Establishment of a Stable Human Cell Line, HPL-A3, for Use in Reporter Gene Assays of Cytochrome P450 3A Inducers

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We have established a stable human cell line, termed HPL-A3, by co-transfection of a human pregnane X receptor (hPXR) expression vector and a reporter plasmid (p3A4-hPXRE-Luc) containing a luciferase gene and a promoter/enhancer region of the human *cytochrome P450 3A4* (CYP3A4) gene into a human hepatoma-derived cell line, HepG2. We then examined the usefulness of HPL-A3 for a chemically activated luciferase expression (CALUX) assay of human CYP3A inducers. The induction of CALUX in HPL-A3 by hPXR activators, including rifampicin, occurred in time- and concentration-dependent fashions, whereas no such induction was observed using rat/mouse PXR activators, such as pregnenolone-16 α -carbonitrile and dexamethasone. The hPXR activator-mediated induction of CYP3As, especially CYP3A4, was observed at levels of both mRNA and enzyme activity. Furthermore, there were positive correlations between chemical-mediated inductions of CALUX and CYP3A4 mRNA levels. In addition, the induction of CALUX by dihydropyridine calcium channel blockers, which are known to act as CYP3A inducers in rats, was observed in HPL-A3 cells. Interestingly, expression levels of not only hPXR but also of vitamin D receptor (VDR), a transcription factor that positively regulates CYP3A subfamily genes, were significantly increased in HPL-A3 cells compared with those in the parental cell line, HepG2. Consequently, VDR ligand (1,25-dihydroxyvitamin D₃)-mediated inductions of CALUX and CYP3A4 mRNA were observed in the cells. These findings verified the usefulness of HPL-A3 for the screening of CYP3A inducers, which can activate the hPXR and/or hVDR.

Key words cytochrome P450 3A4; pregnane X receptor; vitamin D receptor; chemically activated luciferase expression; calcium channel blocker

Human cytochrome P450 3A (CYP3A) subfamily enzymes, especially CYP3A4, play important roles in the metabolism of xenobiotics, including drugs, agricultural chemicals and endocrine disruptors.^{1,2)} Accordingly, studies on xenobiotic-mediated induction/inhibition of CYP3A enzymes are important for understanding the therapeutic/toxic effects of xenobiotics, including drugs. We can now assess the inhibitory effects of xenobiotics on the activity of human CYP3A enzymes by *in vitro* assays using commercially available human hepatic microsomes and/or enzymes.³⁾ However, only a few *in vitro* induction assays are established, because there are only a limited number of cell lines capable of xenobiotic-mediated induction of CYP3A enzymes.⁴⁾ The lack of such responsiveness of cultured cell lines to xenobiotics is caused by a considerable decrease in the expression level of pregnane X receptor (PXR),⁵⁾ a main transcription factor for the CYP3A subfamily genes.^{6,7)} Likewise, vitamin D receptor (VDR)⁸⁾ and constitutive androstane receptor (CAR),⁹⁾ which also act as a transcriptional activators of CYP3A subfamily genes, are expressed at low levels or not at all in human hepatoma-derived cell lines, including HepG2.^{5,10)}

Recently, several stable cell lines for a human PXR (hPXR)-based reporter gene assay for the screening of CYP3A4 inducers have been established by co-transfection into human cell

lines of an hPXR expression vector and a plasmid containing a reporter gene and hPXR-binding elements.^{11–15)} However, the available data on the xenobiotic-mediated induction of CYP3A enzymes at the levels of mRNA and activity in the reported cell lines are limited.

In the present study, to further develop an improved tool for the assessment of human CYP3A inducers, we have established a human cell line applicable for not only the reporter gene assay but also for assays of mRNA levels and enzyme activities. We confirmed the usefulness of the established cell line, termed HPL-A3, in assays of human CYP3A inducers.

MATERIALS AND METHODS

Chemicals Rifampicin (RIF), clotrimazole (CLO), pregnenolone-16 α -carbonitrile (PCN), dexamethasone (DEX), tamoxifen citrate (TAM), nimodipine (NIM) and 1,25-dihydroxyvitamin D₃ (VD₃) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nicardipine hydrochloride (NIC), nifedipine (NIF), nisoldipine (NIS), nitrendipine (NIT), and dimethylsulfoxide (DMSO) were obtained from Wako Pure Chemical (Osaka, Japan). The structures of chemicals used in this study are shown in Fig. 1. All the chemicals examined were dissolved in DMSO.

Construction of Reporter Plasmid The nuclear hormone receptor binding motif (ER6, –172 to –149) and distal nuclear receptor (dNR)-binding elements, such as dNR-1 (–7733 to –7719) and dNR-2 (–7489 to –7472), in a promoter/enhancer region of the human CYP3A4 gene play a crucial role in the PXR-mediated induction of the gene.¹⁶⁾ Therefore, an hPXR reporter plasmid, termed p3A4-hPXRE-Luc, containing a

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Fig. 1. Structures of Chemicals Used in This Study

luciferase gene and the promoter region (−362 to +53) and enhancer region (−7839 to −7208) of human *CYP3A4* gene was prepared using a previously reported method¹⁶⁾ with slight modifications.

In this study, human genomic DNA (Batch number; M00021571, Novagen, WI, U.S.A.) was used as a template for the amplification of the promoter/enhancer regions by polymerase chain reaction (PCR). A luciferase reporter plasmid, p3A4-hPXRE-Luc, was constructed through five steps as follows: (1) The promoter fragment A (−362 to +53) containing an ER-6 motif was prepared by *Bgl*II and *Bam*HI digestion of the promoter region (−1084 to +53), which was amplified by PCR with a primer set: forward sequence (bases −1085 to −1065, 5'-TCA TTG CTG GCT GAG GTG GTT-3') and reverse sequence (bases +29 to +53, 5'-CAT GGA TCC TGT TGC TCT TTG CTG GGC TAT GTG C-3'), which has an engineered *Bam*HI digestion site, (2) The plasmid (p3A4-Luc) was constructed by insertion of the promoter fragment A into the *Bgl*II site of the PicaGene Basic Vector 2 (pGV-B2, NipponGene, Tokyo, Japan), (3) The enhancer fragment B (−7836 to −7208) containing dNR-1 and dNR-2 was prepared by PCR with a primer set: forward sequence (bases −7839 to −7810, 5'-GAA GAT CTA TTC TAG AGA GAT GGT TCA TTC CTT TCA-3'), which has an engineered *Bgl*II digestion site, and reverse sequence (bases −7208 to −7235, 5'-TCT CGT CAA CAG GTT AAA GGA GAA TGG T-3'), (4) The plasmid (hPXRE-pGEM-T) was prepared by insertion of the enhancer fragment B into the pGEM-T easy vector (Promega, Madison,

MI, U.S.A.) containing a *Bam*HI site by the TA-cloning method, and (5) p3A4-hPXRE-Luc was prepared by insertion of the *Bam*HI-*Bgl*II digested hPXRE-pGEM-T fragment, containing enhancer fragment B, into the *Bgl*II site of p3A4-Luc. The obtained p3A4-hPXRE-Luc plasmid was used for the establishment of the cell line applicable to the hPXR-based chemically activated luciferase expression (CALUX) assay.

Construction of hPXR Expression Plasmid The full-length coding region of the human *PXR* gene was amplified from cDNA of a human hepatocellular carcinoma cell line, HepG2, by PCR with a primer set: forward sequence, 5'-GCC GCC ACC ATG GAG GTG AGA CCC AAA GAA AGC-3'; reverse sequence, 5'-AGC CGC TCA GCT ACC TGT GAT GCC GAA CAA-3'. An hPXR expression plasmid was prepared by the insertion of the full-length hPXR cDNA into the pTARGET Mammalian Expression vector (Promega) according to the manufacturer's instructions.

Establishment of a Cell Line for the hPXR-Based Reporter Gene Assay The reporter plasmid, p3A4-hPXRE-Luc, and the hPXR expression plasmid were co-transfected into HepG2 cells using Lipofectamine reagent (Life Technologies, Carlsbad, CA, U.S.A.) in Opti-MEM-I Reduced Serum Media (Life Technologies) for 24h. After the co-transfection, a portion (10⁴ cells/10 mL culture medium) of cell suspension was seeded in 100-mm culture dishes (Corning, Corning, NY, U.S.A.) and then cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo, Japan) supplemented with 5% fetal calf serum and G418 (400 μg/mL medium) for

2 weeks. The G418-resistant cells were selected and further cloned. Consequently, eight clones (HPLs-A3, A7, A11, A12, A13, A14, A17 and A18) expressing hPXR and luciferase were obtained.

Luciferase Assay Luciferase assays were performed as previously described.^{17,18} In brief, each HPL clone (5×10^4 cells/well) was pre-cultured for 48 h in a 24-well plate (Corning) and further incubated for the indicated times after addition of each test chemical to the culture medium. After incubation, cells were treated with 100 μ L of 1 $\times$ Reporter Lysis Buffer (Promega) for 15 min at room temperature, frozen at -80°C for 30 min and defrosted at room temperature. A portion (10 μ L) of the resultant cell lysates was mixed with 50 μ L of PicaGene Luminescence Reagent (Toyo-INK, Tokyo, Japan), and the amount of the luminescence product formed

was immediately measured with a Luminescencer-PSN (ATTO, Tokyo, Japan). The amount of protein in cell lysates was measured using a bicinchoninic acid (BCA)-protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, U.S.A.). Luciferase activity was represented as a luminescence unit per mg protein.

Clone HPL-A3 showed the greatest ability to induce chemical-mediated luciferase activity among the clones examined. Therefore, HPL-A3 was used for further experiments.

Gene Expression of CYP3A4 and Its Positive Transcriptional Regulators Constitutive gene expression levels of the positive transcriptional nuclear receptors (PXR, VDR, and CAR) of the *CYP3A4* gene and of their partner protein, retinoid X receptor α (RXR α), were determined by reverse transcription (RT)-PCR method^{17,18} with the appropriate primer

Table 1. The Primer Sets and Amplification Protocols Used in This Study

Target gene	Primer set	Reaction condition			Cycle	Reference
		Denaturation	Annealing	Elongation		
Conventional PCR						
<i>PXR</i>	5'-TTG TTC GGC ATC ACA GGT AG-3' (forward) 5'-CTT GCC TCT CTG ATG GTC CT-3' (reverse)	95°C, 30 s	60°C, 30 s	72°C, 60 s	25	(19)
<i>CAR</i>	5'-GCA GCT GTG GAA ATC TGT CA-3' (forward) 5'-CAG GTC GGT CAG GAG AGA AG-3' (reverse)	95°C, 30 s	60°C, 30 s	72°C, 60 s	35	(20)
<i>VDR</i>	5'-CTC TTC AGA CAT GAT GGA CTC G-3' (forward) 5'-GGA TGC TGT AAC TGA CCA GG-3' (reverse)	95°C, 30 s	60°C, 30 s	72°C, 60 s	28	(21)
<i>RXRα</i>	5'-AGC TTG TGT CCA AGA TGC G-3' (forward) 5'-ACT TGT GCT TGC AGT AGG CC-3' (reverse)	95°C, 30 s	60°C, 30 s	72°C, 60 s	25	(21)
<i>GAPDH</i>	5'-TGT TGC CAT CAA TGA CCC CTT-3' (forward) 5'-AGC ATC GCC CCA CTT GAT TTT G-3' (reverse)	95°C, 30 s	60°C, 30 s	72°C, 60 s	18	(22)
Real-time PCR						
<i>CYP3A4</i>	5'-ATA AGT AAG GAA AGT AGT GAT GGC TCT CA-3' (forward) 5'-TCA AAC ATA CAA AAG CCC TTA TGG TA-3' (reverse)	95°C, 30 s	60°C, 30 s	72°C, 60 s	40	(23)
<i>GAPDH</i>	5'-TGT TGC CAT CAA TGA CCC CTT-3' (forward) 5'-AGC ATC GCC CCA CTT GAT TTT G-3' (reverse)	95°C, 30 s	60°C, 30 s	72°C, 60 s	40	(22)

sets.^{19–23} The primer sets and amplification protocol used are shown in Table 1.

In brief, HPL-A3 or its parent cell line, HepG2, was cultured for 48h in a 60-mm dish (Corning) (5×10^5 cells/dish). Total RNA was then isolated from the cells using Isogen (NipponGene). A portion (4 μ g) of the prepared total RNA was converted to cDNA in an RT-reaction mixture (20 μ L) containing Moloney Murine Leukemia Virus Reverse Transcriptase (Life Technologies) and Random Hexamers (Life Technologies). PCR was performed in a reaction mixture (25 μ L) containing the prepared cDNA (equivalent to 32 ng of total RNA), the corresponding primer sets (each 12.5 pmol), and AmpliTaq Gold (0.625 units, Life Technologies) with a GeneAmp PCR system model 9600 (Life Technologies). The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining under UV light.

To determine chemical-altered *CYP3A4* expression, real-time PCR analysis was performed using an Applied Biosystems 7300 (Life Technologies) in 25 μ L of reaction mixture containing the prepared cDNA (equivalent to 32 ng of total RNA), 12.5 μ L of the Power SYBR Green PCR Master Mix (Life Technologies), the corresponding primer sets (each 12.5 pmol). After the reaction, dissociation curve analysis was carried out to confirm the amplification of a single PCR product. The amounts of *CYP3A4* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNAs were assessed by the relative standard curve method. *CYP3A4* and *GAPDH* fragments, obtained by conventional PCR with the corresponding primer sets (Table 1), were purified using a Qiaquick PCR Purification Kit (QIAEN, Valencia, CA, U.S.A.) and used as standard cDNAs. The level of *CYP3A4* mRNA was normalized to that of *GAPDH*, an internal control.

Chemical-Mediated Induction of CYP3A Enzyme Activity The total activity of CYP3A subfamily enzymes was determined using a P450-Glo™ CYP3A4 Biochemical and Cell-Based Assay (Promega), as described in the instruction manual. In brief, HPL-A3 cells (10^4 cells/well) were pre-cultured for 48h in a 96-well plate (Corning) and further treated with a chemical for the indicated times. After chemical treatment, cells were cultured for 4h in serum-free DMEM containing Luciferin-PFBE (50 μ M), a substrate of CYP3A subfamily enzymes (including CYP3A4, CYP3A5, and CYP3A7).²⁴ Thereafter, a portion (50 μ L) of the culture supernatant was mixed with an equal volume of Luciferin Detection Reagent, and the mixture was incubated at room temperature for 20 min under light shielding. The amount of luciferin formed was measured using a Luminescencer-PSN. In addition, the protein contents of cell lysates were measured with a BCA-protein Assay Kit. The CYP3A enzyme activity was represented as a luminescence unit per mg protein.

Statistics Statistically significant differences between control group (treatment with a vehicle alone) and each chemical-treated group were assessed using Student's *t*-test. Time- and concentration-dependent effects were analyzed using one-way analysis of variance (ANOVA) and Dunnett's *post hoc* test. Furthermore, the correlation between two parameters was evaluated by regression analysis. These analyses were performed using ystat2002, a Microsoft Excel-based Statistical Program (Igaku Tosho Shuppan, Tokyo, Japan).

RESULTS

Selection of a Stable Cell Clone Applicable to hPXR-Based CALUX Assays G418-resistant cells were selected from HepG2 cells co-transfected with the reporter plasmid, p3A4-hPXRE-Luc, and the hPXR expression vector and further cloned. From 19 G418-resistant clones obtained, eight clones (HPLs-A3, A7, A11, A12, A13, A14, A17 and A18) that stably expressed luciferase were selected using the CALUX assay with RIF (10 μ M), a representative hPXR activator. The concentration of RIF used was determined on the basis of previously reported data.^{12,14} Clone HPL-A3 showed the greatest response to the RIF-mediated CALUX induction among the eight clones examined, although the other clones examined, with the exception of two clones (HPL-A14 and HPL-A18), showed moderate responses (Table 2). Therefore, HPL-A3 was selected and used for further experiments.

Time- and Concentration-Dependent Changes in the RIF-Mediated Induction of CALUX HPL-A3 was treated with RIF (10 μ M), and the induced luciferase activities were measured at the indicated times. The luciferase activity increased in a time-dependent fashion up to 24h after RIF-treatment and thereafter gradually decreased (Fig. 2A). Therefore, the reaction time was fixed at 24h, and the concentration-effect of RIF on the induction of luciferase was further examined. RIF-mediated increases in luciferase activity occurred in a concentration-dependent fashion up to 100 μ M (Fig. 2B).

RIF-Mediated Induction of CYP3A Subfamily Enzymes We first examined the induction of CYP3A enzyme activity according to the CYP3A4 p450-Glo™ Biochemical and Cell-Based Assay. The constitutive CYP3A activity in HPL-A3 was 0.107 ± 0.036 RLU/ μ g protein, while in its parental cell line, HepG2, no CYP3A activity was detected. The time-dependent change in enzyme activity after treatment of HPL-A3 with RIF (10 μ M) was then examined. The RIF-mediated increase in enzyme activity occurred in a time-dependent fashion up to 48h, and the increased level at 48h was maintained for up to 96h (Fig. 3A). Considering such time-dependent induction, the concentration-effect of RIF was further assessed at 24h. The RIF-mediated increase in CYP3A enzyme activity occurred in a concentration-dependent fashion up to 30 μ M (Fig. 3B), while

Table 2. RIF-Mediated Induction of CALUX Activity in the Established Clones

Clone	Luciferase activity (luminescence unit/mg protein)		Induction ratio (RIF/CONT)
	CONT	10 μ M RIF	
HPL-A3	4171 $\pm$ 152	66958 $\pm$ 2390**	16.05
HPL-A7	159186 $\pm$ 1369	533185 $\pm$ 8035**	3.35
HPL-A11	371 $\pm$ 38	1020 $\pm$ 45**	2.75
HPL-A12	275 $\pm$ 17	1340 $\pm$ 187**	4.87
HPL-A13	32895 $\pm$ 2164	149212 $\pm$ 7809**	4.54
HPL-A14	7844 $\pm$ 6307	7743 $\pm$ 3278	0.99
HPL-A17	105 $\pm$ 2	121 $\pm$ 5*	1.16
HPL-A18	107 $\pm$ 11	118 $\pm$ 5	1.10

The eight G418-resistant HepG2 clones, which stably express both PXR and luciferase, were treated with RIF or vehicle alone (CONT, 0.1% DMSO) for 24h. Total cell lysates were prepared and used for the CALUX assay. The values represent the mean $\pm$ S.E. in vehicle- and RIF-treated groups ($n=3$), respectively. ***Significant differences from the corresponding vehicle-treated groups were assayed by Student's *t*-test; * $p < 0.05$, ** $p < 0.01$.

Fig. 2. Time-Course or Dose-Dependent Changes in CALUX in HPL-A3 after Treatment with RIF

HPL-A3 cells were treated with RIF (10 μM) or with vehicle alone (0.1% DMSO) for the indicated times (A) or were treated with RIF at the indicated concentrations for 24 h (B), and total cell lysates were used for the CALUX assay. The luciferase activity was measured as described in the Materials and Methods, and the data are represented as a ratio relative to corresponding controls. Solid and open circles represent the mean in RIF- and vehicle-treated groups, respectively ($n=4$), and the bars show their standard errors. **Significant differences from the corresponding controls assayed by ANOVA and Dunnett's test; ** $p<0.01$.

no such significant increase was observed at 100 μM.

The constitutive level of *CYP3A4* expression was measured by a real-time RT-PCR method. The constitutive *CYP3A4* expression level in HPL-A3 was 1.6-fold higher than in HepG2. Subsequently, the time-dependent change in the level of *CYP3A4* expression after treatment with RIF (10 μM) was examined. The expression level was increased in a time-dependent fashion up to 24 h (Fig. 4A). The increased level was maintained for up to 48 h and thereafter gradually decreased. *CYP3A4* expression at 24 h was about 5-fold higher than the corresponding control level. In addition, 24 h after the RIF treatment, the expression levels of *CYP3A5* and *CYP3A7* were likewise increased 3–3.5-fold compared with the corresponding control levels (data not shown). A concentration-effect of RIF on expression of *CYP3A4* was further examined at 24 h after RIF-treatment. *CYP3A4* expression was increased in a concentration-dependent fashion up to 100 μM (Fig. 4B). Moreover, there was a significantly positive correlation between the CALUX (luciferase activity) and the gene expression of *CYP3A4* (Fig. 4C).

Usefulness of HPL-A3 for Screening Human CYP3A Inducers It has been reported that species specificity in the induction of CYP3A subfamily enzymes by xenobiotics is likely to be a consequence of differences at the level of PXR activation.^{25,26} Therefore, we comparatively examined the effects of the human and/or rodent PXR ligands on the inductions of CALUX and *CYP3A4* mRNA in HPL-A3. In this experiment, human PXR activators (RIF and CLO), human/mouse/rat PXR activators (TAM and NIC), and mouse/rat

Fig. 3. Time-Course or Dose-Dependent Changes in the Activity of CYP3A Subfamily Enzymes in HPL-A3 after Treatment with RIF

HPL-A3 cells were treated with RIF (10 μM) or with vehicle alone (0.1% DMSO) for the indicated times (A) or were treated with RIF at various concentrations for 24 h (B). CYP3A enzyme activity was assessed by p450-Glo CYP3A4 assays, and the data are represented as a ratio relative to the control (treated with vehicle alone). Solid and open circles represent the mean in RIF- and vehicle-treated groups, respectively ($n=3$), and the bars show their standard errors. **Significant differences from the corresponding controls assayed by ANOVA and Dunnett's test; ** $p<0.01$.

PXR activators (DEX and PCN) were used as representative species-selective PXR activators.^{27–30} The working concentration of each chemical (10 μM) was determined on the basis of previously reported data.^{14,28,30}

Treatments of HPL-A3 with RIF, CLO, TAM and NIC for 24 h resulted in significant increases in CALUX, represented as luciferase activity (Fig. 5A). On the other hand, no significant increases by DEX and PCN were observed. Significant increases in the level of *CYP3A4* expression was likewise observed in HPL-A3 treated with RIF, CLO, TAM or NIC, but not with DEX and PCN (Fig. 5B). Furthermore, there were significantly positive correlations between CALUX and the level of *CYP3A4* expression in the chemical-treated HPL-A3 cells (Fig. 5C).

Induction of CALUX by Calcium Channel Blockers We have previously reported that several dihydropyridine calcium channel blockers (CCBs), especially NIC, showed abilities to induce hepatic CYP3A subfamily enzymes in rats and mice.^{31–33} Therefore, the effects on CALUX of CCBs, such as NIC, NIF, NIM, NIT or NIS, were assessed using HPL-A3 cells. The CALUX assay was performed 24 h after incubation with each CCB. The increases in CALUX in response to all the CCBs used occurred in a concentration-dependent fashion (Fig. 6). In addition, NIS showed cytotoxic effects on HPL-A3 cells at concentrations of more than 10 μM. When the effects on the induction of CALUX were compared among CCBs at 3 μM, they were active in the following order: NIM>NIC, NIT>NIS, NIF.

Gene Expression of Positive Transcriptional Regulators

Fig. 4. Time-Course or Dose-Dependent Changes in the Level of CYP3A4 mRNA in HPL-A3 after Treatment with RIF

HPL-A3 cells were treated with RIF (10 μM) or with a vehicle alone (0.1% DMSO) for the indicated times (A) or were treated with RIF at the indicated concentrations for 24 h (B), and the total RNA from the cells was used for real-time RT-PCR analysis. The level of CYP3A4 mRNA was normalized to that of GAPDH mRNA and represented as a ratio relative to the corresponding controls. The correlations between luciferase activities (as shown in Fig. 2B) and the levels of CYP3A4 mRNA after the treatment with RIF at various concentrations for 24 h were assessed by regression analysis (C). Solid and open circles represent the means of RIF- and vehicle-treated groups ($n=3$), respectively, and the bars show their standard errors. ***Significant differences from the corresponding controls assayed by ANOVA and Dunnett's test; * $p<0.05$, ** $p<0.01$.

of the CYP3A4 Gene It has been reported that not only PXR but also CAR and VDR are associated with chemical-mediated activation of CYP3A4 expression.^{8,9} Therefore, we examined the constitutive gene expression levels of PXR, CAR, and VDR in HPL-A3 cells, and their levels were compared with those in HepG2. Likewise, the gene expression levels of RXRα, a partner protein for PXR, CAR, and VDR, were comparatively examined. As expected, the constitutive expression level of PXR in HPL-A3, which is transfected with an hPXR expression vector, was higher than in the HepG2 parental cell line (Fig. 7). On the other hand, only low level expression of CAR was observed in either HPL-A3 or HepG2. No clear difference in the expression level of RXRα between HPL-A3 and HepG2 was observed. The expression level of VDR was surprisingly clearly increased in HPL-A3 compared with HepG2.

VD₃-Mediated Induction of CALUX and CYP3A4 Expression Since HPL-A3 was predicted to express VDR, the

Fig. 5. Effects of Various Chemicals on the Induction of CALUX and CYP3A4 mRNA in HPL-A3

HPL-A3 cells were treated with each chemical (10 μM) for 24 h. Total cell lysates and total RNA were used for CALUX assay and real-time RT-PCR analysis, respectively. The luciferase activity is represented as a ratio relative to the control (treated with vehicle alone) (A). The level of CYP3A4 mRNA was normalized to that of GAPDH mRNA and represented as a ratio relative to the corresponding controls (B). The correlations between luciferase activities and the levels of CYP3A4 mRNA after the chemical-treatment for 24 h were assessed by regression analysis (C). ***Significant differences from the corresponding controls assayed by ANOVA and Dunnett's test; * $p<0.05$, ** $p<0.01$.

Fig. 6. Dose-Dependent Changes in CALUX in HPL-A3 Cells after Treatment with Calcium Channel Blockers

HPL-A3 cells were treated with a calcium channel blocker (NIC, NIF, NIM, NIT or NIS) at the indicated concentrations for 24 h, and total cell lysates were used for the CALUX assay. The luciferase activity is shown as a ratio relative to the control (treated with vehicle alone). The values represent the mean in each group ($n=3$), and the bars show their standard errors. **Significant differences from the corresponding controls assayed by ANOVA and Dunnett's test; ** $p<0.01$.

Fig. 7. Gene Expression of Positive Transcriptional Regulators of the *CYP3A4* Gene in HPL-A3 Cells and the Parental Cell Line, HepG2

Total RNA was prepared from HPL-A3 or HepG2 cells and used for conventional RT-PCR analyses of PXR, VDR, CAR and RXRα mRNAs. *GAPDH* was used as an internal standard. The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining under UV light.

effects of VD_3 , a VDR ligand, on the induction of CALUX and *CYP3A4* mRNA were examined. The working concentration ($0.1 \mu M$) of VD_3 was determined on the basis of previously reported data.^{8,34}

After VD_3 -treatment, luciferase activity was increased in a time-dependent fashion up to 24 h (data not shown). Therefore, the reaction time was fixed at 24 h, and the concentration-effect of VD_3 on the induction of CALUX was examined. CALUX induction occurred in a concentration-dependent fashion in the range of 0.01 – $0.1 \mu M$ (Fig. 8A). In addition, a significant increase in *CYP3A4* expression was confirmed in HPL-A3 treated with VD_3 ($0.1 \mu M$) for 24 h (Fig. 8B).

DISCUSSION

In the present study, we established an HPL-A3 cell line by co-transfection of the p3A4-hPXRE-Luc reporter plasmid and an hPXR expression plasmid into HepG2 cells, and the applicability of the cell line to the hPXR-based CALUX assay was first assessed using RIF, a representative hPXR activator. The time- and concentration-dependent inductions of CALUX and *CYP3A4* expression by RIF were clearly observed in HPL-A3, and significantly positive correlation between CALUX (luciferase activity) and *CYP3A4* expression was also observed. In addition, RIF-mediated induction of CYP3A enzyme activity occurred in a concentration-dependent fashion up to $30 \mu M$, but at a higher concentration ($100 \mu M$), the increased activity was reduced. These phenomena can be attributed to the biochemical properties of RIF; it has the ability not only to induce CYP3A subfamily enzymes³⁵) but also to inhibit the activities of several CYP enzymes, including CYP3A4.³⁶

CALUX in HPL-A3 was further increased not only by hPXR-selective activators (RIF and CLO) but also by human/mouse/rat PXR activators (TAM and NIC), while no such significant increases by mouse/rat PXR activators (DEX and PCN) were observed. These results suggest that HPL-A3 is adaptable for use in hPXR-based reporter gene assays.

We have previously reported that CCBs, such as NIC, NIF, NIM, NIT and NIS, show abilities to induce hepatic CYP3A subfamily enzymes in rats.³⁷) These CCBs are also known to have affinity to hPXR and to show the ability to activate hPXR-based reporter genes.^{27,38–40}) Therefore, we examined

Fig. 8. Effects of VD_3 on the Induction of CALUX and *CYP3A4* mRNA in HPL-A3

HPL-A3 cells were treated with VD_3 at the indicated concentrations for 24 h, and their total cell lysates were used for the CALUX assay. RIF ($10 \mu M$) was used as a positive control. The luciferase activity is represented as a ratio relative to the control (treated with vehicle alone) (A). The real-time RT-PCR analysis for *CYP3A4* mRNA was performed using cells treated with VD_3 ($0.1 \mu M$) or RIF ($10 \mu M$) for 24 h (B). The level of *CYP3A4* mRNA was normalized to that of *GAPDH* mRNA and represented as a ratio relative to the corresponding controls. The solid columns represent the mean in each group ($n=3$), and the bars show their standard errors. *** Significant differences from the corresponding controls assayed by ANOVA and Dunnett's test; * $p<0.05$, ** $p<0.01$.

the capacities of NIC, NIF, NIM, NIT and NIS for hPXR activation using HPL-A3, and confirmed that all the CCBs used act as hPXR activators. Furthermore, we demonstrated that they were active in the following order: NIM>NIC, NIT>NIS, NIF. This order was similar to that in a previous report on the induction of hepatic *CYP3A1* in rats.³⁷)

To further understand the mechanisms for the xenobiotic-mediated induction of CALUX and *CYP3A4* expression in HPL-A3, we examined the gene expression of transcriptional factors, such as PXR, CAR, VDR and their partner protein (RXRα), that are involved in the regulation of *CYP3A4* expression. As expected, the expression level of PXR was significantly increased in HPL-A3, which is transfected with an hPXR expression vector, compared with that in the HepG2 parental cell line. The gene expression level of VDR was likewise increased. However, the mechanism for the increase in gene expression of VDR remains unclear, although the increase might be dependent on the insertion sites of the hPXR expression vector and/or the reporter plasmid p3A4-PXRE-Luc in the genome of host cells. In addition, no clear changes in the expression levels of CAR or RXRα by the insertion of the expression vector and reporter plasmid were observed in HPL-A3.

Because a high level of VDR expression is predicted in HPL-A3, we further examined whether VD_3 , a representative ligand of hVDR, can induce CALUX and *CYP3A4* mRNA in HPL-A3 cells, and we indeed demonstrated VD_3 -mediated inductions. Although the expression level of VDR is not especially high in human hepatocytes,^{41,42}) it has been reported that the activation of VDR led to modifications in the expression of hepatic CYPs, such as CYP3A4,⁸) CYP7A1 and CYP24A1,¹⁰) and to altered hepatic lipid metabolism.⁴³) The previous and present findings therefore suggest that HPL-A3 is a useful tool for the screening of hVDR activators/inhibitors and for the analysis of hVDR function in the liver.

In conclusion, we have established an HPL-A3 cell line, which is a useful tool for the screening of not only human CYP3A inducers but also the activators/inhibitors of CYP3A transcription factors, such as hPXR and hVDR. Studies on the altered expression of CYP3A subfamily enzymes, especially

CYP3A4, by xenobiotics such as drugs are important for understanding the therapeutic/toxic effects of drugs and environmental chemicals. Therefore, the established HPL-A3 cell line will greatly contribute to the development of these studies.

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