and HFL cells**
HepG2 and HFL cells were coinfected with Ad-CYP3A4-Luc (30 MOI) and Ad-*lacZ* (50 MOI) or Ad-PXR (50 MOI) and treated with vehicle (DMSO), 40 μM RIF, 10 μM RU486, or 5 μM CTZ for 48 h. After treatment, CYP3A4 promoter activity was analyzed by luciferase gene assay as described in Materials and Methods. Data are presented as the ratios of CYP3A4 promoter activity as relative light units (RLU)/mg to protein and normalized at 1.0 for Ad-*lacZ* infection and DMSO treatment alone. Columns and vertical bars represent means and SD ($n = 4–6$). Significantly different from DMSO group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

## Discussion

CYP3As gene expression is modulated by a large variety of endogenous and exogenous substances, including drugs, and induction of CYP3As expression has been analyzed extensively using experimental animals. However, it has been difficult to estimate alterations in drug metabolism by inducers of drug-metabolizing enzymes in the human fetus due to the problems of differences among species. Recently, primary cultures of human hepatocytes, HepG2 and HepaRG cells, have been used to study drug metabolism and enzyme induction. On the other hand, there have been few reports regarding the expression and induction of drug-metabolizing enzymes in human fetal hepatocytes in culture.

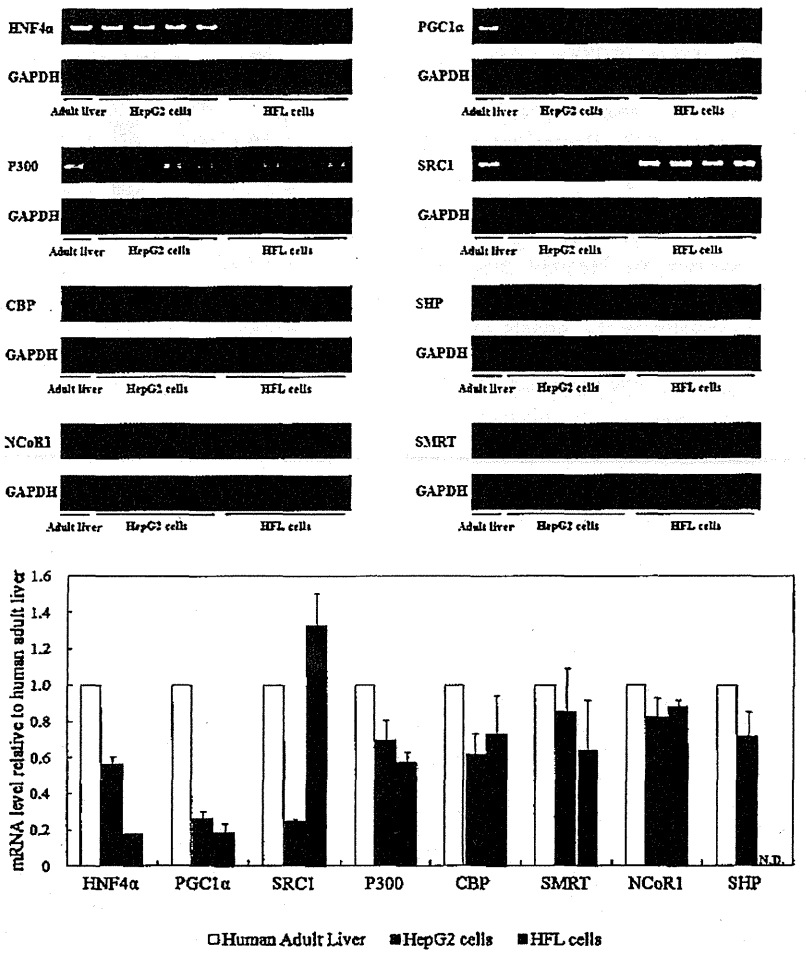


**Fig. 4. Expression of coactivators and corepressors mRNA in HepG2 and HFL Cells**
mRNA was analyzed by semiquantitative RT-PCR as described in Materials and Methods. Images of ethidium bromide-stained agarose gels are shown. Data are presented as ratios of coactivators or corepressors to GAPDH and normalized at 1.0 for human adult liver. Columns and vertical bars represent means and SD ($n = 1$ or 4).

We reported previously that CYP3A4 and CY3A7 mRNA expression levels were markedly upregulated by DEX in HFL cells.[11,21] We showed that GR plays a critical role in DEX-mediated CYP3As induction in HFL cells. However, RIF-mediated CYP3As induction was not observed, and PXR mRNA was not detected in HFL cells by RT-PCR.[11] We considered that the very low level or absence of PXR expression may impair RIF-mediated CYP3As induction.

Upon ligand binding, PXR forms a heterodimer with RXRα and transactivates ER-6 and DR-3 regions upstream of CYP3A4. Takada *et al.* performed reporter gene assays in HepG2 cells using a CYP3A4-luciferase fusion vector (pCYP3A4-362-7.7k) containing ER-6 and DR-3 regions and concluded that these regions were important in PXR-mediated CYP3A4 transactivation. Furthermore, they described the possibility of another *cis*-element mediating the basal transactivation by PXR in addition to ER-6 and DR-3 regions.[20] Accordingly, PXR and RXRα are essential for PXR ligand-mediated CYP3A4 induction. RXRα expression was confirmed in HFL cells, but PXR was not detected (**Fig. 1**). The present study was performed to examine the effects of PXR overexpression on PXR ligand-mediated CYP3As induction in HFL cells. The HepG2 cell line was established from a liver tumor biopsy specimen from a 15-year-old white male subject.[31] The induction of CYP in HepG2 cells has been studied as a model of human liver.[32,33] This cell line, however, has features of hepatoblast-like cells, because the cells express CYP3A7[34] and secrete AFP[31] into the cell culture medium, both proteins that are found predominantly in human fetal hepatocytes.[35] PXR mRNA was not detectable in HFL cells, although it was expressed in HepG2 cells. PXR has been identified as the major receptor responsible for transcriptional activation of CYP3A gene expression.[36] Another cellular factor, GRα,[37,38] is known to influence xenobiotic-mediated transcription induction, through cooperative activation of PXR expression. The level of GRα mRNA expression was almost identical in HepG2 and HFL cells. These CYP enzymes in HFL cells were

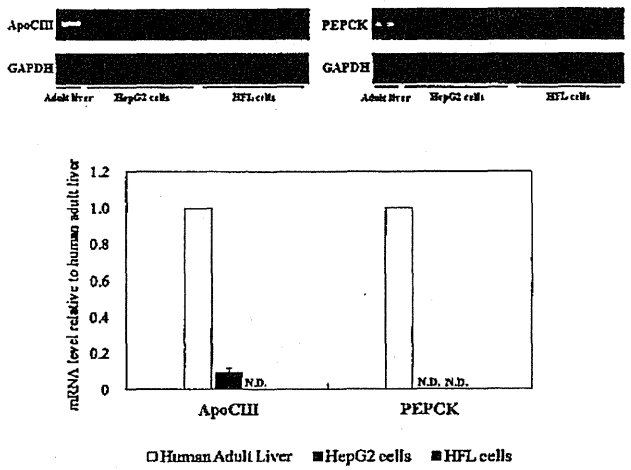


**Fig. 5. Expression of HNF4α and PGC1α target gene mRNA in HepG2 and HFL cells**

mRNA was analyzed by semiquantitative RT-PCR as described in Materials and Methods. Images of ethidium bromide-stained agarose gels are shown. Data are presented as ratios of ApoCIII or PEPCK to GAPDH and normalized at 1.0 for human adult liver. Columns and vertical bars represent means and SD ($n = 1$ or 4). ApoCIII and PEPCK mRNA were not detected in HFL cells.

significantly induced by DEX. We suggested previously that one reason why CYP3A4 and CYP3A7 mRNA expression were not induced by RIF in HFL cells may be that PXR expression level is very low in these cells as compared with HepG2 cells.[11] In this study, we used HepG2 cells as a positive control for HFL cells.

PXR overexpression, without PXR ligands, caused increases in CYP3As expression in HepG2 and HFL cells (**Fig. 2**). Similar results were observed in the reporter gene assays (**Fig. 3**). Support for this observation was provided in a report by Zang *et al.*[39] These results indicated that PXR plays an important role in basal CYP3As expression. RIF, RU486, CTZ, and DEX are known to be PXR ligands. These ligands caused the induction of CYP3As with or without PXR overexpression in HepG2 cells (**Figs. 2** and **3**) and the expression levels were further increased by PXR overexpression in HepG2 cells (**Figs. 2** and **3**).

Previously, we suggested that PXR underexpression impaired RIF-mediated CYP3A4 induction in HFL cells. However, these results suggested that factors other than PXR underexpression may control RIF-mediated CYP3A4 induction in HFL cells. DEX, which is known to be a GR ligand, increased CYP3As mRNA expression, unrelated to PXR overexpression. Interestingly, in HFL cells CTZ-mediated CYP3A4 induction was not detected by RT-PCR (**Fig. 2**), while CTZ markedly increased CYP3A4 transactivation under conditions where PXR was overexpressed (**Fig. 3**). In addition, CTZ-mediated CYP3A4 induction was lower than that induced by RIF in HepG2 cells (**Fig. 2**). Furukawa *et al.* reported that CTZ strongly activated CYP3A4 promoter activity while RIF did not in HepG2 cells using a CYP3A4-luciferase reporter construct (−362/+11).[40] These results indicated that the mechanisms of RIF- and CTZ-mediated CYP3A4 induction may be different.

Recently, transcription factors that mediate CYP3As expression have been identified. Various studies have shown that HNF4α interacts strongly with the p160 family coactivators (SRC1, 2, and 3) and that HNF4α activity is enhanced by the action of CBP/P300.[41–45] On the other hand, SHP has been shown to decrease PXR-mediated transactivation and another corepressor-silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), nuclear receptor corepressor 1 (NCoR1), inhibits PXR transactivation.[13,30,46] HNF4α is one of the most important liver-enriched transcription factors involved in hepatocyte differentiation. HNF4α increases PXR-mediated CYP3A4, CYP2C8, and CYP2C9 transactivation.[47–50] Furthermore, PGC1α has been shown to increase the HNF4α-mediated transactivation of CYP7A1, PEPCK, and glucose-6-phosphatase genes.[27–29]

Interaction with PXR, HNF4α, SRC1, and PGC1α has been shown to enhance RIF-mediated CYP3A4 transactivation in HepG2 cells.[50] Martinez-Jiménez *et al.* demonstrated that in HepG2 cells, PGC1α and SRC1 expression levels were lower to allow maximal function of HNF4α. HNF4α was expressed at levels as high as in human liver but its activity on target genes (ApoCIII, ApoAV, PEPCK, AldoB, OTC, and CYP7A1) was very low or absent in HepG2 cells. They concluded that PGC1α and SRC1 are key coactivators for the proper function of HNF4α.[51] We found similar results in HepG2 cells, in that PGC1α and SRC1 expression levels were 20−25% of those in the human adult liver (**Fig. 4**). HNF4α mRNA was detected in both HepG2 and HFL cells, but its expression level was lower in HFL cells than in HepG2 cells (**Fig. 4**). PGC1α mRNA expression level in HFL cells was similar to that in HepG2 cells, but lower than that in the human adult liver (**Fig. 4**). Corepressor SMRT and NCoR1 mRNA expression levels in HFL cells were similar to those in HepG2 cells, but SHP was not detectable in HFL cells (**Fig. 4**). These results suggested that lower levels of HNF4α and PGC1α expression may impair RIF-mediated CYP3A4 induction under conditions where PXR is overexpressed in HFL cells. The expression levels of the HNF4α target genes ApoCIII and PEPCK are indirectly enhanced by PGC1α,[26,27,52,53] and ApoCIII and PEPCK mRNA expression levels were very low or below the limit of detection in HepG2 cells (**Fig. 5**). Similarly, neither ApoCIII nor PEPCK mRNA expression was detected in HFL cells (**Fig. 5**). These results suggested that low levels of HNF4α and PGC1α expression may impair HNF4α target gene expression in HFL cells.

The PXR promoter has been reported to contain the HNF4α binding site that is required for expression of PXR in fetal hepatocytes. Further, HNF4α regulates the expression of xenobiotic-related PXR target genes through control of PXR in fetal liver development.[49] These reports suggested

that HNF4α is important for the expression of PXR and PXR target genes.

The above results suggested that low levels of HNF4α and PGC1α expression may be responsible for PXR dysfunction as RIF-mediated CYP3As induction was insufficient in HFL cells compared with HepG2 cells.

**Acknowledgments:** We are grateful to Dr. Y. Yamazoe (Department of Environmental and Health Science, Tohoku Pharmaceutical University) and Dr. K. Nagata (Division of Drug Metabolism and Molecular Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University) for providing adenovirus expression vectors for *lacZ*, PXR, and CYP3A4-362-7.7k-Luc.

## References

1) Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C. and Nebert, D. W.: P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, 6: 1–42 (1996).
2) Thummel, K. E. and Wilkinson, G. R.: In vitro and in vivo drug interactions involving human CYP3A. *Annu. Rev. Pharmacol. Toxicol.*, 38: 389–430 (1998).
3) Domanski, T. L., Finta, C., Halpert, J. R. and Zaphiropoulos, P. G.: cDNA cloning and initial characterization of CYP3A43, a novel human cytochrome P450. *Mol. Pharmacol.*, 59: 386–392 (2001).
4) Shimada, T., Yamazaki, H., Mimura, M., Inui, Y. and Guengerich, F. P.: Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J. Pharmacol. Exp. Ther.*, 270: 414–423 (1994).
5) Li, A. P., Kaminski, D. L. and Rasmussen, A.: Substrates of human hepatic cytochrome P450 3A4. *Toxicology*, 104: 1–8 (1995).
6) Kitada, M.: Cytochrome P-450 in human fetal liver. Properties of P-450 HFLa, a form of cytochrome P-450 in human fetal livers. *Yakugaku Zasshi*, 111: 158–169 (1991).
7) Lu, C. and Li, A. P.: Species comparison in P450 induction: effects of dexamethasone, omeprazole, and rifampin on P450 isoforms 1A and 3A in primary cultured hepatocytes from man, Sprague-Dawley rat, minipig, and beagle dog. *Chem. Biol. Interact.*, 134: 271–281 (2001).
8) Vignati, L. A., Bogni, A., Grossi, P. and Monshouwer, M.: A human and mouse pregnane X receptor reporter gene assay in combination with cytotoxicity measurements as a tool to evaluate species-specific CYP3A induction. *Toxicology*, 199: 23–33 (2004).
9) Komoroski, B. J., Zhang, S., Cai, H., Hutzler, J. M., Frye, R., Tracy, T. S., Strom, S. C., Lehmann, T., Ang, C. Y., Cui, Y. Y. and Venkataramanan, R.: Induction and inhibition of cytochromes P450 by the St. John's wort constituent hyperforin in human hepatocyte cultures. *Drug Metab. Dispos.*, 32: 512–518 (2004).
10) Gibson, G. G., Plant, N. J., Swales, K. E., Ayrton, A. and El-Sankary, W.: Receptor-dependent transcriptional activation of cytochrome P4503A genes: induction mechanisms, species differences and interindividual variation in man. *Xenobiotica*, 32: 165–206 (2002).
11) Maruyama, M., Matsunaga, T., Harada, E. and Ohmori, S.: Comparison of Basal Gene Expression and Induction of CYP3As in HepG2 and Human Fetal Liver Cells. *Biol. Pharm. Bull.*, 30: 2091–2097 (2007).
12) Wang, H. and LeCluyse, E. L.: Role of orphan nuclear receptors in the regulation of drug-metabolising enzymes. *Clin. Pharmacokinet.*, 42: 1331–1357 (2003).
13) Li, T. and Chiang, J. Y.: Rifampicin induction of CYP3A4 requires pregnane X receptor crosstalk with hepatocyte nuclear factor 4alpha and coactivators, and suppression of small heterodimer partner gene expression. *Drug Metab. Dispos.*, 34: 756–764 (2006).
14) Usui, T., Saitoh, Y. and Komada, F.: Induction of CYP3As in HepG2 cells by several drugs. Association between induction of CYP3A4 and expression of glucocorticoid receptor. *Biol. Pharm. Bull.*, 26: 510–517 (2003).
15) Pascussi, J. M., Drocourt, L., Gerbal-Chaloin, S., Fabre, J. M., Maurel, P. and Vilarem, M. J.: Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes. Sequential role of glucocorticoid receptor and pregnane X receptor. *Eur. J. Biochem.*, 268: 6346–6358 (2001).
16) Bertilsson, G., Heidrich, J., Svensson, K., Asman, M., Jendeberg, L., Sydow-Backman, M., Ohlsson, R., Postlind, H., Blomquist, P. and Berkenstam, A.: Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc. Natl. Acad. Sci. USA*, 95: 12208–12213 (1998).
17) Blumberg, B., Sabbagh, W., Jr., Bolado, J., Jr., van Meter, C. M., Ong, E. S. and Evans, R. M.: SXR, a novel steroid and xenobiotic sensing nuclear receptor. *Genes Dev.*, 12: 3195–3205 (1998).
18) Lehmann, J. M., McKee, D. D., Watson, M. A., Wilson, T. M., Moore, J. T. and Kliewer, S. A.: The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J. Clin. Invest.*, 102: 1016–1023 (1998).
19) Kliewer, S. A., Moore, J. T., Wade, L., Staudinger, J. L., Watson, M. A., Jones, S. A., McKee, D. D., Oliver, B. B., Willson, T. M., Zetterstrom, R. H., Perlmann, T. and Lehmann, J. M.: An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell*, 92: 73–82 (1998).
20) Takada, T., Ogino, M., Miyata, M., Shimada, M., Nagata, K. and Yamazoe, Y.: Differences in transactivation between rat CYP3A1 and human CYP3A4 genes by human pregnane X receptor. *Drug Metab. Pharmacokinet.*, 19: 103–113 (2004).
21) Matsunaga, T., Maruyama, M., Harada, E., Katsuyama, Y., Sugihara, N., Ise, H., Negishi, N., Ikeda, U. and Ohmori, S.: Expression and induction of CYP3As in human fetal hepatocytes. *Biochem. Biophys. Res. Commun.*, 318: 428–434 (2004).
22) Sladek, F. M., Zhong, W. M., Lai, E. and Darnell, J. E., Jr.: Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev.*, 4: 2353–2365 (1990).
23) Li, J., Ning, G. and Duncan, S. A.: Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha. *Genes Dev.*, 14: 464–474 (2000).
24) Hayhurst, G. P., Lee, Y. H., Lambert, G., Ward, J. M. and Gonzalez, F. J.: Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol. Cell. Biol.*, 21: 1393–1403 (2001).
25) Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M. and Spiegelman, B. M.: A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell*, 92: 829–839 (1998).
26) Rhee, J., Inoue, Y., Yoon, J. C., Puigserver, P., Fan, M., Gonzalez, F. J. and Spiegelman, B. M.: Regulation of hepatic fasting response by PPARgamma coactivator-1alpha (PGC-1): requirement for hepatocyte nuclear factor 4alpha in gluconeogenesis. *Proc. Natl. Acad. Sci. USA*, 100: 4012–4017 (2003).
27) Yoon, J. C., Puigserver, P., Chen, G., Donovan, J., Wu, Z., Rhee, J., Adelmant, G., Stafford, J., Kahn, C. R., Granner, D. K., Newgard, C. B. and Spiegelman, B. M.: Control of hepatic

gluconeogenesis through the transcriptional coactivator PGC-1. *Nature*, 413: 131–138 (2001).
28) Herzog, B., Hall, R. K., Wang, X., Waltner-Law, M. and Granner, D. K.: Peroxisome proliferator-activated receptor gamma coactivator-1alpha, as a transcription amplifier, is not essential for basal and hormone-induced phosphoenolpyruvate carboxykinase gene expression. *Mol. Endocrinol.*, 18: 807–819 (2004).
29) Bhalla, S., Ozalp, C., Fang, S., Xiang, L. and Kemper, J. K.: Ligand-activated Pregnane X Receptor Interferes with HNF-4 Signaling by Targeting a Common Coactivator PGC-1$\alpha$. *J. Biol. Chem.*, 279: 45139–45147 (2004).
30) Ourlin, J. C., Lasserre, F., Pineau, T., Fabre, J. M., Sa-Cunha, A., Maurel, P., Vilarem, M. J. and Pascussi, J. M.: The small heterodimer partner interacts with the pregnane X receptor and represses its transcriptional activity. *Mol. Endocrinol.*, 17: 1693–1703 (2003).
31) Aden, D. P., Fogel, A., Plotkin, S., Damjanov, I. and Knowles, B. B.: Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature*, 282: 615–616 (1979).
32) Sumida, A., Fukuen, S., Yamamoto, I., Matsuda, H., Naohara, M. and Azuma, J.: Quantitative analysis of constitutive and inducible CYPs mRNA expression in the HepG2 cell line using reverse transcription-competitive PCR. *Biochem. Biophys. Res. Commun.*, 267: 756–760 (2000).
33) Sumida, A., Yamamoto, I., Zhou, Q., Morisaki, T. and Azuma, J.: Evaluation of induction of CYP3A mRNA using the HepG2 cell line and reverse transcription-PCR. *Biol. Pharm. Bull.*, 22: 61–65 (1999).
34) Schuetz, E. G., Schuetz, J. D., Strom, S. C., Thompson, M. T., Fisher, R. A., Molowa, D. T., Li, D. and Guzelian, P. S.: Regulation of human liver cytochromes P-450 in family 3A in primary and continuous culture of human hepatocytes. *Hepatology*, 18: 1254–1262 (1993).
35) Stevens, J. C., Hines, R. N., Gu, C., Koukouritaki, S. B., Manro, J. R., Tandler, P. J. and Zaya, M. J.: Developmental expression of the major human hepatic CYP3A enzymes. *J. Pharmacol. Exp. Ther.*, 307: 573–582 (2003).
36) Lehmann, J. M., McKee, D. D., Watson, M. A., Willson, T. M., Moore, J. T. and Kliewer, S. A.: The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J. Clin. Invest.*, 102: 1016–1023 (1998).
37) Pascussi, J. M., Drocourt, L., Fabre, J. M., Maurel, P. and Vilarem, M. J.: Dexamethasone induces pregnane X receptor and retinoid X receptor-alpha expression in human hepatocytes: synergistic increase of CYP3A4 induction by pregnane X receptor activators. *Mol. Pharmacol.*, 58: 361–372 (2000).
38) Pascussi, J. M., Gerbal-Chaloin, S., Fabre, J. M., Maurel, P. and Vilarem, M. J.: Dexamethasone enhances constitutive androstane receptor expression in human hepatocytes: consequences on cytochrome P450 gene regulation. *Mol. Pharmacol.*, 58: 1441–1450 (2000).
39) Zhang, W., Purchio, A., Chen, K., Burns, S. M., Contag, C. H. and Contag, P. R.: In vivo activation of the human CYP3A4 promoter in mouse liver and regulation by pregnane X receptors. *Biochem. Pharmacol.*, 65: 1889–1896 (2003).
40) Furukawa, M., Okubo, T., Ogino, M., Yamazaki, T., Shimada, M., Nagata, K. and Yamazoe, Y.: Adenovirus vector-mediated reporter system for in vivo analyses of human CYP3A4 gene activation. *J. Biochem.*, 131: 71–78 (2002).
41) Iordanidou, P., Aggelidou, E., Demetriades, C. and Hadzopoulou-Cladaras, M.: Distinct amino acid residues may be involved in coactivator and ligand interactions in hepatocyte nuclear factor-4alpha. *J. Biol. Chem.*, 280: 21810–21819 (2005).
42) Sladek, F. M., Ruse, M. D., Jr., Nepomuceno, L., Huang, S. M. and Stallcup, M. R.: Modulation of transcriptional activation and coactivator interaction by a splicing variation in the F domain of nuclear receptor hepatocyte nuclear factor 4alpha1. *Mol. Cell. Biol.*, 19: 6509–6522 (1999).
43) Wang, J. C., Stafford, J. M. and Granner, D. K.: SRC-1 and GRIP1 coactivate transcription with hepatocyte nuclear factor 4. *J. Biol. Chem.*, 273: 30847–30850 (1998).
44) Dell, H. and Hadzopoulou-Cladaras, M.: CREB-binding protein is a transcriptional coactivator for hepatocyte nuclear factor-4 and enhances apolipoprotein gene expression. *J. Biol. Chem.*, 274: 9013–9021 (1999).
45) Eeckhoute, J., Formstecher, P. and Laine, B.: Maturity-onset diabetes of the young Type 1 (MODY1)-associated mutations R154X and E276Q in hepatocyte nuclear factor 4alpha (HNF4alpha) gene impair recruitment of p300, a key transcriptional co-activator. *Mol. Endocrinol.*, 15: 1200–1210 (2001).
46) Johnson, D. R., Li, C. W., Chen, L. Y., Ghosh, J. C. and Chen, J. D.: Regulation and binding of pregnane X receptor by nuclear receptor corepressor silencing mediator of retinoid and thyroid hormone receptors (SMRT). *Mol. Pharmacol.*, 69: 99–108 (2006).
47) Chen, Y., Kissling, G., Negishi, M. and Goldstein, J. A.: The nuclear receptors constitutive androstane receptor and pregnane X receptor cross-talk with hepatic nuclear factor 4alpha to synergistically activate the human CYP2C9 promoter. *J. Pharmacol. Exp. Ther.*, 314: 1125–1133 (2005).
48) Ferguson, S. S., Chen, Y., LeCluyse, E. L., Negishi, M. and Goldstein, J. A.: Human CYP2C8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4alpha. *Mol. Pharmacol.*, 68: 747–757 (2005).
49) Kamiya, A., Inoue, Y. and Gonzalez, F. J.: Role of the hepatocyte nuclear factor 4alpha in control of the pregnane X receptor during fetal liver development. *Hepatology*, 37: 1375–1384 (2003).
50) Tirona, R. G., Lee, W., Leake, B. F., Lan, L. B., Cline, C. B., Lamba, V., Parviz, F., Duncan, S. A., Inoue, Y., Gonzalez, F. J., Schuetz, E. G. and Kim, R. B.: The orphan nuclear receptor HNF4alpha determines PXR- and constitutive androstane receptor-mediated xenobiotic induction of CYP3A4. *Nat. Med.*, 9: 220–224 (2003).
51) Martinez-Jiménez, C. P., Gomez-Lechon, M. J., Castell, J. V. and Jover, R.: Underexpressed coactivators PGC1alpha AND SRC1 impair hepatocyte nuclear factor 4alpha function and promote dedifferentiation in human hepatoma cells. *J. Biol. Chem.*, 281: 29840–29849 (2006).
52) Rhee, J., Ge, H., Yang, W., Fan, M., Handschin, C., Cooper, M., Lin, J., Li, C. and Spiegelman, B. M.: Partnership of PGC-1alpha and HNF4alpha in the regulation of lipoprotein metabolism. *J. Biol. Chem.*, 281: 14683–14690 (2006).
53) Prieur, X., Schaap, F. G., Coste, H. and Rodríguez, J. C.: Hepatocyte nuclear factor-4alpha regulates the human apolipoprotein AV gene: identification of a novel response element and involvement in the control by peroxisome proliferator-activated receptor-gamma coactivator-1alpha, AMP-activated protein kinase, and mitogen-activated protein kinase pathway. *Mol. Endocrinol.*, 19: 3107–3125 (2005).
54) Linja, M. J., Porkka, K. P., Kang, Z., Savinainen, K. J., Janne, O. A., Tammela, T. L., Vessella, R. L., Palvimo, J. J. and Visakorpi, T.: Expression of androgen receptor coregulators in prostate cancer. *Clin. Cancer Res.*, 10: 1032–1040 (2004).

Drug Metab. Pharmacokinet. 28 (1): 38–43 (2013). 

# Review

# Clinical Evidence of Pharmacokinetic Changes in Thalidomide Therapy

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

**Summary:** The teratogenic effects of thalidomide have been studied for more than 50 years. However, there have been few studies of the pharmacokinetic changes occurring during thalidomide therapy. Thalidomide was originally developed as a sedative. However, thalidomide induces multiple birth defects when used in pregnant women. Thalidomide is now used in the treatment of multiple myeloma (MM) and erythema nodosum leprosum (ENL) in Japan. Rational use of thalidomide is problematic due to a lack of basic research regarding its mechanism of action and serum concentration/effect relationships. There are a number of hypotheses for pharmacokinetic changes in thalidomide therapy. Genetic factors including single nucleotide polymorphisms (SNPs) that change cytochrome P450 (CYP) activity and epigenetic regulation that modifies CYP expression levels may contribute to the changes in pharmacokinetics and adverse drug reactions (ADRs) of thalidomide. Environmental factors include the pharmacological context of drug-drug interactions and the physiological context of liver diseases. Liver and kidney diseases do not play important roles in pharmacokinetic changes or ADRs in thalidomide therapy. To date, most research has focused on teratogenic activity, while the impact of polymorphisms in genes encoding drug metabolic enzymes and drug-drug interactions could mediate ADRs. Here, we discuss clinical evidence of pharmacokinetic changes in thalidomide therapy.

**Keywords: thalidomide; CYP2C19; CYP3A5; polymorphism; inter-individual variability**

## Introduction

Thalidomide was once used as a nonbarbiturate sedative-hypnotic with only low toxicity, but was withdrawn from the market because of its teratogenic side effects in the early 1960s.[1] Cereblon, a protein encoded by a candidate gene for mild mental retardation, was shown to be a primary target of thalidomide teratogenicity due to the inhibition of ubiquitin ligase activity.[2] Thalidomide has attracted renewed interest in recent years for use on a restricted basis in certain countries as a novel antineoplastic agent with immunomodulatory and antiangiogenic activities.[3–6] This drug is now commonly used in the treatment of multiple myeloma (MM).[7–9] Thalidomide has also been shown to have some activity against renal cell carcinoma, Kaposi's sarcoma, agnogenic myeloid metaplasia, Waldenström's macroglobulinemia, and myelodysplastic syndrome.[10,11] However, limited efficacy data are available in these conditions compared to myeloma, and its use in these settings is still investigational. Studies of its use in several other malignant and nonmalignant disorders are currently ongoing.[12] The incidence of toxicity is correlated with the dose of the drug. Patients receiving 200 mg or less seem to tolerate the treatment well with minimal side effects. Conversely, almost all patients taking thalidomide at doses of more than 400 mg/day experience drug-related toxicity.[12] Thalidomide is eliminated mainly through nonenzymatic hydrolysis and urinary excretion in humans, although animal studies have suggested that it may also be metabolized by the hepatic CYP enzyme system.[13–16] (*S*)-Thalidomide was reported to induce apoptosis.[17] However, (*S*)-thalidomide was reported to undergo rapid conversion into the racemate *in vitro*.[18,19] The transition of (*R*)- and (*S*)-thalidomide could influence the therapeutic effects. The increasing use of thalidomide raises the possibility of metabolic interactions with other prescription medications.[20] Clinically important interactions between thalidomide and coadministered therapeutic agents may

*Received August 17, 2012; Accepted October 26, 2012*
*J-STAGE Advance Published Date: November 20, 2012, doi:10.2133/dmpk.DMPK-12-RV-089*
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affect the activation or detoxification of thalidomide and other drugs, giving rise to attenuation or amplification of biological effects and/or toxicities. While metabolic studies have been performed in animals,[13] to date there have been only a few human studies. This review summarizes the clinical evidence of pharmacokinetic changes in thalidomide therapy.

## Metabolism

**Metabolism of thalidomide in humans:** Thalidomide is an optical isomer, and (*S*)-thalidomide was reported to induce apoptosis.[17] However, rapid conversion occurs from (*S*)-thalidomide into the racemate *in vitro*.[18,19] It has been suggested that the transition of (*R*)- and (*S*)-thalidomide in the blood influences the therapeutic effects. Although thalidomide is mainly eliminated through nonenzymatic hydrolysis and urinary excretion in humans, animal studies have suggested that it may also be metabolized by the hepatic CYPs.[13–15] Thalidomide was oxidized to 5-hydroxythalidomide and 5′-hydroxythalidomide by NADPH-fortified liver microsomes from humans as observed in monkeys, rats, mice, rabbits, and dogs (**Fig. 1**).[16] (*R*)-Thalidomide was hydroxylated more efficiently than (*S*)-thalidomide. Thalidomide has been reported to be metabolized by human recombinant CYP2C19, 3A4, and 3A5 *in vitro*.[21] CYP3A is known to be the main enzyme involved in the metabolism of many currently available medications. It is commonly accepted that CYP3A5 substrate specificity is similar to that of CYP3A4, although some differences in catalytic properties of thalidomide metabolism have been found.[21] Human CYP2C19, 3A4, and 3A5 mediate thalidomide 5-hydroxylation and further oxidation leading to a glutathione conjugate, which may be of relevance in the pharmacological actions of thalidomide (**Fig. 1**).[22] These metabolic pathways were confirmed by humanized TK-NOG mice, prepared by the introduction of thymidine kinase followed by induction with ganciclovir, and transplantation of human liver cells.[21] Experiments in these humanized mice could be expected to reveal the pharmacokinetic differences between humans and other animals. As thalidomide metabolites were not stable, degradation was avoided by rapid chilling and acidification of the samples. This property makes it more difficult to perform kinetic analysis of thalidomide. After incubation of thalidomide with the S9 fraction from human liver, formation of the 5-hydroxy and 5′-hydroxy metabolites was observed. The 5′-hydroxy metabolite was found in plasma samples from eight healthy male volunteers who had received thalidomide orally, but the concentrations were low.[23] Thalidomide does not undergo significant metabolism by human CYP and clinically important interactions between thalidomide and drugs that are also metabolized by this enzyme system are unlikely (Table 1). The major route of thalidomide breakdown in humans and animals is through spontaneous hydrolysis with subsequent elimination in the urine.[24] As both enzymatic metabolism and renal excretion play minor roles in the elimination of thalidomide, the risk of drug interactions seems to be low.[1]

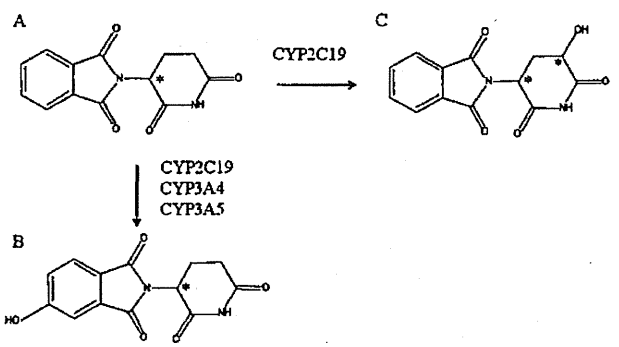


**Fig. 1. Structures of thalidomide and two hydroxylated metabolites**
These two hydroxylated metabolites are further oxidized leading to a glutathione conjugate. A, Thalidomide; B, 5-Hydroxy thalidomide; C, 5′-Hydroxy thalidomide.

## CYPs and Transporters

**Genetic polymorphisms and thalidomide pharmacokinetic changes:** CYP is one of the most important groups of enzymes

**Table 1. Summary of thalidomide-related pharmacokinetic changes from drug-drug interactions**

| Substrate and inhibitor | Effect | Mechanism of pharmacokinetic change |
|---|---|---|
| Drugs affecting the pharmacokinetics of thalidomide | | |
| Cyclophosphamide | inhibition | Cyclophosphamide increased the thalidomide t(1/2) and AUC in plasma and tumor tissue in mice[54] |
| CPT-11 | no change | CPT-11 did not significantly alter the pharmacokinetics of thalidomide[55] |
| | no change | Concurrent administration of CPT-11/thalidomide did not influence pharmacokinetics[56] |
| Thalidomide affecting the pharmacokinetics of other drugs | | |
| Cyclosporin A | enhancement | Cyclosporin metabolism was enhanced by thalidomide in CYP3A5 and in liver microsomes expressing CYP3A5 in the presence of thalidomide[21] |
| | enhancement | Cyclosporin A clearance was enhanced in CYP3A5 and liver microsomes expressing CYP3A5 by thalidomide[34] |
| CPT-11 and SN-38 | no change | Thalidomide inhibited CPT-11 metabolism, unlikely to be clinically significant[57,58] |
| | inhibition | Thalidomide increased the AUC of CPT-11[38,56] |
| | inhibition | Thalidomide decreased the AUC and t(1/2) of SN-38[38,56] |
| | inhibition | Thalidomide decreased metabolism of CPT-11 into SN-38[58] |
| Midazolam | inhibition | Midazolam 4-hydroxylation activities were suppressed by thalidomide[34] |
| | enhancement | 1′-Hydroxylation and total midazolam oxidation were enhanced in the presence of thalidomide[34] |
| | enhancement | Midazolam hydroxylation was enhanced by thalidomide in CYP3A5 and in liver microsomes expressing CYP3A5 in the presence of thalidomide[21] |
| (*S*)-Mephenytoin | inhibition | Thalidomide inhibited (*S*)-mephenytoin 4′-hydroxylation activities of recombinant CYP2C19 and human liver microsomes[34] |
| Testosterone | enhancement | Testosterone 6$\beta$-hydroxylation were enhanced in the presence of thalidomide[34] |
| DMXAA | inhibition | Thalidomide reduced clearance of DMXAA[59] |
| | no change | (*S*)-Thalidomide did not alter plasma DMXAA AUC in rats[60] |

in thalidomide metabolism. Many CYPs are polymorphic and catalytic alterations of allelic variant proteins can affect the metabolic activities of many drugs. The *CYP2C19*, *3A4*, and *3A5* genes, the gene products of which catalyze thalidomide hydroxylation, are particularly polymorphic. *In vitro* studies using cDNA expression systems are useful tools for evaluating functional alterations of the allelic variants of CYP, particularly for low-frequency alleles.[25–28] There was some influence of genetic polymorphism in *CYP2C19* on the blood concentration of thalidomide in MM, amyloid light chain amyloidosis, and polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes (POEMS) syndrome related to MM in Japanese patients.[29] It has been reported that decreased formation of thalidomide metabolites would be expected with defective alleles of *CYP2C19* compared to wild-type in clinical treatment with thalidomide plus dexamethasone.[30] Association studies of genetic variation and treatment effect may serve as predictive markers for the effects of treatment and can also uncover biological pathways behind drug effects. The SNPs have been studied in relation to high-dose treatment, thalidomide- and bortezomib-based therapy, maintenance treatment with interferon-$\alpha$, and therapy-related adverse effects caused by treatment. In thalidomide- and bortezomib-based therapy, candidate genes include tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and genes involved in the nuclear factor kappa B (NF$\kappa$B) pathway, respectively. In maintenance treatment with interferon-$\alpha$, a polymorphism in gene NF$\kappa$B1 is a candidate for prediction of efficacy. Adverse drug reactions (ADRs) include infection, osteonecrosis of the jaw, venous thrombotic events (VTE), and peripheral neuropathy (PN). A SNP in the *CYP2C8* gene was strongly associated with osteonecrosis of the jaw.[31] Several SNPs in genes encoding proteins involved in DNA repair, apoptosis, and inflammation as well as genes involved in nervous system function are associated with VTE induced by thalidomide and PN induced by bortezomib. Further studies of SNPs in clinical trials are needed.[31]

**Relation of transporters to the pharmacokinetic changes of thalidomide:** Since studies in patients have indicated that the oral absorption of thalidomide is considerably variable at high doses, the contributions of transporters to the pharmacokinetics/pharmacodynamics of thalidomide were examined by Zhou *et al.*[32] and Zimmermann *et al.*[33] using human colon cancer cell lines, which have been widely used to investigate drug permeability. Thalidomide did not induce P-glycoprotein (P-gp) expression in LS180 cells. The uptake of rhodamine 123 in CCRF cells overexpressing P-gp was not influenced by coincubation with thalidomide. Transport through Caco-2 monolayers was linear and the permeability was similar in both directions. There were no differences between the thalidomide enantiomers. From this study, thalidomide was concluded to be neither a substrate nor an inhibitor or inducer of P-gp, and P-gp-related drug-drug interactions with thalidomide are unlikely.[33] Zhou *et al.* reported that the uptake of thalidomide by Caco-2 cells was very limited (up to 2.1%).[32] The transport of thalidomide appeared to be linear up to 1 h. The permeability coefficients (Papp) of thalidomide at 2.5–300 μM from apical (AP) to basolateral (BL) and from BL to AP were $2 \times 10^{-5}$–$6 \times 10^{-5}$ cm/s, with a marked decrease in Papp values from AP to BL at increased thalidomide concentration. The transport of thalidomide was dependent on sodium, temperature, and pH, as replacement of extracellular sodium chloride or reducing temperature and apical pH resulted in significant decreases in the Papp values. Additional data indicated that transport of thalidomide is energy-dependent, as it was significantly ($p < 0.05$) inhibited by the ATP inhibitors sodium azide and 2,4-dinitrophenol. In addition, DL-glutamic acid, cytidine, dipyridamole, papaverine, quinidine, and cyclophosphamide significantly ($p < 0.05$) inhibited the transport of thalidomide, while the P-gp inhibitor verapamil and other nucleosides and nucleotides, such as thymidine and guanine, had no effect. These results suggested that thalidomide may be rapidly transported by a saturable energy-dependent transporter in Caco-2 monolayers.[32]

**Drug-drug interaction of thalidomide:** The increasing use of thalidomide raises the possibility of metabolic interactions with other prescription medications. Clinically important interactions between thalidomide and coadministered therapeutics may affect the activation or detoxification of thalidomide and other drugs giving rise to attenuation or amplification of biological effects and/or toxicities. While metabolic studies have been performed in animals,[13] to date there have been only a few human studies (**Table** 1). The possible drug interactions that could be mediated by thalidomide were investigated in human liver microsomes. (*S*)-Mephenytoin 4′-hydroxylation activity was inhibited by thalidomide in recombinant CYP2C19 and human liver microsomes with apparent $IC_{50}$ of approximately 270 μM for CYP2C19.[34] Interestingly, midazolam 4-hydroxylation activity was suppressed by the presence of thalidomide, but 1′-hydroxylation activities, total midazolam oxidation activity, and testosterone 6$\beta$-hydroxylation activities were enhanced in the presence of thalidomide. Recombinant CYP3A5 altered kinetics at clinical concentrations of thalidomide. CYP3A4 was affected only at higher thalidomide concentrations. Enhancement of midazolam hydroxylation by thalidomide was seen in liver microsomes from *CYP3A5*1* subjects. Cyclosporin A clearance was similarly enhanced by thalidomide in recombinant CYP3A5 and liver microsomes expressing CYP3A5. Close interaction between thalidomide and the heme of CYP3A5 was observed in docking studies. As total midazolam metabolism or cyclosporin A clearance may be increased by thalidomide in a dose-dependent manner, unexpected drug interactions could occur *via* heterotropic cooperativity of CYP3A5.[34] Both (*S*)-thalidomide and diclofenac increased the plasma DMXAA AUC in mice. In the case of diclofenac, this may be due to direct competitive inhibition of DMXAA metabolism, but this mechanism is not always applied to (*S*)-thalidomide. The *in vivo* predictive model is inappropriate for the (*S*)-thalidomide-DMXAA interactions when based on direct inhibition of metabolism in mice and humans.[35] Thalidomide tends not to affect the pharmacokinetics of orally administered hormonal contraceptives.[36] In contrast, conversion of CPT-11 into the active metabolite SN-38 was significantly inhibited by thalidomide. The possibility of an interaction of thalidomide with CPT-11 metabolism may explain the previously described improvement in tolerability of CPT-11 therapies.[37]

**Factors contributing to adverse effects of thalidomide:** Given the increasing clinical use of thalidomide, it is becoming important to study the adverse effects of this drug. In addition to teratogenicity, thalidomide has certain unique and frequent toxicities, such as constipation, sedation, fatigue, and neuropathy. Matsuzawa *et al.*[29] evaluated the genotype, age, sex, diagnosis, and concurrent CYP2C19 substrate medications as factors important for the adverse effects of thalidomide. Age and sex did not show clear effects on thalidomide dose in the Japanese population. In patients taking concurrent drugs, thalidomide dosages were not reduced

in patients taking CYP2C19 substrates. *CYP2C19* genetic polymorphism may be one of the factors underlying the individual differences in thalidomide toxicity. The mean (*R*)-thalidomide AUC was 35.9% lower in the *CYP2C19*1/*3* and **1/*2* groups than in the *CYP2C9*2/*2* group, and the mean (*S*)-thalidomide AUC was 33.5% lower in the *CYP2C19*1/*3* and **1/*2* groups than the *CYP2C9*2/*2* group. All patients developed adverse reactions to thalidomide. Major adverse drug effects were constipation, somnolence, and peripheral neuropathy. Only one patient with the *CYP2C19*2/*2* genotype taking thalidomide developed dyspnea as a side effect, but it improved following the termination of thalidomide.[29] Peripheral neuropathy is a common side effect of thalidomide and often calls for the cessation of therapy when the symptoms are severe.[12] The thalidomide-related peripheral neuropathy associated with ABCA1 (rs363717), ICAM1 (rs1799969), PPARD (rs2076169), SERPINB2 (rs6103), and SLC12A6 (rs7164902) SNPs and an individual's risk of developing peripheral neuropathy after thalidomide treatment can be mediated by polymorphisms in genes governing repair mechanisms and inflammation in the peripheral nervous system.[38] No association was observed between the number of functional *CYP2C19* and *CYP2D6* alleles and outcome in a population of 166 MM patients treated with thalidomide.[39] There were also no associations between the numbers of functional *CYP2C19* and *CYP2D6* alleles and neurological adverse reactions to thalidomide. Further studies in larger numbers of patients may be needed to determine the roles of polymorphic *CYP* alleles in treatment outcome.[39] VTE with the subsequent risk of pulmonary embolism (PE) is a major concern in the treatment of MM patients with thalidomide. Deep venous thrombosis (DVT) and/or PE occurs in only about 1–3% of patients receiving single-agent thalidomide for myeloma.[40-46] The risk of thalidomide-induced thrombosis is highest in newly diagnosed patients when the drug is given in combination with dexamethasone, doxorubicin, or other chemotherapeutic drugs. The risk is elevated in the elderly and in patients with an underlying inherited or acquired thrombotic predisposition. The use of routine prophylactic warfarin or low molecular weight heparin or aspirin for all patients receiving thalidomide in combination with dexamethasone is currently under consideration.[12] The susceptibility to the development of VTE in response to thalidomide therapy is likely to be influenced by both genetic and environmental factors. SNPs associated with thalidomide-related VTE were enriched in genes and pathways important in drug transport/metabolism, DNA repair, and cytokine balance.[46]

## Other Factors

**Effects of liver or kidney dysfunction:** The absorption and elimination of thalidomide are not significantly different in patients with hepatic dysfunction.[47] Thalidomide is mainly hydrolyzed and passively excreted, and its pharmacokinetics are not expected to be altered in patients with impaired liver or kidney function.[48] The inter- and intra-patient variability in liver or kidney dysfunction was low. There was no correlation between thalidomide clearance and renal function. Although clearance during dialysis is doubled, thalidomide dose need not be changed for patients with decreased kidney function. There is also no need for a supplementary dose due to hemodialysis.[49] The serum concentration of thalidomide in MM patients with renal insufficiency was investigated in Japanese patients.[50] The serum concentration of thalidomide 12 and 16 h after administration in patients with MM on hemodialysis (HD) taking 100–200 mg/day were similar with or without HD. The thalidomide concentration was not significantly increased by renal insufficiency. In this study, there is no correlation between the concentration of thalidomide and its clinical effect. In Japanese patients, the thalidomide dosage need not be modified for renal insufficiency or HD.[50]

**Effects of food on the pharmacokinetics of thalidomide:** Although food often delays and/or decreases drug absorption, the absorption of a few drugs is increased by food. The effects of food on the oral pharmacokinetics of thalidomide and the relative bioavailability of two oral thalidomide formulations were determined by Teo *et al.*[24] Five male and eight female healthy volunteers received a single oral dose of 200 mg thalidomide in capsule form under fasting and non-fasting conditions. A high-fat breakfast delayed the onset of absorption of thalidomide by 0.5–1.5 h.

**Gender difference on the pharmacokinetics of thalidomide:** Although there were no statistically significant differences in any of the pharmacokinetic parameters of thalidomide pharmacokinetics between men and women, tendencies toward gender differences in the pharmacokinetics of thalidomide were observed for some of the parameters.[24] For example, women tended to have slightly larger $C_{max}$ and AUC values than men. These differences can be explained by the greater body weight of the male subjects. The mean half-life and mean residence time were also slightly larger for females than for males.[24]

**Differences in pharmacokinetic parameters among formulations:** Terminal half-life showed two- to three-fold differences among tested formulations and is clear evidence for absorption rate limitations.[51] Fujita *et al.* compared the dissolution profile and plasma thalidomide concentrations of Japanese and British capsules and Mexican tablets. The dissolution rate of the Japanese capsule was the fastest, followed by the British and Mexican formulations. The pharmacokinetic profiles of the Japanese and British capsules were similar, while the 100 mg Japanese thalidomide capsule showed a 1.6-fold higher maximum plasma concentration than the 200 mg Mexican thalidomide tablet, greatly shortened $T_{max}$, and an apparent half life that was only one-third that of the Mexican tablet. Thus, pharmacokinetic changes may occur in plasma thalidomide concentration when switching between different formulations.[52]

## Conclusions

In conclusion, this review provided insights into the contribution of gene variations, drug-drug interactions, liver and kidney dysfunction, and thalidomide formulations to pharmacokinetic changes of thalidomide (**Table 2**). *CYP2C19* poor metabolizer (PM) patients

**Table 2. Effects of various factors on thalidomide pharmacokinetics in humans**

| Factors | Thalidomide pharmacokinetics | References |
|---|---|---|
| Gender | No significant difference (slightly higher $C_{max}$ and AUC in females) | 24) |
| Age | No significant difference | 29) |
| Renal function | No significant difference | 48–50) |
| Hepatic function | No significant difference | 47) |
| Food | High-fat meal delayed the absorption of thalidomide | 24) |
| Transporter polymorphisms | Unreported (*in vivo* data) | |
| CYP polymorphisms | No significant difference (larger AUC in *CYP2C9*2/*2* genotype) | 29) |
| Formulations | Pharmacokinetic changes occur when switching to other formulations | 51, 52) |

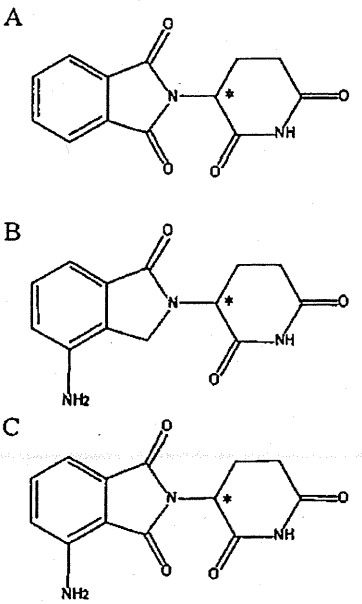


**Fig. 2. Structures of IMiDs**
A, Thalidomide; B, Lenalidomide; C, Pomalidomide.

tend to have high serum thalidomide concentrations and high risks of adverse drug effects, such as constipation, somnolence, and peripheral neuropathy. Further studies to clarify the mechanisms underlying the pharmacokinetic changes of thalidomide are required. Several next-generation antiepileptic drugs with improved tolerability profiles and reduced potential for drug interactions have been added to the therapeutic armamentarium. Thalidomide analogs termed immunomodulatory drugs (IMiDs, **Fig. 2**) have been developed that are more effective and have less toxicity than thalidomide. Thalidomide and its co-stimulatory IMiD analogs are currently being assessed in patients with advanced myeloma and some solid tumors, with promising effects.[9] However, despite the promising effects of thalidomide on a broad range of serious diseases, further careful studies on the pharmacological and pharmacodynamic properties of thalidomide are necessary.[12] Although thalidomide and IMiDs show similar biological activities, IMiDs are more potent than thalidomide and achieve responses at lower doses. Lenalidomide, a thalidomide derivative, has also been shown to have a different toxicity profile.[53] Pharmacokinetic and clinical interactions between lenalidomide and other drugs seemed to occur, as *in vitro* data indicated that lenalidomide is a P-gp substrate.[53]

Overall, these advances in studies of thalidomide pharmacokinetics have expanded the opportunities for individualization of drug therapy with antiepileptic drugs, to enhance effectiveness and minimize the risk of ADRs.

## References


1) Eriksson, T., Björkman, S. and Höglund, P.: Clinical pharmacology of thalidomide. *Eur. J. Clin. Pharmacol.*, **57**: 365–376 (2001).
2) Ito, T., Ando, H., Suzuki, T., Ogura, T., Hotta, K., Imamura, Y., Yamaguchi, Y. and Handa, H.: Identification of a primary target of thalidomide teratogenicity. *Science*, **327**: 1345–1350 (2010).
3) Juliusson, G., Celsing, F., Turesson, I., Lenhoff, S., Adriansson, M. and Malm, C.: Frequent good partial remissions from thalidomide including best response ever in patients with advanced refractory and relapsed myeloma. *Br. J. Haematol.*, **109**: 89–96 (2000).
4) Calabrese, L. and Resztak, K.: Thalidomide revisited: pharmacology and clinical applications. *Expert Opin. Investig. Drugs*, **7**: 2043–2060 (1998).
5) Seldin, D. C., Choufani, E. B., Dember, L. M., Wiesman, J. F., Berk, J. L., Falk, R. H., O'Hara, C., Fennessey, S., Finn, K. T., Wright, D. G., Skinner, M. and Sanchorawala, V.: Tolerability and efficacy of thalidomide for the treatment of patients with light chain-associated (AL) amyloidosis. *Clin. Lymphoma*, **3**: 241–246 (2003).
6) Palladini, G., Perfetti, V., Perlini, S., Obici, L., Lavatelli, F., Caccialanza, R., Invernizzi, R., Comotti, B. and Merlini, G.: The combination of thalidomide and intermediate-dose dexamethasone is an effective but toxic treatment for patients with primary amyloidosis (AL). *Blood*, **105**: 2949–2951 (2005).
7) Rajkumar, S. V.: Thalidomide in the treatment of multiple myeloma. *Expert Rev. Anticancer Ther.*, **1**: 20–28 (2001).
8) Kyle, R. A. and Rajkumar, S. V.: Therapeutic application of thalidomide in multiple myeloma. *Semin. Oncol.*, **28**: 583–587 (2001).
9) Kyle, R. A.: Current therapy of multiple myeloma. *Intern. Med.*, **41**: 175–180 (2002).
10) Cavenagh, J. D. and Oakervee, H.: UK Myeloma Forum and the BCSH Haematology/Oncology Task Forces. Thalidomide in multiple myeloma: current status and future prospects. *Br. J. Haematol.*, **120**: 18–26 (2003).
11) Bertolini, F., Mingrone, W., Alietti, A., Ferrucci, P. F., Cocorocchio, E., Peccatori, F., Cinieri, S., Mancuso, P., Corsini, C., Burlini, A., Zucca, E. and Martinelli, G.: Thalidomide in multiple myeloma, myelodysplastic syndromes and histiocytosis: analysis of clinical results and of surrogate angiogenesis markers. *Ann. Oncol.*, **12**: 987–990 (2001).
12) Ghobrial, I. M. and Rajkumar, S. V.: Management of thalidomide toxicity. *J. Support. Oncol.*, **1**: 194–205 (2003).
13) Meyring, M., Mühlbacher, J., Messer, K., Kastner-Pustet, N., Bringmann, G., Mannschreck, A. and Blaschke, G.: In vitro biotransformation of (R)- and (S)-thalidomide: application of circular dichroism spectroscopy to the stereochemical characterization of the hydroxylated metabolites. *Anal. Chem.*, **74**: 3726–3735 (2002).
14) Teo, S. K., Sabourin, P. J., O'Brien, K., Kook, K. A. and Thomas, S. D.: Metabolism of thalidomide in human microsomes, cloned human cytochrome P-450 isozymes, and Hansen's disease patients. *J. Biochem. Mol. Toxicol.*, **14**: 140–147 (2000).
15) Ando, Y., Price, D. K., Dahut, W. L., Cox, M. C., Reed, E. and Figg, W. D.: Pharmacogenetic associations of CYP2C19 genotype with in vivo metabolisms and pharmacological effects of thalidomide. *Cancer Biol. Ther.*, **1**: 669–673 (2002).
16) Ando, Y., Fuse, E. and Figg, W. D.: Thalidomide metabolism by the CYP2C subfamily. *Clin. Cancer Res.*, **8**: 1964–1973 (2002).
17) Liu, W. M., Strauss, S. J., Chaplin, T., Shahin, S., Propper, D. J., Young, B. D., Joel, S. P. and Malpas, J. S.: S-thalidomide has a greater effect on apoptosis than angiogenesis in a multiple myeloma cell line. *Hematol. J.*, **5**: 247–254 (2004).
18) Knoche, B. and Blaschke, G.: Investigations on the *in vitro* racemization of thalidomide by high-performance liquid chromatography. *J. Chromatogr. A*, **666**: 235–240 (1994).
19) Nishimura, K., Hashimoto, Y. and Iwasaki, S.: (S)-form of alpha-methyl-N(alpha)-phthalimidoglutarimide, but not its (R)-form, enhanced phorbol ester-induced tumor necrosis factor-alpha production by human leukemia cell HL-60: implication of optical resolution of thalidomidal effects. *Chem. Pharm. Bull. (Tokyo)*, **42**: 1157–1159 (1994).
20) Furuta, T., Ohashi, K., Kobayashi, K., Iida, I., Yoshida, H., Shirai, N., Takashima, M., Kosuge, K., Hanai, H., Chiba, K., Ishizaki, T. and Kaneko, E.: Effects of clarithromycin on the metabolism of omeprazole in relation to CYP2C19 genotype status in humans. *Clin. Pharmacol. Ther.*, **66**: 265–274 (1999).
21) Yamazaki, H., Nakamoto, M., Shimizu, M., Murayama, N. and Niwa, T.: Potential impact of cytochrome P450 3A5 in human liver on drug interactions with triazoles. *Br. J. Clin. Pharmacol.*, **69**: 593–597 (2010).
22) Chowdhury, G., Murayama, N., Okada, Y., Uno, Y., Shimizu, M., Shibata, N., Guengerich, F. P. and Yamazaki, H.: Human liver microsomal cytochrome P450 3A enzymes involved in thalidomide 5-hydroxylation and formation of a glutathione conjugate. *Chem. Res. Toxicol.*, **23**: 1018–1024 (2010).
23) Eriksson, T., Björkman, S., Roth, B. and Höglund, P.: Intravenous formulations of the enantiomers of thalidomide: pharmacokinetic and initial pharmacodynamic characterization in man. *J. Pharm. Pharmacol.*, **52**: 807–817 (2000).
24) Teo, S. K., Scheffler, M. R., Kook, K. A., Tracewell, W. G., Colburn, W. A., Stirling, D. I. and Thomas, S. D.: Effect of a high-fat meal on thalidomide pharmacokinetics and the relative bioavailability of oral formulations in healthy men and women. *Biopharm. Drug Dispos.*, **21**:

33–40 (2000).
25) Hiratsuka, M.: In vitro assessment of the allelic variants of cytochrome P450. *Drug Metab. Pharmacokinet.*, **27**: 68–84 (2012).
26) Miyazaki, M., Nakamura, K., Fujita, Y., Guengerich, F. P., Horiuchi, R. and Yamamoto, K.: Defective activity of recombinant cytochromes P450 3A4.2 and 3A4.16 in oxidation of midazolam, nifedipine, and testosterone. *Drug Metab. Dispos.*, **36**: 2287–2291 (2008).
27) Parikh, A., Gillam, E. M. and Guengerich, F. P.: Drug metabolism by Escherichia coli expressing human cytochromes P450. *Nat. Biotechnol.*, **15**: 784–788 (1997).
28) Iwata, H., Fujita, K., Kushida, H., Suzuki, A., Konno, Y., Nakamura, K., Fujino, A. and Kamataki, T.: High catalytic activity of human cytochrome P450 co-expressed with human NADPH-cytochrome P450 reductase in Escherichia coli. *Biochem. Pharmacol.*, **55**: 1315–1325 (1998).
29) Matsuzawa, N., Nakamura, K., Matsuda, M., Ishida, F. and Ohmori, S.: Influence of cytochrome P450 2C19 gene variations on pharmacokinetic parameters of thalidomide in Japanese patients. *Biol. Pharm. Bull.*, **35**: 317–320 (2012).
30) Li, Y. H. and Hou, J.: Effect of *CYP2C19* gene polymorphism on efficacy of thalidomide-based regimens for the treatment of multiple myeloma. *Zhonghua Xue Ye Xue Za Zhi*, **28**: 651–654 (2007).
31) Vangsted, A., Klausen, T. W. and Vogel, U.: Genetic variations in multiple myeloma II: association with effect of treatment. *Eur. J. Haematol.*, **88**: 93–117 (2012).
32) Zhou, S., Li, Y., Kestell, P., Schafer, P., Chan, E. and Paxton, J. W.: Transport of thalidomide by the human intestinal caco-2 monolayers. *Eur. J. Drug Metab. Pharmacokinet.*, **30**: 49–61 (2005).
33) Zimmermann, C., Gutmann, H. and Drewe, J.: Thalidomide does not interact with P-glycoprotein. *Cancer Chemother. Pharmacol.*, **57**: 599–606 (2006).
34) Okada, Y., Murayama, N., Yanagida, C., Shimizu, M., Guengerich, F. P. and Yamazaki, H.: Drug interactions of thalidomide with midazolam and cyclosporine A: heterotropic cooperativity of human cytochrome P450 3A5. *Drug Metab. Dispos.*, **37**: 18–23 (2009).
35) Zhou, S., Kestell, P., Tingle, M. D., Ching, L. M. and Paxton, J. W.: In vitro and in vivo kinetic interactions of the antitumour agent 5,6-dimethylxanthenone-4-acetic acid with thalidomide and diclofenac. *Cancer Chemother. Pharmacol.*, **47**: 319–326 (2001).
36) Scheffler, M. R., Colburn, W., Kook, K. A. and Thomas, S. D.: Thalidomide does not alter estrogen-progesterone hormone single dose pharmacokinetics. *Clin. Pharmacol. Ther.*, **65**: 483–490 (1999).
37) Allegrini, G., Di Paolo, A., Cerri, E., Cupini, S., Amatori, F., Masi, G., Danesi, R., Marcucci, L., Bocci, G., Del Tacca, M. and Falcone, A.: Irinotecan in combination with thalidomide in patients with advanced solid tumors: a clinical study with pharmacodynamic and pharmacokinetic evaluation. *Cancer Chemother. Pharmacol.*, **58**: 585–593 (2006).
38) Johnson, D. C., Corthals, S. L., Walker, B. A., Ross, F. M., Gregory, W. M., Dickens, N. J., Lokhorst, H. M. and Goldschmidt, H.: Genetic factors underlying the risk of thalidomide-related neuropathy in patients with multiple myeloma. *J. Clin. Oncol.*, **29**: 797–804 (2011).
39) Vangsted, A. J., Soeby, K., Klausen, T. W., Abildgaard, N., Andersen, N. F., Gimsing, P., Gregersen, H., Vogel, U., Werge, T. and Rasmussen, H. B.: No influence of the polymorphisms CYP2C19 and CYP2D6 on the efficacy of cyclophosphamide, thalidomide, and bortezomib in patients with Multiple Myeloma. *BMC Cancer*, **10**: 404–411 (2010).
40) Rajkumar, S. V., Hayman, S., Gertz, M. A., Dispenzieri, A., Lacy, M. Q., Greipp, P. R., Geyer, S., Iturria, N., Fonseca, R., Lust, J. A., Kyle, R. A. and Witzig, T. E.: Combination therapy with thalidomide plus dexamethasone for newly diagnosed myeloma. *J. Clin. Oncol.*, **20**: 4319–4323 (2002).
41) Barlogie, B., Desikan, R., Eddlemon, P., Spencer, T., Zeldis, J., Munshi, N., Badros, A., Zangari, M., Anaissie, E., Epstein, J., Shaughnessy, J., Ayers, D., Spoon, D. and Tricot, G.: Extended survival in advanced and refractory multiple myeloma after single-agent thalidomide: identification of prognostic factors in a phase II study of 169 patients. *Blood*, **98**: 492–494 (2001).
42) Osman, K., Comenzo, R. and Rajkumar, S. V.: Deep venous thrombosis and thalidomide therapy for multiple myeloma. *N. Engl. J. Med.*, **344**: 1951–1952 (2001).
43) Zangari, M., Anaissie, E., Barlogie, B., Badros, A., Desikan, R., Gopal, A. V., Morris, C., Toor, A., Siegel, E., Fink, L. and Tricot, G.: Increased risk of deep-vein thrombosis in patients with multiple myeloma receiving thalidomide and chemotherapy. *Blood*, **98**: 1614–1615 (2001).
44) Bowcock, S. J., Rassam, S. M., Ward, S. M., Turner, J. T. and Laffan, M.: Thromboembolism in patients on thalidomide for myeloma. *Hematology*, **7**: 51–53 (2002).
45) Camba, L., Peccatori, J., Pescarollo, A., Tresoldi, M., Corradini, P. and Bregni, M.: Thalidomide and thrombosis in patients with multiple myeloma. *Haematologica*, **86**: 1108–1109 (2001).
46) Johnson, D. C., Corthals, S., Ramos, C., Hoering, A., Cocks, K., Dickens, N. J., Haessler, J., Goldschmidt, H., Child, J. A., Bell, S. E., Jackson, G., Baris, D., Rajkumar, S. V., Davies, F. E., Durie, B. G., Crowley, J., Sonneveld, P., Van Ness, B. and Morgan, G. J.: Genetic associations with thalidomide mediated venous thrombotic events in myeloma identified using targeted genotyping. *Blood*, **112**: 4924–4934 (2008).
47) Shiah, H. S., Chao, Y., Chen, L. T., Yao, T. J., Huang, J. D., Chang, J. Y., Chen, P. J., Chuang, T. R. and Chin, Y. H.: Phase I and pharmacokinetic study of oral thalidomide in patients with advanced hepatocellular carcinoma. *Cancer Chemother. Pharmacol.*, **58**: 654–664 (2006).
48) Teo, S. K., Colburn, W. A., Tracewell, W. G., Kook, K. A., Stirling, D. I., Jaworsky, M. S., Scheffler, M. A., Thomas, S. D. and Laskin, O. L.: Clinical pharmacokinetics of thalidomide. *Clin. Pharmacokinet.*, **43**: 311–327 (2004).
49) Eriksson, T., Höglund, P., Turesson, I., Waage, A., Don, B. R., Vu, J., Scheffler, M. and Kaysen, G. A.: Pharmacokinetics of thalidomide in patients with impaired renal function and while on and off dialysis. *J. Pharm. Pharmacol.*, **55**: 1701–1706 (2003).
50) Arai, A., Hirota, A., Fukuda, T., Tohda, S., Mori, Y., Terada, Y., Sasaki, S. and Miura, O.: Analysis of plasma concentration of thalidomide in Japanese patients of multiple myeloma with renal dysfunction. *Rinsho Ketsueki*, **50**: 295–299 (2009).
51) Teo, S. K., Colburn, W. A. and Thomas, S. D.: Single-dose oral pharmacokinetics of three formulations of thalidomide in healthy male volunteers. *J. Clin. Pharmacol.*, **39**: 1162–1168 (1999).
52) Fujita, Y., Yamamoto, K., Aomori, T., Murakami, H. and Horiuchi, R.: Comparison of dissolution profile and plasma concentration-time profile of the thalidomide formulations made by Japanese, Mexican and British companies. *Yakugaku Zasshi*, **128**: 1449–1457 (2008).
53) Hofmeister, C. C., Yang, X., Pichiorri, F., Chen, P., Rozewski, D. M., Johnson, A. J., Lee, S., Liu, Z., Garr, C. L., Hade, E. M., Ji, J., Schaaf, L. J., Benson, D. M., Jr., Kraut, E. H., Hicks, W. J., Chan, K. K., Chen, C. S., Farag, S. S., Grever, M. R., Byrd, J. C. and Phelps, M. A.: Phase I trial of lenalidomide and CCI-779 in patients with relapsed multiple myeloma: evidence for lenalidomide-CCI-779 interaction via P-glycoprotein. *J. Clin. Oncol.*, **29**: 3427–3434 (2011).
54) Chung, F., Wang, L. C., Kestell, P., Baguley, B. C. and Ching, L. M.: Modulation of thalidomide pharmacokinetics by cyclophosphamide or 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in mice: the role of tumour necrosis factor. *Cancer Chemother. Pharmacol.*, **53**: 377–383 (2004).
55) Yang, X. X., Hu, Z. P., Chan, S. Y., Duan, W., Ho, P. C., Boelsterli, U. A., Ng, K. Y., Chan, E., Bian, J. S., Chen, Y. Z., Huang, M. and Zhou, S. F.: Pharmacokinetic mechanisms for reduced toxicity of irinotecan by coadministered thalidomide. *Curr. Drug Metab.*, **7**: 431–455 (2006).
56) Villalona-Calero, M., Schaaf, L., Phillips, G., Otterson, G., Panico, K., Duan, W., Kleiber, B., Shah, M., Young, D., Wu, W. H. and Kuhn, J.: Thalidomide and celecoxib as potential modulators of irinotecan's activity in cancer patients. *Cancer Chemother. Pharmacol.*, **59**: 23–33 (2007).
57) Ramírez, J., Wu, K., Janisch, L., Karrison, T., House, L. K., Innocenti, F., Cohen, E. E. and Ratain, M. J.: The effect of thalidomide on the pharmacokinetics of irinotecan and metabolites in advanced solid tumor patients. *Cancer Chemother. Pharmacol.*, **68**: 1629–1632 (2011).
58) Allegrini, G., Di Paolo, A., Cerri, E., Cupini, S., Amatori, F., Masi, G., Danesi, R., Marcucci, L., Bocci, G., Del Tacca, M. and Falcone, A.: Irinotecan in combination with thalidomide in patients with advanced solid tumors: a clinical study with pharmacodynamic and pharmacokinetic evaluation. *Cancer Chemother. Pharmacol.*, **58**: 585–593 (2006).
59) Chung, F., Liu, J., Ching, L. M. and Baguley, B. C.: Consequences of increased vascular permeability induced by treatment of mice with 5,6-dimethylxanthenone-4-acetic acid (DMXAA) and thalidomide. *Cancer Chemother. Pharmacol.*, **61**: 497–502 (2008).
60) Zhou, S., Kestell, P., Tingle, M. D., Ching, L. M. and Paxton, J. W.: A difference between the rat and mouse in the pharmacokinetic interaction of 5,6-dimethylxanthenone-4-acetic acid with thalidomide. *Cancer Chemother. Pharmacol.*, **47**: 541–544 (2001).

# Differentiation of Monkey Embryonic Stem Cells to Hepatocytes by Feeder-Free Dispersion Culture and Expression Analyses of Cytochrome P450 Enzymes Responsible for Drug Metabolism

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Received October 3, 2012; accepted November 15, 2012; advance publication released online December 8, 2012

**We reported previously that monkey embryonic stem cells (ESCs) were differentiated into hepatocytes by formation of embryoid bodies (EBs). However, this EB formation method is not always efficient for assays using a large number of samples simultaneously. A dispersion culture system, one of the differentiation methods without EB formation, is able to more efficiently provide a large number of feeder-free undifferentiated cells. A previous study demonstrated the effectiveness of the Rho-associated kinase inhibitor Y-27632 for feeder-free dispersion culture and induction of differentiation of monkey ESCs into neural cells. In the present study, the induction of differentiation of cynomolgus monkey ESCs (cmESCs) into hepatocytes was performed by the dispersion culture method, and the expression and drug inducibility of cytochrome P450 (CYP) enzymes in these hepatocytes were examined. The cmESCs were successfully differentiated into hepatocytes under feeder-free dispersion culture conditions supplemented with Y-27632. The hepatocytes differentiated from cmESCs expressed the mRNAs for three hepatocyte marker genes (α-fetoprotein, albumin, CYP7A1) and several CYP enzymes, as measured by real-time polymerase chain reaction. In particular, the basal expression of cmCYP3A4 (3A8) in these hepatocytes was detected at mRNA and enzyme activity (testosterone 6β-hydroxylation) levels. Furthermore, the expression and activity of cmCYP3A4 (3A8) were significantly upregulated by rifampicin. These results indicated the effectiveness of Y-27632 supplementation for feeder-free dispersed culture and induction of differentiation into hepatocytes, and the expression of functional CYP enzyme(s) in cmESC-derived hepatic cells.**

**Key words** embryonic stem cell; differentiation; hepatocyte; monkey; cytochrome P450; feeder-free dispersed culture

Investigation of drug metabolism with human hepatocytes is important in the early stages of drug development. However, primary human hepatocytes are short-lived and cannot be maintained in culture over the long term. In addition, there are large donor-dependent variations in drug metabolism. On the other hand, human embryonic stem cells (ESCs) are able to replicate infinitely and differentiate into various types of somatic cells including germ cells.[1] Thus, they represent an attractive source to provide large numbers of cells that can be utilized for the development of candidate drug-screening strategies in place of primary cells.[2] However, ethical and legal restrictions have limited the availability of human ESCs. The phenotype of human ESCs is known to closely resemble that of monkey ESCs but not mouse ESCs with regard to morphology, leukemia inhibitory factor responsiveness, gene expression profiles, and some disease models.[1,3–6] Thus, monkey ESCs are a more suitable model for preclinical research of drug development. In particular, hepatocytes derived from monkey ESCs may be useful for pharmacokinetic studies, such as investigation of drug–drug interactions and the inducibility of drug-metabolizing enzymes, including cytochrome P450 (CYP).

We reported previously that monkey ESCs were successfully differentiated into hepatocytes by the formation of embryoid bodies (EBs) and treatment with specific growth factors and cytokines critical for hepatic differentiation.[7] EBs can mimic the inductive microenvironment required for liver organogenesis[8–10] and develop into many different cell types in culture. However, this EB formation method is not always appropriate for assays with large numbers of samples, such as high-throughput screening, because the formation of EBs is inefficient. A dispersion culture system, one of the differentiation methods without EB formation, can more efficiently provide a large number of feeder-free undifferentiated cells. The Rho-associated kinase (ROCK) inhibitor Y-27632 enables expansion from single-cell culture of human ESCs under dispersion culture conditions because the ROCK inhibitor markedly reduces dissociation-induced apoptosis of human ESCs.[11] Furthermore, Takehara *et al.*[12] conducted direct neural stem cell induction from cynomolgus monkey (*Macaca fascicularis*) ESCs (cmESCs) using Y-27632 and demonstrated the effectiveness of Y-27632 supplementation for feeder-free culture and induction of differentiation. However, it is not clear whether this dispersion culture method is effective for differentiation of monkey ESCs into hepatocytes.

In the present study, we carried out induction of hepatocyte differentiation from cmESCs by the dispersion culture method and examined expression and drug inducibility of CYP in the differentiated cells.

## MATERIALS AND METHODS

**Materials** Growth Factor Reduced BD Matrigel Matrix (Matrigel reduced) was obtained from BD Biosciences

The authors declare no conflict of interest.

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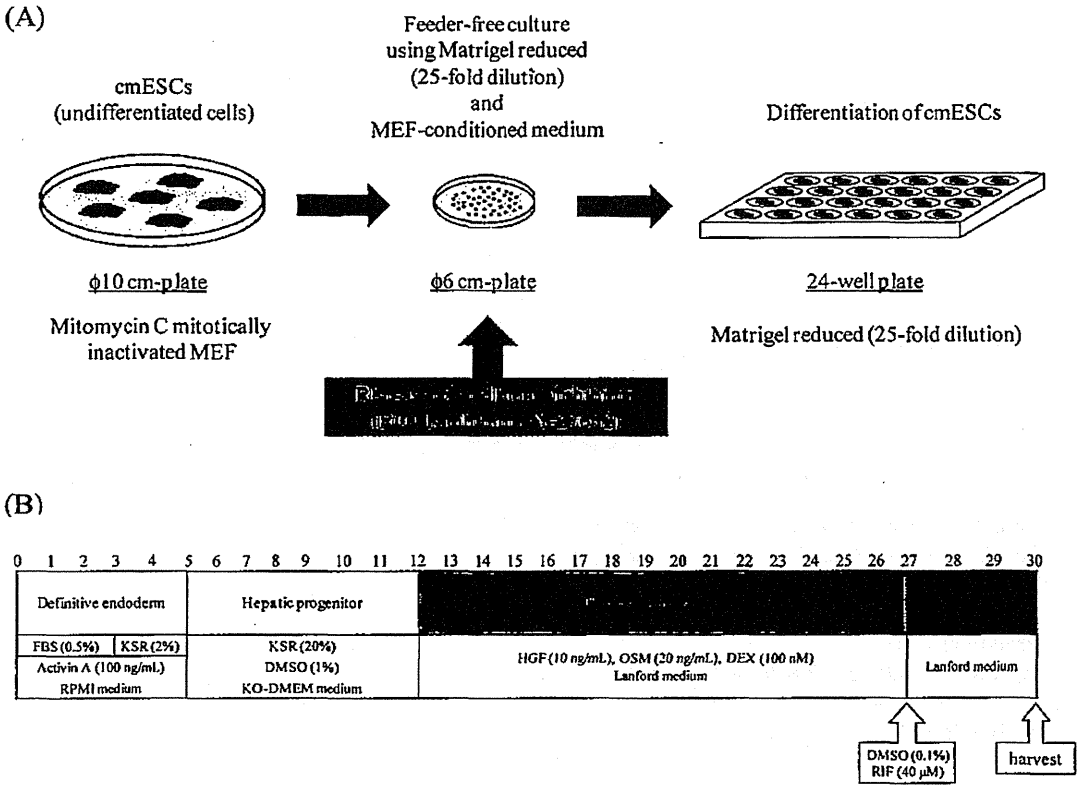


Fig. 1. Scheme of *in Vitro* Differentiation of cmESCs into Hepatocytes

(A) Illustration of the feeder-free dispersion culture of undifferentiated cmESCs. (B) Schematic procedure of differentiation of cmESCs into hepatocytes and drug treatment.

(Bedford, MA, U.S.A.); mitomycin C, Dulbecco's modified Eagle's medium (DMEM), William's E medium with GlutaMAX without phenol red, MEM non-essential amino acid solution (100×), and 6$\beta$-hydroxytestosterone from Sigma (St. Louis, MO, U.S.A.); murine embryonic fibroblasts (MEF) from Oriental Yeast (Tokyo, Japan); RPMI1640 medium supplemented with GlutaMAX, KnockOut™ DMEM, KnockOut Serum Replacement (KSR), minimum essential medium (MEM), L-glutamine, 0.25% (w/v) trypsin-ethylenediaminetetraacetic acid (EDTA), and SuperScript™ III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) from Invitrogen Life Technologies (Carlsbad, CA, U.S.A.); fetal bovine serum (FBS) from Equitech-Bio, Inc. (Kerrville, TX, U.S.A.); recombinant human activin A and recombinant human hepatocyte growth factor (HGF) from Funakoshi Co., Ltd. (Tokyo, Japan); modified Lanford medium from Charles River Laboratories Japan Inc. (Yokohama, Japan); recombinant human basic fibroblast growth factor (bFGF), Y-27632, oncostatin M (OSM), dexamethasone (DEX), rifampicin (RIF), testosterone, and dimethyl sulfoxide (DMSO) from Wako Pure Chemicals (Osaka, Japan); [$^2H_7$]6$\beta$-hydroxytestosterone from BD Gentest (Franklin Lakes, NJ, U.S.A.); illustra RNAspin Mini RNA Isolation kit from GE Healthcare (Tokyo, Japan); SYBR® Green real-time PCR Master Mix from TaKaRa Bio (Otsu, Japan). All other reagents used were of the highest quality available.

**ESC Culture and Differentiation** The cmESCs (CMK6) were generously provided by Tanabe Seiyaku Co., Ltd. (Osaka, Japan)[4] and maintained according to the method reported previously[7] except that recombinant human bFGF was added to ES medium. Feeder-free dispersed culture was carried out as follows (Fig. 1A). The cmESCs were cultured in the presence of 10 $\mu$M Y-27632 for 1 h before detaching the cells from the feeder layer. After detachment of the cmESCs, contaminating MEF were removed by incubating the cell suspension on gelatin-coated plates (BD Falcon, Franklin Lakes, NJ, U.S.A.) at 37°C for 2 h. The cmESC clumps were recovered from the suspension by centrifugation, incubated in 0.25% (w/v) trypsin-EDTA solution at 37°C for 5 min, and dissociated into single cells by pipetting. The cells were passed through a Cell Strainer (40 $\mu$m mesh; BD Falcon) and seeded onto culture plates 6 cm in diameter (BD Falcon) coated with Matrigel reduced (25-fold dilution). The cmESCs were cultured in medium conditioned by contact with MEF with 4 ng/mL recombinant human bFGF and 10 $\mu$M Y-27632 for the first 24 h. The medium was changed for MEF-conditioned medium for cmESCs without Y-27632.

When cmESCs reached approximately 70% confluence, differentiation was initiated by replacing RPMI1640 medium supplemented with GlutaMax containing 0.5% FBS and 100 ng/mL activin A (Fig. 1B). After 72 h, the medium was changed to RPMI1640 medium supplemented with GlutaMax containing 2% KSR and 100 ng/mL activin A, and culture was continued for 48 h. The cells were passaged onto 24-well plates coated with Matrigel reduced (25-fold dilution) and cultured in KnockOut™ DMEM containing 20% KSR, 1 mM L-glutamine, 1% MEM nonessential amino acids, and 1% DMSO for 7 d. Finally, the cells were cultured in modified Lanford medium containing 10 ng/mL HGF, 20 ng/mL OSM, and 100 nM DEX. The medium was changed daily during differentiation.

Table 1. Primers Used for Real-Time PCR Analysis

| Genes | Forward primer (5'–3') | Reverse primer (5'–3') | Product (bp) |
|---|---|---|---|
| AFP | ACTATTGGCCTGTGGTGAGG | CACCCTGAGCTTGACACAGA | 224 |
| ALB | CTTCCTGGGCATGTTTTTGT | GGCTCTTCCACAAGAGGTTG | 177 |
| CYP1A1 | CTAGACACAGTGATTGGCAGGTC | GGTTGACCCATAGCTTCTGGTCA | 232 |
| cmCYP2B6 (2B30) | GGGGCATTGAAGAAGAATGA | ATTTTGCCCACACCACTCTC | 188 |
| cmCYP2C9 (2C43) | TGATTCCCAAGGGTACAACC | AAATTGCCACCTTCATCCAG | 118 |
| cmCYP2D6 (2D17) | AGATCGACGACGTGATAGGG | GTCCCCTTAGGGATGAGGAA | 178 |
| cmCYP3A4 (3A8) | CCAAGAAGCTTTTAAGATTTGATTTC | ATCTACTCGGTGCTTTTGTGTA | 191 |
| cmCYP3A5 (3A66) | TTTGCCCAATAAGGCACCTG | GGTTGGAATCACCACCATTG | 181 |
| CYP7A1 | ATTTGGTGCCAATCCTCTTG | CATCCTTTGGGTCAATGCTT | 215 |
| AhR | ACTCCACTTCAGCCACCATC | CTCGTGCACAGTTCTGCTTC | 146 |
| PXR | AAGGATGCAAGGGCTTTTTC | TTCTTCATGCCGCTCTCC | 151 |
| GAPDH | GTCAGTGGACCTGACCT | TGCTGTAGCCAAATTCGTTG | 245 |

AFP, α-fetoprotein; ALB, albumin; AhR, aryl hydrocarbon receptor; PXR, pregnane X receptor; GADPH, glyceraldehyde-3-phosphate dehydrogenase.

**Drug Treatment** To clarify the effects of RIF on expression of CYP, cmESC-derived hepatocytes were treated with 40 μM RIF for 72 h (Fig. 1B). The compound was dissolved in DMSO, which was added to the modified Lanford medium at a final concentration of 0.1%.

**Primary Hepatocyte Culture** Primary cynomolgus monkey hepatocytes (primary cmHCs, Batch HEP 18605) were obtained from BIOPREDIC International (Renes, France). The primary cmHCs were thawed according to the manufacturer's instructions. Briefly, the primary cmHCs were cultured on 24-well plates (BD Falcon) in William's E medium with GlutaMax without phenol red for 72 h, and the medium was changed daily.

**Real-time PCR Analysis** Total RNA was isolated from the cells and the liver of an adult male monkey (Ina Research Inc., Ina, Japan) using the illustra RNAspin Mini RNA Isolation kit according to the manufacturer's protocol. First-strand cDNA was generated from 5 μg of total RNA. Reverse transcription reaction was performed using the SuperScript™ III First-Strand Synthesis System for RT-PCR in accordance with the manufacturer's instructions. For detection of mRNA expression levels, CYP mRNAs were analyzed by SYBR® Green real-time quantitative PCR. All PCR procedures were performed using the ABI Prism 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, U.S.A.). PCR was performed in a mixture consisting of 10 μL of SYBR® Green real-time PCR Master Mix, 0.4 μL of 10 μM forward and reverse primers, 0.4 μL of dye, 7.8 μL of water, and 1 μL template cDNA in a total of 20 μL. The primers used are summarized in Table 1. The levels of these mRNAs were normalized relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

**Measurement of Cellular Activity of Testosterone 6β-Hydroxylation** Following drug treatment, cmESC-derived hepatocytes were incubated with 100 μM testosterone in modified Lanford medium for 6 h. On the other hand, primary cmHCs were incubated with 100 μM testosterone in MEM for 6 h. After incubation, each medium was collected and 6β-hydroxytestosterone was measured by LC-MS/MS under the conditions described below.

**Instrument** An Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump, a degasser linked to a CTC HTS PAL New Wash System Autosampler (AMR Inc., Tokyo, Japan) was used.

Table 2. Timetable for HPLC

| Time (min) | Solvent A (%) | Solvent B (%) |
|---|---|---|
| 0 | 10 | 90 |
| 3 | 10 | 90 |
| 6 | 90 | 10 |
| 9 | 90 | 10 |
| 9.1 | 10 | 90 |
| 16 | 10 | 90 |

Solvents A: 10 mM ammonium acetate in water. Solvents B: 0.1% formic acid in methanol.

Mass spectrometry was performed on an API 4000 triple quadruple instrument (Applied Biosystems/Sciex, Foster City, CA, U.S.A.) equipped with a TurboIonSpray® electrospray ionization (ESI) interface. Data processing was performed with the Analyst 1.4.2 software package (Applied Biosystems/Sciex).

**Chromatographic Conditions** Chromatographic separation was performed on a reversed-phase CAPCELL PAK C18 MG III column (50×4.6 mm i.d., 5 μm; Shiseido Co., Inc., Tokyo, Japan). The column temperature was kept constant at 40°C. The mobile phase consisted of a mixture of 10 mM ammonium acetate in water (A) with 0.1% formic acid in methanol (B) and was delivered at a flow rate of 0.5 mL/min. A stepwise gradient was used as shown in Table 2.

**Mass Spectrometry Conditions** The mass spectrometer was operated using the ESI source in positive ion mode. To optimize all of the MS parameters, standard solutions (100 ng/mL) and internal standard were infused into the mass spectrometer at a flow rate of 250 μL/min. The ion spray voltage (IS) was set at 4500 V. The TurboIonSpray probe temperature was maintained at 600°C. The instrument parameters *viz.*, nebulizer gas, curtain gas, auxiliary gas, and collision gas, were set at 60, 15, 80, and 5, respectively. Compound parameters *viz.*, declustering potential, collision energy, entrance potential, and collision exit potential, were 40, 20, 10, and 15, respectively, for 6β-hydroxytestosterone and [$^2H_7$]6β-hydroxytestosterone. Zero air was used as the source gas, while nitrogen was used as both curtain and collision gas. The mass spectrometer was operated in ESI positive ion mode and detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of *m/z* 305 precursor ion [M+H] to the *m/z* 269 product ion for 6β-hydroxytestosterone (retention time 8.7 min) and *m/z* 312 precursor ion [M+H] to the *m/z* 276 product ion for

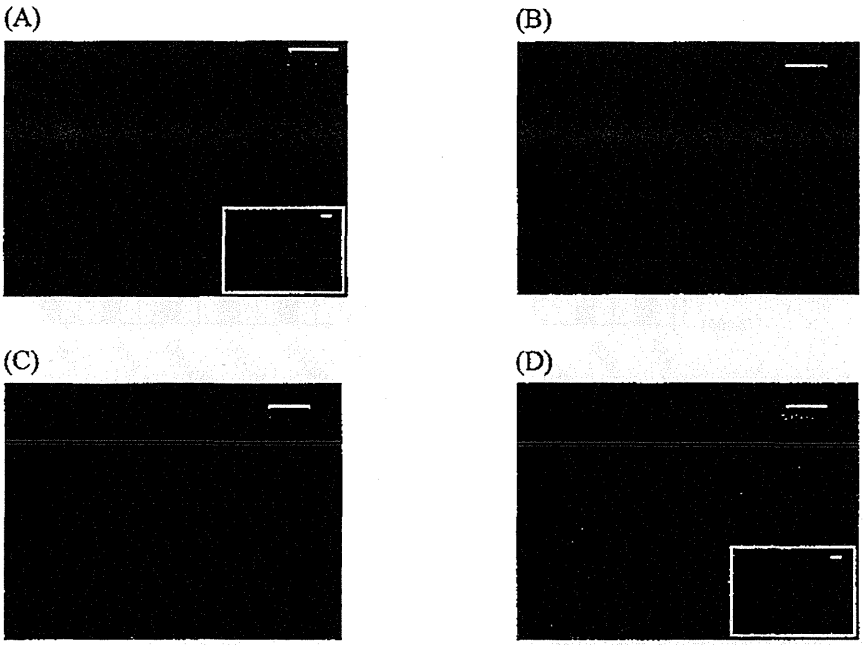


Fig. 2. Morphology of cmESCs in Individual Culture Processes

(A) Undifferentiated cmESCs. (B) Suspended cmESCs incubated on gelatin-coated plates for 2 h. (C) cmESCs cultured on plates 6 cm in diameter coated with Matrigel reduced (25-fold dilution) for 24 h. (D) cmESC-derived cells at 27 d after the initiation of hepatocyte differentiation. The cells were visualized by phase microscopy. Bars, 200 $\mu$m; 30 $\mu$m for A and D insets.

[$^2H_7$]6$\beta$-hydroxytestosterone (8.7 min). Quadrupoles Q1 and Q3 were set to unit resolution. Data acquisition and quantification were performed using Analyst software version 1.4.2 (Applied Biosystems, MDS Sciex, Toronto, ON, Canada).

**Calibration Standards** Calibration standards to cover the assay range of 10–5000 nM 6$\beta$-hydroxytestosterone were prepared by adding 10 $\mu$L of 0.1, 0.5, 1, 5, 10, and 50 $\mu$M working standards to 0.1 mL aliquots of control reaction mixture.

**Statistical Analysis** Statistical significance was assessed using the unpaired $t$ test. In all analyses, $p < 0.05$ was taken to indicate statistical significance.

## RESULTS

**Morphology of cmESCs at Individual Culture Steps** A typical colony of undifferentiated cmESCs is shown in Fig. 2A. As reported previously for primate ESCs, undifferentiated cmESCs formed tightly packed and flat colonies.[4] Each cell had a high nucleus/cytoplasm ratio and prominent nucleolus. In an effort to circumvent the problem of apoptosis in cmESC culture, the single-cell dispersed culture was performed under feeder-free cell culture conditions using Y-27632. The undifferentiated cmESCs were cultured in the presence of 10 $\mu$M Y-27632 for 1 h before detaching the cells from the feeder layer. After the cmESC colonies were dissociated by trypsin and suspended, the cells were seeded on gelatin-coated plates. In this procedure, contaminating MEF adhered to the plate bottom, whereas the cmESCs did not (Fig. 2B). The cmESC clumps were recovered from the suspension and dissociated into single cells by pipetting. The single cells were cultured on culture plates 6 cm in diameter coated with Matrigel reduced (25-fold dilution) in the presence of 10 $\mu$M Y-27632 for first 24 h. The cmESCs proliferated on the feeder-free culture plates (Fig. 2C). Twenty-seven days after initiation of hepatocyte differentiation, cells showed characteristic morphologies of hepatocytes, *i.e.*, polygonal in shape and multiple nuclei (Fig. 2D). Y-27632 was effective for cmESC survival under dispersion culture conditions.

**Expression of Hepatocyte Markers and CYP Enzymes in Primary cmHCs and cmESC-Derived Hepatocytes** The mRNA expression levels of hepatocyte marker genes and CYP enzymes in primary cmHCs and differentiated cells from cmESCs were measured by a real-time PCR method. As shown in Fig. 3, the mRNAs of hepatocyte marker genes, $\alpha$-fetoprotein (AFP), albumin (ALB), and CYP7A1, were detected in cmESC-derived hepatocytes together with those of CYP1A1, cmCYP2B6 (2B30), cmCYP2C9 (2C43), cmCYP2D6 (2D17), cmCYP3A4 (3A8), cmCYP3A5 (3A66), pregnane X receptor (PXR), and aryl hydrocarbon receptor (AhR). The mRNA levels of AFP and CYP7A1 in differentiated cells from cmESCs were 16- and 21-fold, respectively, higher than those in primary cmHCs, although the expression level of ALB was approximately 80-fold lower in cmESC-derived hepatocytes than in primary cmHCs. The mRNA levels of CYP1A1, cmCYP2B6 (2B30), cmCYP2C9 (2C43), cmCYP2D6 (2D17), cmCYP3A4 (3A8), cmCYP3A5 (3A66), and PXR in the cells differentiated from cmESCs were 386-, 7.4-, 284-, 1.6-, 136-, 5.9-, and 13-fold, respectively, lower than those in primary cmHCs. In contrast, the expression level of AhR mRNA in cmESC-derived hepatocytes was 2.2-fold higher than that in primary cmHCs.

**Testosterone 6$\beta$-Hydroxylase Activity of Primary cmHCs and cmESC-Derived Hepatocytes** Testosterone 6$\beta$-hydroxylase activity as a marker of CYP3A, especially cmCYP3A4 (3A8), was evaluated with primary cmHCs and cmESC-derived hepatocytes. Testosterone 6$\beta$-hydroxylase activity was detected in the cells differentiated from cmESCs (Fig. 4). The activity was about one sixth that of the primary