FUNCTIONAL CHARACTERIZATION OF FOUR NATURALLY OCCURRING VARIANTS OF HUMAN PREGNANE X RECEPTOR (PXR): ONE VARIANT CAUSES DRAMATIC LOSS OF BOTH DNA BINDING ACTIVITY AND THE TRANSCONTIVATION OF THE CYP3A4 PROMOTER/ENHANCER REGION

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ABSTRACT:

Metabolism of administered drugs is determined by expression and activity of many drug-metabolizing enzymes, such as the cytochrome P450 (P450s) family members. Pregnane X receptor (PXR) is a master transcriptional regulator of many drug/xenobiotic-metabolizing enzymes, including P450s and drug transporters. In this study, we describe the functional analysis of four naturally occurring human PXR (hPXR) variants (R98C, R148Q, R381W, and I403V) that we have recently identified. By a reporter gene assay using the CYP3A4 promoter/enhancer reporter in COS-7 or HepG2 cells, it was found that the R98C variant failed to transactivate the CYP3A4 reporter. The R381W and I403V variants also showed varying degrees of reduction in transactivation, depending on the dose of PXR activators, rifampicin, clotrimazole, and paclitaxel. The transcriptional activities of the R148Q variant were not significantly different from that of the wild-type hPXR. The electrophoretic mobility shift assay revealed that only the R98C variant lacked DNA binding. Furthermore, the cellular localization of the hPXR proteins was analyzed. All four variants as well as the wild-type hPXR localized exclusively to the nucleus, regardless of the presence or absence of rifampicin. These data suggest that the R98C, R381W, and I403V hPXR variants, especially R98C, may influence the expression of drug-metabolizing enzymes and transporters, which are transactivated by PXR.

The fate of administered drugs is determined by the expression and activity of many drug-metabolizing enzymes, such as the cytochrome P450 (P450s) family members. Human pregnane X receptor (hPXR) [also called steroid and xenobiotic receptor or pregnane-activated receptor (PAR)] encoded by NR1I2, is a member of the orphan nuclear receptor family (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). hPXR is a master transcriptional regulator of many genes involved in the detoxification of xenobiotics, including CYP3A4 and ABCB1 (MDR1), and bile acid homeostasis (Goodwin et al., 2002). It is activated by many drugs, such as rifampicin and clotrimazole (Luo et al., 2002). NRI12 is located on chromosome 3q13-21 and consists of 10 exons, including one noncoding exon (Zhang et al., 2001; Fukuen et al., 2002). Two major alternatively spliced forms, PAR1 and PAR2, derived from nine exons, have been identified. PAR1 is translated from the leucine residue in exon 2 and composed of 434 amino acid residues. PAR2 is translated from the methionine residue in exon 1b and composed of 473 amino acid residues (Lehmann et al., 1998). The functional domains of the hPARI protein consist of DNA binding (DBD) (amino acid residues 41–107) and ligand binding (LBD) (amino acid residues 141–434) domains (Lehmann et al., 1998; Honkakoski et al., 2000). The DBD is characterized by highly conserved amino acid residues among mouse, rat, rabbit, and human species, including two conserved groups of four cysteine residues, which form two zinc fingers (Honkakoski et al., 2000; Khorasanizadeh Rastinejad, 2001;
Kliewer et al., 2002). The LBD folds to form a hydrophobic pocket into which the ligands bind and also contains dimerization and transcription activation motifs, including the well characterized activation function-2 helix in the LBD C terminus (Kliewer et al., 2002).

CYP3A4 transcriptional activation is the best studied system that is dependent on hPXR binding. hPXR forms a heterodimer with human 9-cis retinoic acid receptor-α (hRXRα) and binds to DNA, such as everted repeat-6 (ER6; 5'-gatcaataTGAACTcaaaggAGGTCAgtg-3') in the 5'-flanking region of the human CYP3A4 promoter/enhancer (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). CYP3A4 is the most abundant form among the CYP3A family members in the human liver and is involved in the metabolism of nearly 50% of all the drugs currently prescribed (Gibson et al., 2002). CYP3A4 expression varies up to 100-fold in humans, and the interindividual variation correlates with the metabolizing rate of drugs such as midazolam (Lamba et al., 2002). Several single nucleotide polymorphisms have been reported in the CYP3A4 promoter region. The most common variant, CYP3A4*1B, has an A-392G transition in the 5'-flanking region with an allele frequency ranging from 0% (Chinese and Japanese) to 45% (African Americans). CYP3A4*1B was reported not to be linked with alterations in CYP3A4 transcriptional activation and CYP3A substrate metabolism (Spurdle et al., 2002). The existence of the effect of SNPs on CYP3A4 expression in the distal enhancer/promoter region, including PXR-responsive element and XREM ( xenobiotic-responsive enhancer module), is currently unknown. Thus, SNPs inducing changes in the function or expression of hPXR are thought to be potential sources for variation in CYP3A4 expression. In Caucasian and American African populations, two reports on variations inducing amino acid changes in the hPXR gene have been published (Hustert et al., 2001; Zhang et al., 2001). Zhang et al. (2001) have reported an hPXR variant, R122Q, which reduces DNA binding activity and results in a slight attenuation of the rifampicin-induced CYP3A4 promoter activity. Hustert et al. (2001) have reported V140M, D163G, and A370T in the hPXR LBD. Among those, D163G had reduced basal and rifampicin-induced activities toward a reporter gene construct containing the CYP3A4 proximal promoter and distal enhancer.

We recently sequenced all the hPXR exons from 205 Japanese subjects and found four nonsynonymous SNPs (292C > T (the first C of the CUG codon of PAR1 is defined as position 1); R98C, 443G > A; R148Q, 1141C > T; R381W, 1207G > A; I403V) (Koyano et al., 2002). The variant R98C showed complete loss of the ligand-binding activity and also contains dimerization and transcriptional activation of CYP3A4 enhancer/promoter by the three hPXR variants was significantly reduced compared with the wild-type hPXR. The variant R98C showed complete loss of the transcriptional and the DNA binding activity to ER6. The other two variants also showed varying degrees of reduction in transcriptional activation, depending on the dose of PXR activators, rifampicin, clotrimazole, and paclitaxel.

### Materials and Methods

#### Cell Culture.

HepG2 cells, which are derived from a human hepatoma, were assayed since hPXR has a critical function in vivo in human hepatocytes. In addition, COS-7, an African green monkey kidney cell line with low endogenous hPXR levels, was used for transfection of the hPXR expression plasmids. HepG2 or COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 100 U/ml penicillin and streptomycin under an atmosphere of 5% CO2 at 37°C. For treatment of the PXR activators, cells were cultured in DMEM supplemented with FBS (10%) that was pretreated with dextrose-coated charcoal.

#### PXR Activators.

Rifampicin, clotrimazole, and paclitaxel, which are known to activate human PXR (Luo et al., 2002), were purchased from Sigma-Aldrich (St. Louis, MO).

#### Construction of Plasmids.

A full-length human PXR cDNA (hPAP1, encoding amino acids 1–434) was amplified by PCR from human liver Marathon-Ready cDNA (BD Biosciences Clontech, Palo Alto, CA). Although the native translation initiation codon for hPAP1 is CTG, the CTG codon was substituted with the ATG codon to increase the translation efficiency according to a previous report (Lehmann et al., 1998). The hPXR cDNA expression plasmid was then constructed by the GATEWAY Cloning System using the pDEST 12.2 mammalian expression plasmid according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). The variant hPXR (R98C, R148Q, R381W, and I403V) expression plasmids were constructed by the Quick-Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All coding regions of the wild-type and variant hPXRs were verified by DNA sequencing. The empty vector plasmid used as a control was prepared by removing the NotI/SalI fragment from pDEST 12.2.

A chimeric CYP3A4 luciferase reporter plasmid was constructed by amplifying the CYP3A4 proximal promoter region by the PCR from human genomic DNA using primers 5'-GACAGCCACACCTCAAGCTGGGCTATGTGC-3' and 5'-ACAGGTTGGTCTTTTGCTGGGCTATGTGC-3'. Next, the BglII/HindIII-digested PCR fragment (~362+53) was cloned into the corresponding sites of the pGL3-Basic vector (Promega, Madison, WI) to generate pCYP3A4–362/53Luc. The CYP3A4 XREM (Goodwin et al., 2002) was amplified by PCR from human genomic DNA using primers 5'-TATTCTGACAGATGGTCTTATCTTCTTTA-3' and 5'-CGAGATTCTCGTCAAGACACGT-3'. The resulting construct, pCYP3A4XREM–362/53Luc, contained two fragments of the CYP3A4 5'-flanking region (~735 to ~7208) was cloned into the Nhel/BglII sites of pCYP3A4–362/53Luc. The Western blot analysis

#### Western Blot Analysis.

Four micrograms of each hPXR expression plasmid (wild-type, R98C, R148Q, R381W, and I403V) were transfected into HepG2 cells (1.6 x 10⁶ cells/10-cm culture dish) by LipofectAMINE reagent (Invitrogen). COS-7 cells were transfected with PolyFect transfection reagent (Qiagen GmbH, Hilden, Germany). Forty-eight hours after transfection, the cell pellets were boiled in Laemmli sample buffer. Twenty micrograms of total cell proteins were separated by electrophoresis in a 12% SDS-polyacrylamide gel (Tefuco Co., Tokyo, Japan) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% skim milk and incubated with polyclonal rabbit anti-human PXR antibody as a primary antibody, which was generated by immunizing New Zealand White rabbits with a synthetic peptide, EERRALIKRKKSERTGT, corresponding to hPAP1 amino acids 119–135, and then incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) as a secondary antibody. hPXR protein was visualized with the WestFemto maximum sensitivity substrate (Pierce Chemical, Rockford, IL) according to the manufacturer’s protocol. The signals were assessed by densitometric analysis and quantified by three independent experiments.

#### Luciferase Reporter Assay.

HepG2 or COS-7 cells (1.4 x 10⁶ cells/3-cm culture dish) were transfected with 0.2 µg each of the hPXR expression plasmids, including the wild-type and the variants, together with 0.4 µg of the pcDNA3.1 luciferase reporter (pCYP3A4XREM–362/53Luc and 0.4 µg of pHRL-TK plasmids (Promega), the latter of which encoded Renilla luciferase as an internal control. Twenty-four hours after transfection, the cells were treated with the vehicle (dimethyl sulfoxide) or various concentrations of the PXR activators and then cultured for an additional 24 h. The cells were washed with PBS, and the lysates were prepared using the Dual-Glo Luciferase Assay System (Promega). Luciferase activity was then measured using an ARVO SX Multilabel Counter (PerkinElmer Wallac, Turku, Finland). All transfection efficiencies were normalized to the Renilla luciferase activity.

#### Electrophoretic Mobility Shift Assay (EMSA).

EMSA was performed essentially as described (Lehmann et al., 1998). hRXRα, wild-type, and variant hPXR proteins were in vitro transcribed and translated by the TNTQuick Coupled Transcription/Translation System (Promega) according to the manufacturer’s protocol. Reactions (20 µl) were performed in 10 mM Tris (pH 8.0),...
The human, mouse, rat, and rabbit PXR amino acid sequences were aligned. The predicted DBD is boxed, and the predicted LBD is shaded. The locations of R98, R148, R381, and I403 are indicated in white against black.

The human vitamin D receptor (hVDR; NR1I1) and the human constitutive androstane receptor (NR1I3), indicating that this amino acid substitution may have a significant functional effect. R148, R381, and I403 map to the ligand binding domain (amino acids 141–434). Next, the functional properties of these amino acid substitutions were examined with a focus on the induction of CYP3A4 transcription.

**Expression of Variant hPXR Proteins in HepG2 and COS-7 Cells.** HepG2 or COS-7 cells were transiently transfected with the hPXR variant (R98C, R148Q, R381W, and I403V) expression plasmids. Western blotting was used to estimate the expressed level of each hPXR variant protein, which was similar between the variant and wild-type hPXRs in both HepG2 and COS-7 cells, indicating that these substitutions do not affect protein stability and translational efficiency (Fig. 2 and data not shown).

**Reduced Transactivation of the CYP3A4 Promoter/Enhancer by PXR Variants.** To evaluate the effect on the transactivation of hPXR variants, HepG2 and COS-7 cells were transiently transfected with a CYP3A4 luciferase reporter plasmid containing the CYP3A4 distal and proximal PXR-responsive elements, together with each variant expression plasmid in the presence or absence of rifampicin, which is an hPXR ligand. The R98C variant protein failed to transactivate the CYP3A4 luciferase reporter plasmid, despite the presence of 20 μM rifampicin both in HepG2 [Fig. 3a; 2.9 ± 0.6% (p < 0.005) of the wild-type luciferase activity] and in COS-7 cells [data not shown; 3.4 ± 1.4% (p < 0.005) of the wild-type luciferase activity]. The other variants, R148Q, R381W, and I403V, showed almost the same transactivation ability as that of the wild-type at high doses of rifampicin (10–20 μM), whereas they showed reduced ability at a lower dose of rifampicin (1 μM) in HepG2 cells [75.9 ± 7.7%, 32.1 ± 2.3% (p < 0.005), and 62.6 ± 10.1% (p < 0.005) of the wild-type luciferase activity, respectively], but not in COS-7 cells (data not shown). When clotrimazole was used as a PXR activator, R148Q showed a transactivation ability similar to that of WT with a slight reduction at a higher dose of the activator (1 μM, 116.9 ± 21.8%; 10 μM, 84.8 ± 14.6%). However, R381W and I403V showed reduced transactivation at both 1 μM [50.3 ± 15.4% (p < 0.005) and 59.9 ± 17.3% (p < 0.05), respectively] and 10 μM [68.7 ± 8.8% (p < 0.005) and 60.6 ± 7.0% (p < 0.005), respectively] of clotrimazole (Fig. 3b). As for paclitaxel as a PXR activator, R148Q showed almost the same transactivation ability as that of the WT (1 μM, 94.9 ± 25.9%; 10 μM, 98.8 ± 13.4%) (Fig. 3c). In contrast, R381W and I403V showed reduced transactivation at a higher dose of paclitaxel [68.2 ± 3.4% (p < 0.01) and 70.2 ± 8.4% (p < 0.05), respectively], R98C could not be activated by clotrimazole or paclitaxel (Fig. 3, b and c).

**DNA Binding Properties of the hPXR Variants.** hPXR forms a complex with hRXRα as a heterodimer and the PXR/RXR heterodimer formation is required for DNA binding of hPXR to ER6,
which is identified as a PXR binding site in the CYP3A4 promoter (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). To examine DNA binding properties of the variants, EMSA was performed using in vitro synthesized hPXR variants and hRXR/H9251 proteins with the 32P-labeled ER6 probe or mutant ER6 (mtER6) as a probe. The amount of each variant protein used for EMSA was examined by Western blotting (Fig. 4a). The wild-type hPXR in combination with hRXR showed a shifted band (Fig. 4b, lane 2). No protein-DNA complex was observed without the wild-type hPXR protein in the reaction mixture (Fig. 4b, lane 1). Competition experiments using 50-, 500-, and 1000-fold excess of unlabeled ER6 oligonucleotide or 1000-fold excess of unlabeled mtER6 oligonucleotide confirmed that the shifted bands contain PXR/RXR heterodimer-32P-ER6 oligonucleotide probe complexes. In the EMSAs, R98C variant protein did not bind to ER6 even in the presence of

![Fig. 4. DNA binding activity for each hPXR variant to the CYP3A4 ER6 element.](image_url)

**a.** in vitro synthesized hPXR variant proteins (5 μl) were analyzed by Western blotting with anti-hPXR antibody and subsequently used in the EMSA. b, the 32P-labeled CYP3A4 PXR binding element (ER6) oligonucleotide was incubated with hPXR wild-type (WT), R98C, R148Q, R381W, or I403V along with hRXR in the absence (no competitor; lanes 2, 6, 10, 14, and 18, respectively) or presence of unlabeled ER6 (50-fold excess, lanes 3, 7, 11, 15, and 19; 500-fold excess, lanes 4, 8, 12, 16, and 20; 1000-fold excess, lanes 5, 9, 13, 17, and 21, respectively) or unlabeled mtER6 (1000-fold excess, lanes 22–26). No shifted band was observed without the WT protein (lane 1). After electrophoresis, complexes were analyzed by BAS-1500.

![Fig. 3. Effect of each hPXR variant on CYP3A4 promoter/enhancer reporter activity.](image_url)

**a.** HepG2 cells were transfected with either empty, wild-type (WT), R98C, R148Q, R381W, or I403V expression plasmids with the pGL3-CYP3A4 reporter and the Renilla luciferase (RL) plasmids. Cotransfected cells were incubated in the absence (0) or presence (0.3, 1, 3, 10, and 20 μM) of rifampicin for 24 h. b, HepG2 cells were transfected with either empty, WT, R98C, R148Q, R381W, or I403V expression plasmids with the pGL3-CYP3A4 reporter and the RL plasmids. Cotransfected cells were incubated in the absence (0) or presence (1 and 10 μM) of paclitaxel for 24 h as indicated in the figure. The CYP3A4 reporter-driven luciferase activities were normalized by the RL activity. Bars represent mean ± S.D. (n = 3) (*, p < 0.05, **, p < 0.01, ***, p < 0.005 by the Fisher’s protected least significant difference method).
Functional Characterization of hPXR Variants

Fig. 5. Cellular localization of hPXR and its variant proteins in HepG2 cells.

HepG2 cells were transfected with the wild-type hPXR (WT) and each variant (R98C, R148Q, R381W, and I403V) expression plasmid and in the absence (-); the left two columns) or presence (+; the right two columns) of 10 μM rifampicin (RIF). The transfected cells were immunostained with anti-hPXR antibody and Alexa 594-conjugated secondary antibody. Photographs in the left column show immunoreacted WT and the variant proteins (red). Photographs in the right column show nuclei stained with DAPI (blue). The empty plasmid-transfected cells (negative control) were also immunostained (empty).

Intracellular Distribution of the hPXR Variant Proteins. To examine whether hPXR and its variants are localized in the nucleus in the presence or absence of rifampicin, immunostaining of HepG2 and COS-7 cells transiently expressing hPXR or variant proteins was performed. The wild-type hPXR protein was exclusively localized in the nucleus both in the presence and absence of 10 μM rifampicin (Fig. 5). This result indicated that hPXR protein does not require the exogenous ligand for its nuclear localization under the assay conditions. Moreover, the variant proteins were also localized in the nucleus (Fig. 5). Similar data were obtained using COS-7 cells (data not shown). These data indicated that the four variants are intact in terms of the protein nuclear localization, although they have a reduced transactivation ability for the CYP3A4 enhancer/promoter.

Discussion

PXR is a master transcriptional regulator of drug- and xenobiotic-metabolizing enzymes, including a variety of P450 enzymes and drug transporters. We had identified four variations (R98C, R148Q, R381W, and I403V) in the NR1I2 gene encoding hPXR in the Japanese population. In this study, the functional aspects of these variant proteins were described.

R98 is located in the hPXR DBD and conserved among human, mouse, rat, and rabbit species (Fig. 1), and also in the closely related orphan nuclear receptors, including hVDR and human constitutive androstane receptor. On the basis of crystal structures and mutagenesis analysis of several nuclear receptors, the 70-amino acid DBD was shown to consist of two zinc finger subdomains followed by a C-terminal extension, which is important for correct DNA binding (Honakoski and Negishi, 2000). Since R98 is located in the C-terminal extension and immediately after the fourth cysteine residue of the second zinc finger of the DBD, it is possible that the R98C variant has aberrant DNA binding. Zhang et al. (2001) reported that R122Q with an amino acid substitution in the DBD had decreased DNA binding activity and a slight attenuation in hPXR transcriptional activity in response to rifampicin. Figures 3 and 4 revealed that the R98C variant caused a more drastic loss of both its DNA binding and transcriptional activities than those of R122Q, suggesting that R98C may induce serious defects in drug metabolism and transport.

R148, R381, and I403 mapped to the helix-1, the spacer region between helix-9 and -10, and the helix-10 of the LBD, respectively (Fig. 1) (Jones et al., 2000). Amino acid residue 148 is not conserved among the four species, whereas R381 and I403 are conserved. Based on the published crystal structure of the human PXR LBD (Watkins et al., 2001), the residues R148, R381, and I403 are not expected to be directly involved in ligand binding because they are not with the ligand binding cavity. However, the two variants, R381W and I403V, showed a significantly reduced ability at 0.3 or 1 μM rifampicin, whereas they showed similar transactivation ability to that of the wild-type hPXR at 10 or 20 μM rifampicin (Fig. 3a). Furthermore, the results on the two additional PXR activators, clotrimazole and paclitaxel, revealed that the transactivation ability of each variant varied depending on the drugs used and their doses (Fig. 3b and c). Although the molecular basis of these altered activities is not clear, these results suggested that the variants R381W and I403V, in addition to R98C, might affect the PXR-dependent drug metabolism and transport. Hustert et al. (2001) reported that the hPXR variants V140M, D163G, and A370T, were located in the LBD, but outside the ligand binding cavity. By a reporter gene assay in LS174T cells using the CYP3A4 promoter/enhancer reporter, they showed that D163G had a reduced basal activity but an increased rifampicin-induced activity, whereas V140M and A370T modestly increased the basal activity and modestly decreased the rifampicin-induced activity at 10 μM rifampicin (Hustert et al., 2001).

The R98C variant protein showed neither DNA binding activity nor transactivation ability on the CYP3A4 reporter plasmid (Figs. 3 and 4). This dramatic loss of the activities of the R98C variant may cause critical changes in drug metabolism and transport. Furthermore, we have tried to determine whether R98C acts as a dominant-negative against the wild-type hPXR because the variant is usually found as a heterozygous. We transiently cotransfected HepG2 cells with the CYP3A4 promoter/enhancer reporter plasmid and equal amounts of R98C and wild-type expression plasmids. The luciferase activity of the HepG2 cell lysate coexpressing R98C and wild-type was almost equal to that expressing the wild-type alone, suggesting that R98C did not act as a dominant negative type against the wild-type hPXR (data not shown).

To examine whether the amino acid substitutions of the proteins induce changes in their cellular localization, immunostaining of HepG2 and COS-7 cells transiently expressing the wild-type or variant hPXR was performed. All four variants and the wild-type hPXR proteins were localized in the nucleus in an exogenous ligand-independent manner under the assay conditions (Fig. 5). More recently, Kawanabe et al. (2003) reported that hPXR overexpressed in cultured HeLa cells spontaneously localized in the nucleus without exposing the cells to exogenous xenoccompounds. Our immunostaining data are consistent with their results. They also identified a nuclear localization signal (amino acid residues 66–92) within the hPXR DBD. Because all four variants identified did not induce any amino acid changes in the nuclear localization signal, it is reasonable that these variations did not affect the protein nuclear localization. As shown in Fig. 4 and
previously (Lehmann et al., 1998), in vitro hPXR/hRXRα heterodimer binds to DNA in the absence of ligand. However, it was reported that hPXR ligands induced the enhanced binding of coactivators, such as steroid receptor coactivator-1, resulting in transactivation of the CYP3A4 promoter/enhancer consisting of PXR-responsive element and XREM (Kliewer et al., 1998; Honkakoski and Negishi, 2000). Thus, our data support that hPXR ligands, such as rifampicin, promote the activation of nuclear hPXR rather than the translocation of the receptor to the nucleus, in contrast to the cases of VDR and glucocorticoid receptor (Racz and Barsony, 1999; Koyano et al., 2003).

In conclusion, we functionally characterized four naturally occurring hPXR variants. The R98C, R381W, and 1403V hPXR variants showed a reduced transcriptional activity, and variant R98C lost both DNA binding and transcriptional activities.

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References


