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SIMULTANEOUS SUBSTITUTION OF PHENYLALANINE-305 AND ASPARTATE-318 OF RAT PXR WITH THE CORRESPONDING HUMAN RESIDUES ABOLISHES THE ABILITY TO TRANSACTIVATE THE CYTOCHROME P4503A23 PROMOTER

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Running Title: Role of Phe-305 and Asp-318 in PCN-mediated activation

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Text Page: 31

Number of Table: 0

Figures: 8

References: 38

Abstract: 243

Introduction: 764

Discussion: 1498

Abbreviation CYP, cytochrome P450; DDE, 1,1-dichlorodiphenyldichloroethylene; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DR3, direct repeat spaced by 3 nucleotides; ER6, everted repeat spaced by 6 nucleotides; PCN, pregnenolone 16α-carbonitrile; PCR, polymerase chain reaction; PXR, pregnane X receptor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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ABSTRACT

The pregnane X receptor (PXR) is a key regulator on the expression of genes involved in the elimination of chemicals. As one of the most divergent members in the nuclear receptor family, PXR is activated in a highly species-dependent manner by certain chemicals. Pregnenolone 16α-carbonitrile (PCN), a glucocorticoid antagonist, efficaciously activates rodent but not human PXR. This study was undertaken to investigate the structural basis for PCN-mediated activation of rat PXR. A series of rat-human chimeric PXRs were prepared to gradually replace the ligand binding domain of human PXR with the corresponding rat sequence at an increasing length of 20 residues. Co-transfection experiments established that the region$_{306\text{-}326}$ acted as a transitional conjunction from none to full PCN responsive status. Site-directed mutagenesis study identified two residues (Phe-305 and Asp-318) that were critical in supporting PCN-mediated activation, and simultaneous substitution of both residues abolished the ability of rat PXR to transactivate the cytochrome P450 3A23 (CYP3A23) promoter. In addition, substitutions on Phe-305, Asp-318 or both markedly reduced the basal transcriptional activity, and the reduction occurred with the CYP3A4 but not CYP3A23 promoter. Further study with CYP3A4 and CYP3A23 hybrid reporter demonstrated that the region harboring the distal PXR element in the CYP3A4 promoter mediated the repressive activity. PXR has been shown to interact with co-repressors in the absence of ligand. The decreased responsiveness toward PCN and reduced basal transcriptional activity suggest that Phe-305 and Asp-318 are involved in both ligand binding and co-repressor interactions.

INTRODUCTION

Metabolism and transport are considered the primary defense systems against chemicals from both foreign and endogenous sources (Venkatakrishnan et al., 2001; Brinkmann and Eichelbaum, 2001; Shou et al., 2001; Skatrud, 2002). Metabolism involves chemical modifications and usually leads to increased polarity of lipophilic compounds, thus favoring excretion into urine and feces. Active transport such as efflux moves compounds against chemical and concentration gradients, thus reducing intracellular concentrations or absorption of chemicals. Therapeutic agents, so-called intended chemicals, are also eliminated through transport and metabolism, and the elimination rate is directly related to their therapeutic effectiveness (Krishna and Mayer, 2000; Billard, 2003; Jansen, 2003). In addition, transport and metabolism are the most common sources for drug-drug interactions when two or more drugs are administered simultaneously. There are three major types of interactions based on the nature of interactions. Drugs competitively inhibit each other, when their elimination is mediated by the same transporter/metabolizing enzyme (e.g., omeprazole *versus* diazepam) (Jones, 2004). Non-competitive inhibition may occur with a drug that inactivates an enzyme, which is responsible for the metabolism of a co-administered drug (e.g., macrolide antibiotics *versus* terfenadine) (Yap and Camm, 2002). The third type of drug-drug interactions results from altered expression of an enzyme/transporter, leading to altered pharmacokinetics for all drugs that are eliminated by this enzyme/transporter (Hollenberg, 2002; Rushmore and Kong, 2002; Niemi, 2003).

Expression of drug-metabolizing enzymes/transporters is largely regulated at the transcriptional level (Kliewer, 1998; Blumberg, 1998; Goodwin et al., 2002; Kast et al., 2002; Rushmore and Kong, 2002). The pregnane X receptor (PXR), also called steroid and xenobiotic receptor, has been recognized as a key regulator that mediates the induction of many chemical elimination genes such as cytochrome P4503A4 (CYP3A4) and multi-drug resistant-1 (MDR-1). PXR structurally belongs to a family of the nuclear receptors and regulates the transcription of target genes in a ligand-dependent manner (Goodwin et al., 2002). Unlike many other nuclear receptors, PXR has a large ligand-binding pocket, which is spherical in

shape, extremely hydrophobic and expandable (Watkins et al., 2001; 2003a; 2003b). Such structural features allow PXR to interact with a wide range of structurally dissimilar chemicals including drugs (e.g., paclitaxel), herbal supplements (e.g., hyperforin), pesticides (e.g., *trans*-nonachlor), and endogenous compounds (e.g., bile acids) (Kliewer and Willson, 2002). PXR-null mice exhibit defects in the induction of drug-metabolizing enzymes and transporters in response to prototypical PXR activators, and these mice are hypersensitive to chemicals (e.g., zoxazolamine) that are substrates for the induced enzymes (e.g., CYP3A) (Xie et al., 2000; Staudinger et al., 2001; Xie et al., 2001). Chemicals that exhibit species-dependent induction also show species-specific activation of PXR (e.g., rodent and human), and the species-specific induction is not observed in the mice that harbor a human PXR transgene (so-called humanized mice) (Xie et al., 2000).

Rifampicin and pregnenolone 16α-carbonitrile (PCN) are the most studied inducers in terms of species-dependent activity (Schustz et al., 1984; Benedetti et al., 1994; Moore et al., 2002; Niemi et al., 2003). Rifampicin is a potent inducer in humans and an efficacious activator of human PXR, and the same is true with PCN in rodents (Kliewer et al., 1998; Lehmann et al, 1998). Rifampicin differs markedly from PCN in terms of general chemical properties. Rifampicin is less hydrophobic and has a markedly larger molecular weight than PCN (MW: 823 *versus* 342). Therefore, it is expected that rifampicin and PCN are not mutually exclusive in terms of PXR activation. In support of this notion, a human PXR mutant containing a substitution with the corresponding rat residue ($L_{308}F$) shows decreased activity toward rifampicin but no changes toward PCN (Tirona et al., 2004). A human PXR mutant ($Q_{285}I$) increases the ability to respond to PCN accompanied by decreased responsiveness to rifampicin. However, the corresponding rat PXR mutant with a reversed substitution ($I_{282}Q$), although less responsive to PCN, shows no increases in responding to rifampicin (Östberg et al., 2002). Finally, rabbit PXR is markedly activated by both PCN and rifampicin (Moore et al., 2002).

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The present study was undertaken to investigate the structural basis for PCN-mediated activation of rat PXR. Experiments with chimeric PXRs and site-directed mutagenesis identified two residues (Phe-305 and Asp-318) that were critical in supporting PCN-mediated activation, and simultaneous substitution of both residues completely abolished the ability to transactivate the CYP3A23 promoter. In addition, substitutions of Phe-305, Asp-318 or both markedly reduced the basal transcriptional activity, and the reduction occurred in the CYP3A4 but not CYP3A23 promoter. PXR has been shown to interact with co-repressors in the absence of ligand. The decreased responsiveness toward PCN and reduced basal transcriptional activity suggest that Phe-305 and Asp-318 are involved in both ligand binding and co-repressor interactions.

MATERIALS AND METHODS

Chemicals and Supplies

DDE, Dexamethasone, HBSS, hyperforin, nifedipine and PCN were purchased from Sigma (St. Louis, MO). DMEM, LipofectAMINE and Plus Reagent were purchased from Invitrogen (Carlsbad, CA). Kits for luciferase detection and the null *Renilla* luciferase plasmid were from Promega (Madison, WI). Delipidated and normal fetal bovine sera were from HyClone (Logan, UT). The goat anti-rabbit IgG conjugated with alkaline phosphatase was from Pierce (Rockford, IL). Nitrocellulose membranes were from Bio-Rad (Hercules, CA). Unless otherwise specified, all other reagents were purchased from Fisher Scientific (Fair Lawn, NJ).

Plasmid constructs

The CYP3A-DR3 reporter was constructed by inserting four copies of CYP3A23 response element (5'-GATCAGAC*AGTTCA*TGA*AGTTCA*TCTAGATC-3') into the pGL3 promoter luciferase vector. The CYP3A23 promoter reporter (CYP3A23-Luc) was prepared by inserting the PCR-amplified genomic fragment (-1445 to +74) into the pGL3 promoterless luciferase vector (Huss and Kasper, 2000). The CYP3A4 promoter reporter (CYP3A4-DP-luc) contained fused fragments (-362 to +53 and -7836 to -7208) was described elsewhere (Goodwin et al., 1999; Song et al., 2004). The reporters are diagrammatically presented in Fig. 1A. The expression constructs encoding rat or human PXR were described previously (Zhang et al., 1999; Song et al., 2004). The chimeric PXR constructs were prepared by a PCR-mediated fusion method as described in Fig. 1B. To facilitate the preparation of the chimeric constructs, the rat PXR plasmid was subjected to site-direct mutagenesis to introduce two restriction endonuclease sites (BamH I and EcoR V). The BamH I site encodes residues 147 and 148, whereas the EcoRV site encodes residues 413 and 414. The resultant construct, designated $rPXR_{L147R/T414I}$, contained two amino acid substitutions (residues 147 and 414). The same endonuclease sites were introduced into the chimeric fragments through the S1 and A2 primers during PCR amplification (Fig. 1B). The chimeric fragments were digested with BamH I/EcoR V, and ligated to the $rPXR_{L147R/T414I}$ plasmid pretreated with

the same enzymes. The rat-human chimeric PXRs were prepared to gradually replace the ligand binding domain of human PXR with the corresponding rat sequence at an increasing length of 20 residues (Fig. 2A). The fusion PCR method was also used to prepare constructs that contained multiple substitutions (e.g., $PXR_{A302S/F305L/P308T/N309A}$), however, the rat PXR plasmid was used as the template for the initial amplification of both PCR fragments. All constructs were subjected to sequencing analyses.

Site-directed mutagenesis

The substitution mutants were prepared with a QuickChange site-directed mutagenesis kit as described previously (Li et al., 2003). Complementary oligonucleotides were synthesized to introduce a substitution. The primers were annealed to the rat PXR construct and subjected to a thermocycler for a total of 15 cycles. The resultant PCR-amplified constructs were then digested with Dpn I to remove the non-mutated parent construct. The mutated PCR-amplified constructs were used to transform XL1-Blue bacteria. Two rounds of site-direct mutagenesis were performed to generate double substitutions: $rPXR_{L147R/T414I}$ and $F_{305}L/D_{318}E$. All mutated constructs were subjected to sequencing analysis to confirm the desired mutation being made without secondary mutations.

Transient co-transfection experiment

Cells (CV-1 and HepG2) were plated in 24-well plates in DMEM media supplemented with 10% delipidated fetal bovine serum at a density of 8×10^4 cells per well. Transfection was conducted by lipofection with LipofectAMINE and Plus reagent as described previously (Song et al., 2004). Transfection mixtures contained 200 ng of a PXR plasmid, 100 ng of a reporter plasmid and 10 ng of null-*Renilla* luciferase plasmid. Cells were transfected for 4 h, and the medium was replaced with fresh medium. After 12 h, the medium was changed again with the same medium containing a chemical or solvent DMSO (final concentration of 0.1%). The transfected cells were incubated for additional 24 h and washed once with PBS and collected by scraping. The collected cells were subjected to 2 cycles of freeze/thaw. The reporter enzyme activities were assayed with a Dual-Luciferase Reporter Assay System

as described by the manufacturer. This system contained two substrates, which were used to determine the activity of two luciferases sequentially. The firefly luciferase activity, which represented the reporter activity, was initiated by mixing aliquot of lysates (10 µl) with Luciferase Assay Reagent II. Then the firefly luminescence was quenched and the *Renilla* luminescence was simultaneously activated by adding Stop & Glo Reagent to the sample tube. The firefly luminescence signal was normalized based on the *Renilla* luminescence signal and the ratio of normalized luciferase activity from chemical- over DMSO-treatment served as fold of induction.

Western analysis

Cell lysates (8 µg) were resolved by 7.5% SDS-PAGE in a mini-gel apparatus and transferred electrophoretically to nitrocellulose membranes. After non-specific binding sites were blocked with 5 % non-fat milk, the blots were incubated with an antibody against rat or human PXR. The antibodies were raised against a peptide derived from respective PXR. The sequence of the peptide from rat PXR was: N-CEEADSALEEPINVEE-C, whereas the sequence of the peptide from human PXR was: N-CELPEVLQAPSR-C. The production and purification of antibodies were described previously (Sachdeva et al., 2003). The primary antibodies were subsequently localized with the goat anti-rabbit IgG conjugated with horseradish peroxidase (Ma et al., 2004). Horseradish peroxidase activity was detected with a chemiluminescent kit (SuperSignal West Pico). The chemiluminescent signal was captured by KODAK Image Station 2000 and the relative intensities were quantified by KODAK 1D Image Analysis Software.

Other analyses Protein concentrations were determined with BCA assay (Pierce) with albumin as standard. Data are presented as mean ± SD of at least three separate experiments, except where results of blots are shown in which case a representative experiment is depicted in the figures. Comparisons between two values were made with Student's test at $p<0.05$.

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RESULTS

The region$_{306-326}$ functions as the switching conjunction for PCN responsiveness

Antiglucocorticoid PCN, a prototypical inducer of CYP3A enzymes in rodents, efficaciously activates rat but not human PXR. The present study was undertaken to locate resides that support PCN-mediated activation. A series of rat-human chimeric PXRs were prepared to gradually replace the ligand binding domain of human PXR with the corresponding rat sequence at an increasing length of 20 residues (Fig. 2A, Left). The chimeric ligand binding domains were generated with fusion PCR as described in Fig. 1B. The responsiveness of chimeric PXRs toward PCN was determined by co-transfection experiments with a reporter containing four copies of PXR DR3 response element (CYP3A23-DR3-Luc) (Fig. 1A).

The activation of chimeric PXRs by PCN is summarized in Fig. 2A. No evident activation was detected with human PXR or chimeric PXR rh147, which contained almost the entire ligand binding domain of human PXR (Fig. 2A). As a matter of fact, all chimeric PXRs, containing a replaced sequence up to residue 306, remained non-responsive to PCN. In contrast, replacement of additional 20 residues (rh326) resulted in an abrupt increase in PCN responsiveness. Actually, similar magnitude of the activation was detected with all chimeric PXRs that had a replaced sequence equal to or longer than that in the chimeric PXR rh326 (Fig. 2A). It should be emphasized that rPXR$_{L147R/T414I}$, containing two amino acid substitutions, responded to PCN similarly as the wild type PXR. In addition, some chimeric PXRs, notably rh326, rh346 and rh366, showed higher basal activity (DMSO) (Fig. 2A).

In order to determine whether chimeric PXR constructs produced similar levels of PXR protein, Western analysis was performed with an antibody against rat or human PXR. As shown in Fig. 2B, transfection of all PXR constructs resulted in a comparable level of expression. In addition to human PXR, three chimeric PXRs including rh147, rh166 and rh186 were detected by the antibody against human PXR. The peptide used for the preparation of the anti-human PXR antibody was derived from residues$_{182-193}$, thus overlapped with the chimeric rh186 by only four residues (Zhang et al., 1999). The detection of this

chimeric PXR by this antibody suggests that these four residues are sufficient to form an immunogenic epitope (Fig. 2B).

Quadruple mutants $PXR_{A302S/F305L/P308T/N309A}$ and $PXR_{K314Q/D318E/L320M/M321L}$ no longer respond to PCN

The study with chimeric PXRs suggested that the $region_{306-326}$ acts as a transitional conjunction from none to full PCN responsive status. In order to further support this conclusion, concentration-dependent activation and site-directed mutagenic studies were performed. For the concentration-dependent activation experiment, the wild type and two chimeric PXRs were tested including rh306 and rh326. As shown in Fig. 3A, both the wild type and chimeric rh326 PXR were activated in a concentration-dependent manner. The wild type PXR was activated slightly higher than rh326 at lower concentrations (< 10 μM), whereas the opposite was true when higher concentrations were used (> 10 μM) (Fig. 3A). In contrast, no activation was detected with rh306 regardless of the concentrations. These results further support the conclusion that the $region_{306-326}$ plays a transitional role in PCN response.

Site-directed mutagenesis was performed to determine whether the transitional role occurs with the wild type PXR. Based on sequence alignment analysis, there are seven residues that differ between rat and human PXR (Fig. 3B, bold form). Two quadruple substitution mutants, $PXR_{A302S/F305L/P308T/N309A}$ and $PXR_{K314Q/D318E/L320M/M321L}$, were prepared to replace the non-conserved amino acids with corresponding human residues. Residues 302 and 305 were included in the mutagenic study because of their proximity to the $region_{306-326}$ and previously implicated role in PCN-mediated activation (Tirona et al., 2004). Residue-325 (cysteine), on the other hand, was excluded from the quadruple substitutions based on our initial observation that this residue played no role (described below). Similarly, the transactivation activity of the quadruple mutants was determined by co-transfection experiments. As expected, increasing concentrations of PCN caused proportional increases in the activation of rat PXR (Fig. 3C). In contrast, none of the quadruple mutants showed evident activation even with the highest concentration of

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PCN (50 μM) (Fig. 3B). Western analysis detected comparable expression of the quadruple mutants as the wild type PXR (data not shown).

Simultaneous substitution of phenylalanine-305 and aspartate-318 abolishes the responsiveness to PCN

The study with the quadruple mutants suggested that one or more residues in each mutant are critical in supporting PCN-mediated activation. In order to directly test this possibility, single residue substitution was made from rat to human. Among nine mutants, only $D_{318}E$ and $F_{305}L$ exhibited a marked reduction on the PCN-mediated activation (Fig. 4A). The reduced activation with $F_{305}L$ was more profound than that with $D_{318}E$ (85 *versus* 60%). The third highest reduction was detected with mutant $K_{314}Q$ (~30%). The rest of the mutants showed some reduction (10-20%) except $A_{302}S$, which was activated comparably as the wild type PXR (Fig. 4A). Apparently, substitutions on these residues caused no changes on the expression (depicted).

The profound reduction with $F_{305}L$ and $D_{318}E$ underscored the importance of Phe-305 and Asp-318 in supporting PCN-mediated activation. We next tested whether these two residues worked additively on responding to PCN. A mutant ($F_{305}L/D_{318}E$) was prepared to simultaneously substitute both residues. As shown in Fig. 4B, the double mutant $F_{305}L/D_{318}E$ no longer responded to PCN regardless of the concentrations of PCN (0-50 μM). In contrast, $F_{305}L$ and $D_{318}E$ were activated in a concentration-dependent manner, although the overall magnitude was markedly lower than that with the wild type PXR (Fig. 4B). Even between $F_{305}L$ and $D_{318}E$, marked differences were detected with $F_{305}L$ being activated to a much lesser extent (Fig. 4B). Interestingly, both $F_{305}L$ and $D_{318}E$ exhibited a relatively lesser reduction with increasing PCN concentrations. For example, at 50 μM, $D_{318}E$ showed a reduction of ~30%, which contrasted to a 60% reduction at 10 μM (Fig. 4B). It should be emphasized that additional mutants were prepared to contain double or triple substitutions as specified in either $PXR_{A302S/F305L/P308T/N309A}$ or $PXR_{K314Q/D318E/L320M/M321L}$. However, no mutants showed a significant reduction unless one of the residues

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was Phe-305 and Asp-318, and mutants containing triple substitutions such as $PXR_{D318E/L320M/M321L}$ no longer responded to PCN as seen with $F_{305}L/D_{318}E$ (data not shown), suggesting that other residues work cooperatively with Phe-305 and Asp-318, and contribute to the overall magnitude of the activation.

Chemical-dependent alteration among mutants $F_{305}L$, $D_{318}E$ and $F_{305}L/D_{318}E$

The inability of $F_{305}L/D_{318}E$ to elicit any transactivation suggested that both residues are critical in supporting PCN-mediated activation. Next we tested whether single or double substitutions altered sensitivity toward other chemicals such as nifedipine, DDE and hyperforin. The selection of the chemicals was largely based on their differences on size and lipophilicity. Based on the activation of the wild type PXR, other chemicals tested were lesser potent than PCN (Fig. 5). For example, when assayed at 50 μM, DDE and nifedipine activated the wild type PXR by only ~10 and 35% of that elicited by PCN, respectively (Figs. 5A-C). The mutants, on the other hand, exhibited differential changes depending on a chemical. PCN and nifedipine caused markedly less activation of all mutants, and the reduced activation was more profound with nifedipine. For example, $D_{318}E$ showed a 35% reduction with PCN but 70% with nifedipine when they were assayed at 50 μM (Figs. 5A and B). In contrast to the reduced activation by PCN and nifedipine, DDE slightly increased the activation of both $F_{305}L$ and $D_{318}E$ (Fig. 5C). With hyperforin, $F_{305}L$ was activated slightly higher than the wild type PXR (e.g., 4 *versus* 3 fold at 5 μM), whereas neither $D_{318}E$ nor $F_{305}L/D_{318}E$ showed any evident activation by this natural compound (Fig. 5D).

Promoter-dependent alteration among $F_{305}L$, $D_{318}E$ and $F_{305}L/D_{318}E$

We next examined the transactivation activity of the mutants toward native promoters regulated by PXR. Two promoter reporters were tested including CYP3A23-Luc and CYP3A4-DP-Luc. The CYP3A23-Luc reporter contained a 1,519 bp-genomic fragment from the proximal promoter region, where a DR3 element is present (Fig. 1A). The CYP3A4-DP-Luc reporter contained a DR3 element in the distal region (-7836 to -7208) and an ER6 element in the proximal promoter (-362 to +53) (Fig. 1A). Both elements

are required for the maximum activation in response to a PXR ligand (Goodwin et al., 1999; Song et al., 2004). In addition to CV-1, hepatoma-derived cell line HepG2 was used for the co-transfection experiment. It should be emphasized that the element reporter (CYP3A23-DR3-Luc) provided a simplified model and indicated a direct functional outcome as a result of the interactions between PXR and its element, whereas the promoter reporters represented a more physiologically-related scenario and indicated better the responsiveness of the native genes (CYP3A23 and CYP3A4), particularly when the hepatic line HepG2 was used.

The results on the transactivation of various reporters are summarized in Fig. 6. Between two cell lines, HepG2 cells generally supported higher activation than CV-1 cells. Among three reporters, the highest transactivation was detected with CYP3A23-DR3-Luc, and the lowest with CYP3A23-Luc. CYP3A23-DR3-Luc and CYP3A23-Luc showed similar transactivation patterns by four PXRs in both CV-1 and HepG2 cells (Figs. 6A and B). In contrast, the transactivation of the CYP3A4-DP-Luc reporter exhibited profound differences depending on the type of cells. In CV-1 cells, the wild type, $F_{305}L$ and $D_{318}E$ PXRs were activated to the same extent (Fig. 6C). In contrast, in HepG2 cells, a profound differential activation was detected. The wild type PXR and $D_{318}E$ mutant were activated to a comparable extent, whereas a 40% reduction was detected with $F_{305}L$. The differences on the transactivation of the CYP3A4-DP-Luc reporter suggest that HepG2 and CV-1 cells support differential expression of genes that are participated in the regulation of CYP3A4 expression (in addition to PXR).

Interestingly in both CV-1 and HepG2 cells, all PXR mutants exhibited markedly lower basal activity (DMSO-treated cells) than the wild type PXR toward the CYP3A4-DP-Luc reporter (Fig. 6C). As a result, the overall activation of mutants $F_{305}L$ and $D_{318}E$ was actually higher than that with the wild type PXR, when the data were expressed as fold of activation (Fig. 6D). For example, $F_{305}L$ was activated lower than the wild type PXR by 35% based on the normalized luciferase activity (Fig. 6C), whereas it was actually activated markedly higher based on the fold of induction (40 *versus* 19 fold) (Fig. 6D).

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Tirona et al. recently reported that mutant $F_{305}L$ was activated higher than the wild type PXR by PCN. They used the CYP3A4 reporter and the data were expressed as fold of activation. Therefore, it is likely that the reported higher fold of activation was actually due to decreased basal activity (Tirona et al., 2004).

The distal region (-7836 to -7208) in the CYP3A4 promoter mediates transcriptional repression

The CYP3A4-DP-Luc was prepared to contain the proximal promoter and a distal region (Fig. 1A). Goodwin et al (1999) reported that both regions are required for maximum activation, however, lack of the distal region no longer responds to PXR-mediated transactivation (Goodwin et al., 1999). We next examined whether the distal region is responsible for the decreased basal activity. The distal region was released from the CYP3A4-DP-Luc reporter and inserted into the CYP3A23-Luc reporter (the upstream of the CYP3A23 proximal promoter). The parent reporter (CYP3A23-Luc) and the reporter containing the distal region (CYP3A23+3A4-D-Luc) were tested for the differential ability to respond to PXR-mediated transactivation. Mutant $F_{305}L$ was also included in this study to determine whether this mutant differs from the wild-type PXR in modulating the basal activity of these reporters. Similarly, the data were expressed as normalized luciferase activities and fold of activation (Fig. 7).

Based on the normalized luciferase activities, the CYP3A23+3A4-D-Luc reporter exhibited markedly lower activation than the CYP3A23-Luc reporter in response to either wild-type PXR or mutant $F_{305}L$ (Figs. 7A, C). As expected, the basal activity of the CYP3A23+3A4-D-Luc reporter was much lower than that of the CYP3A23-Luc reporter (DMSO lanes, labeled as no PCN). Interestingly, the decreased basal activity was observed with both wild-type PXR and mutant $F_{305}L$ with the wild-type PXR showing a higher reduction (Figs. 7A, C). Based on the fold of activation, both reporters were transactivated by PXR and $F_{305}L$, although the CYP3A23+3A4-D-Luc reporter consistently exhibited a slightly or significantly decreased fold of activation depending on the concentrations of PCN (Figs. 7B and D). In addition, the concentration-dependent activation curve by the wild-type PXR differed from that by $F_{305}L$.

At lower concentrations (≤ 10 μM), the wild type PXR caused higher transactivation than mutant $F_{305}L$ on both reporters, whereas the mutant $F_{305}L$ caused higher transactivation at higher concentrations, notably at 50 μM (e.g., 8 *versus* 14 fold for CYP3A23-Luc) (Figs. 7B, D).

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DISCUSSION

PXR is recognized as a key transcription regulator on the expression of genes involved in chemical elimination (Goodwin et al., 2002). Like many other nuclear receptors, PXR contains a DNA binding domain and a ligand-binding domain. Based on the nucleotide substitution rate in the ligand binding domain, PXR represents one of the most divergent members in the nuclear receptor family. As a result, certain chemicals activate PXR in a highly species-dependent manner (Goodwin et al., 2002). Antiglucocorticoid PCN, a prototypical inducer of CYP3A enzymes in rodents, efficaciously activates rat but not human PXR. In this study, we report that Phe-305 and Asp-318 play critical roles in supporting PCN-mediated activation, and simultaneous substitution of both amino acids with their human counterparts abolishes the ability to transactivate the CYP3A23 promoter. In addition, experiments with a series of rat-human chimeric PXRs have established that the region$_{306-326}$ functions as a transitional conjunction switching from none to full-responsive status.

The transitional role of the region$_{306-326}$ in supporting PCN-mediated activation suggests that the N-terminal 188 residues of the ligand binding domain (a total of 293 amino acids) determine the specificity toward and the activation magnitude of PCN. In this study, the chimeric PXR rh326, containing approximately two thirds of the rat ligand binding domain (N-terminus), produces a similar concentration-dependent activation curve as rat PXR (Figs. 2A and 3A). Interestingly, the importance of this region in ligand specificity appears to be a general phenomenon among all PXRs. Rifampicin, for example, is an efficacious activator of human but not rat PXR. A rat-human chimeric PXR containing the N-terminal 184 residues of the rat ligand binding domain remains non-responsive to rifampicin as rat PXR (Tirona et al., 2004). Reversely, a human-rat PXR with a switched order of the chimeric fragments is markedly activated by this antibiotic. In contrast to a single transitional region toward PCN (Fig. 2A), multiple transitional regions are located with rifampicin, and they are all present in the N-terminal two thirds of the ligand binding domain (Tirona et al., 2004).

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The region$_{306-326}$ has several structural features that likely support the transitional role. Based on X-ray crystallographic studies on human PXR, part of this region forms a flexible loop linking the C-terminus of β4 to the N-terminus of α7 (Watkins et al., 2001). The β4, along with other four-stranded β sheet, forms a wall that constitutes one side of the binding cavity. In particular, the flexible loop is involved in the formation of the entrance of the cavity and likely facilitates the expansion of the binding pocket (Watkins et al., 2001). Apparently, the flexibility of this loop is critical for PXRs to accommodate ligands with various sizes such as rifampicin (MW: 823) and PCN (MW: 342). Another important feature of this loop is the ability to adopt ligand-induced conformation (Watkins et al., 2003a). In the hyperforin-PXR structure, part of the loop (residue 314 to 318 based on rat PXR) adopts a helical conformation, whereas such an adoption does not occur in the PXR/SR12813 complex (Watkins et al., 2001; 2003a). The chemical-dependent adoption on the conformation provides another structural basis for the broad ligand specificity associated with PXR.

The flexibility of this region and the expandability of the entire cavity are likely restricted by interactions with other proteins. PXR dimerizes with RXRα and only the heterodimers bind to PXR element (Kliewer et al., 1998). In addition, PXR has been shown to interact with co-repressors and co-activators, and such interactions depend on the presence of a ligand (Gonzalez and Carlberg, 2002; Li et al., 2002). Ligand binding triggers dissociation from a co-repressor and initiates association with a co-activator. X-ray crystallographic analysis reveals several structural differences on PXR when co-crystallized with SR12813 and a co-activator peptide (Watkins et al., 2003b). In the presence of the peptide, PXR appears to form homodimers instead of monomers. Interactions with this peptide also affect the binding mode of SR12813. In the PXR/SR12813/peptide complex, SR12813 lies in the cavity in a single rather than three different orientations observed in the absence of the peptide (Watkins et al., 2001; 2003b). Apparently, PXR and its directly associated proteins represent only part of the transcriptional regulatory complex

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present in a promoter. It is conceivable that other proteins in a promoter likely affect ligand binding and ultimately the transactivation activity of PXR.

The residues in the transitional region$_{306-326}$ likely make direct contacts with a ligand, which in turn alters the sensitivity of PXR. Based X-ray crystallographic structures, hyperforin and SR12831 make direct contacts with residue-320 and -321, respectively (Watkins et al., 2001; 2003a). Interestingly, substitution of either residue causes only a moderate reduction (~20%) on the sensitivity toward PCN (Fig. 4A). In contrast, substitution of residue-305 or -318 ($F_{305}L$ and $D_{318}E$) reduces the transactivation activity by 85 and 60%, respectively (Fig. 4A). X-ray crystallographic study reveals that glutamate-318 is involved in the formation of a salt-bridge with Arg-410 (Watkins et al., 2001). This residue is conserved in rat PXR and is expected to form the same salt bridge with aspartate-318 (Zhang et al., 1999). However, an Arg-Asp salt bridge likely brings closer the structures where each residue resides (the flexible loop and α10). Given the fact that Glu-318 is located at the entrance of the binding cavity, it is conceivable that the substitution of aspartate with glutamate likely enlarges the entrance and increases the sensitivity toward a larger ligand. However, in contrary to this prediction, $D_{318}E$ is activated much lower than the wild type PXR by hyperforin, which is significantly larger than PCN (Figs. 5D and 8).

The size of a ligand, in reference to the size of the entrance, plays only a contributing role to the overall magnitude of activation. Phe-305 (based on rat PXR) is one of the twenty eight residues that line the inner surface of the binding cavity (Watkins et al., 2001). Substitution of this aromatic residue with a leucine likely decreases the hindrance caused by the bulky aromatic ring. However, $F_{305}L$ exhibits reduced activation toward PCN with only a slight increase toward DDE and hyperforin (Fig. 5). It is likely that a relatively bulky residue at the entrance of the cavity imposes certain restriction, thus properly orientate the entry of a ligand, particularly with small chemicals. Another possibility is that a residue (e.g., Leu-308 in human PXR) in the region$_{306-326}$ works in concert with other residues to achieve the overall biological activity. In support of the notion, we have demonstrated that quadruple mutants

$PXR_{A302S/F305L/P308T/N309A}$ and $PXR_{K314Q/D318E/L320M/M321L}$ show no activation activity toward PCN, although individual substitution of these residues cause only slight or moderate reduction with the exception of Phe-305 and Asp-318 (Figs. 3C and 4A). It should be emphasized that substitution of these residues in rat PXR with human counterparts generally results in reduced activation (Fig. 4A), suggesting that these amino acids indeed contribute to the insensitivity of human PXR toward PCN.

It is interesting to notice that the distal region of the CYP3A4 promoter mediates repressive activity on the basal transcription. In this study, we have demonstrated that fusion of this region to the CYP3A23-Luc reporter results in marked decreases on the basal transactivation (Figs. 7A and C). The precise mechanism on the decreased basal activity remains to be determined. Based on the fact that PXR binds to co-repressors in the absence of a ligand (Gonzalez and Carlberg, 2002), it is likely that the distal region provides a better platform for PXR and co-repressor interactions. Alternatively, the distal region serves a binding site for a repressor, and the binding of this repressor and the corresponding function are modulated by PXR-DNA interactions. In addition, the proximal promoter appears to modulate the interactions in the distal region, which are dependent on the type of PXR. For example, the wild-type PXR is more effective than mutant $F_{305}L$ in reducing the basal activity of the CYP3A23+3A4-D-Luc reporter (Figs. 7A and C), whereas the opposite is true with the CYP3A4-DP-Luc reporter (Fig. 6D).

The decreased basal activity likely complicates the interpretation on the role of a residue in basal and ligand-regulated transcription. Reporter activities are usually expressed as fold of activation or normalized activities. Fold of activation but not normalized activity takes into consideration both basal (DMSO-treated) and ligand-regulated activity (e.g., PCN). Interestingly, these two expression methods, in some cases, may lead to opposing conclusions, particularly when a residue is involved in both basal and ligand-regulated activities. For example, the wild-type PXR is more active than mutant $F_{305}L$ in transactivating the CYP3A4-DP-Luc reporter based on the normalized luciferase activity (Fig. 6C), whereas the opposite is true based on the fold of induction (40 *versus* 19 fold) (Fig. 6D). As for the

transactivation of CYP3A23-Luc and CYP3A23+3A4-D-Luc reporters, the wild-type PXR is more active than mutant $F_{305}L$ at low PCN concentrations (e.g., 5 and 10 μM), whereas the opposite is true at high concentrations (e.g., 50 μM) (Figs. 7B and D). These findings suggest that high concentrations of PCN overcome the repression on the basal transcription mediated by this mutant and Phe-305 is involved in both ligand and co-repressor interactions.

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FOOTNOTES

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Fig. 1. Diagrammatic presentation of reporters (A) and procedure for fusion PCR (B)

Fig. 2. The region$_{306\text{-}326}$ functions as the switching conjunction for PCN responsiveness *(A) Identification of the region that supports PCN responsiveness* A series of rat-human chimeric PXRs were prepared by fusion PXR to gradually replace the human ligand binding domain with the corresponding rat sequence at an increasing length of 20 residues. CV-1 cells were transiently transfected by lipofection with construct mixtures containing 200 ng of human, rat or a chimeric PXR plasmid, 100 ng of reporter plasmid (CYP3A23-DR3-luc) and 10 ng of null-luciferase plasmid (reference). After 12-h incubation, the transfected cells were treated with PCN (10 μM) or the same volume of DMSO for 24 h. Cell lysates were prepared and assayed for the luciferase activities with a Dual-Luciferase Reporter Assay System. The firefly luminescence signal was normalized based on the *Renilla* luminescence signal. Data represent the mean of assays in triplicate ± SD. *(B) Western analyses* Cell lysates (8 μg) from the transfected cells were subjected to SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane. The immunoblots were blocked in 5% non-fat dry milk and then incubated with an antibody against human or rat PXR. The primary antibodies were subsequently localized with the goat anti-rabbit IgG conjugated with horseradish peroxidase and chemiluminescent substrate

Fig. 3. Concentration-activation curves of rPXR, rh306, rh326, PXR$_{A302S/F305L/P308T/N309A}$ and PXR$_{K314Q/D318E/L320M/M321L}$ *(A) Concentration-activation experiment with rPXR, rh306 and rh326* CV-1 cells were transiently transfected with construct mixtures containing 200 ng of rat PXR (rPXR), or a chimeric PXR (rh306 and rh326), 100 ng of CYP3A23-DR3-Luc and 10 ng of null-luciferase plasmid. The transfected cells were treated with PCN (0-50 μM) or the same volume of DMSO for 24 h. Luciferase activities of the reporter were determined and normalized based on the *Renilla* luminescence signal. Data represent the mean of assays in triplicate ± SD. *(B) Alignment analysis of rat and human PXRs from residue 301 to 326. (C) Activation of quadruple mutants PXR$_{A302S/F305L/P308T/N309A}$ and*

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$PXR_{K314Q/D318E/L320M/M321L}$ CV-1 cells were transiently transfected with construct mixtures containing 200 ng of rat PXR (rPXR), or a quadruple mutant, 100 ng of CYP3A23-DR3-Luc and 10 ng of null-luciferase plasmid. The transfected cells were then treated with PCN (0-50 μM) or the same volume of DMSO for 24 h. Luciferase activities of the reporter were determined and normalized based on the *Renilla* luminescence signal. Data represent the mean of assays in triplicate ± SD.

Fig. 4. Simultaneous substitution of phenylalanine-305 and aspartate-318 abolishes the responsive-ness to PCN *(A) Identification of residues that support PCN-mediated activation* Rat PXR was subjected to site-directed mutagenesis to introduce a single amino acid substitution. CV-1 cells were transiently transfected with construct mixtures containing 200 ng of rat PXR (rPXR), or a substitution mutant, 100 ng of CYP3A23-DR3-Luc and 10 ng of null-luciferase plasmid. The transfected cells were treated with PCN (10 μM) for 24 h. Luciferase activities of the reporter were determined and normalized based on the *Renilla* luminescence signal. Data represent the mean of assays in triplicate ± SD. For Western blots, cell lysates (8 μg) were detected for the levels of PXR by the antibody against rat PXR as described above. *(B) Concentration-activation experiment of rat PXR and mutants $F_{305}L$, $D_{318}E$ and $F_{305}L/D_{318}E$.* CV-1 cells were transiently transfected with construct mixtures containing 200 ng of rat PXR (rPXR), or a mutant ($F_{305}L$, $D_{318}E$ and $F_{305}L/D_{318}E$), 100 ng of CYP3A23-DR3-Luc and 10 ng of null-luciferase plasmid. The transfected cells were treated with PCN (0-50 μM) for 24 h. Luciferase activities of the reporter were determined and normalized based on the *Renilla* luminescence signal. Data represent the mean of assays in triplicate ± SD.

Fig. 5. Activation of rat PXR, $F_{305}L$, $D_{318}E$ and $F_{305}L/D_{318}E$ by PCN, nifedipine, DDE and hyperforin CV-1 cells were transiently transfected with construct mixtures containing 200 ng of rat PXR (rPXR) or a mutant ($F_{305}L$, $D_{318}E$ and $F_{305}L/D_{318}E$), 100 ng of CYP3A23-DR3-Luc and 10 ng of null-luciferase plasmid. (A) The transfected cells were treated for 24 h with PCN (0-50 μM); (B) the transfected cells

were treated with nifedipine (0-50 μM); (C) the transfected cells were treated with DDE (0-50 μM); and (D) the transfected cells were treated with hyperforin (0-5 μM). Luciferase activities of the reporter were determined, normalized and expressed as fold activation. Data represent the mean of assays in triplicate ± SD.

Fig. 6. Transactivation of CYP3A23-DR3-Luc, CYP3A23-Luc and CYP3A4-DP-Luc in CV-1 and HepG2 cells *(A) Transactivation of CYP3A23-DR3-Luc* CV-1 or HepG2 cells were transfected with construct mixtures containing 200 ng of rPXR, $F_{305}L$, $D_{318}E$ or $F_{305}L/D_{318}E$, 100 ng of CYP3A23-DR3-Luc and 10 ng of null-luciferase plasmid. The transfected cells were treated with 10 μM PCN or the same volume of DMSO for 24 h. Luciferase activities of the reporter were determined and normalized based on the *Renilla* luminescence signal. Data represent the mean of assays in triplicate ± SD. The same experiments were performed with CYP3A23-Luc (B) or CYP3A4-DP-Luc (C + D). The data with CYP3A4-DP-Luc were expressed as normalized luciferase activities (C) or fold of activation (D).

Fig. 7. Transactivation of CYP3A23-Luc and CYP3A23+3A4-D-Luc by the wild-type PXR and mutant $F_{305}L$ *(A + B) Transactivation of CYP3A23-Luc and CYP3A23+3A4-D-Luc by the wild-type PXR* HepG2 cells were transiently transfected with construct mixtures containing 200 ng of rPXR, 100 ng of a reporter and 10 ng of null-luciferase plasmid. The transfected cells were treated with PCN (1-50 μM) or DMSO (0.1%) for 24 h. Luciferase activities of the reporter were determined and normalized based on the *Renilla* luminescence signal (A) or fold of activation (B). Data represent the mean of assays in triplicate ± SD. *(C + D) The same experiments were performed with mutant $F_{305}L$* The data were expressed as normalized luciferase activities (C) or fold of activation (D). Statistical comparison was made between CYP3A23-Luc and CYP3A23+3A4-D-Luc transactivation for Figs. B and D (*$p<0.05$).

Fig. 8. Chemical structure of PCN, nifedipine, DDE and hyperforin

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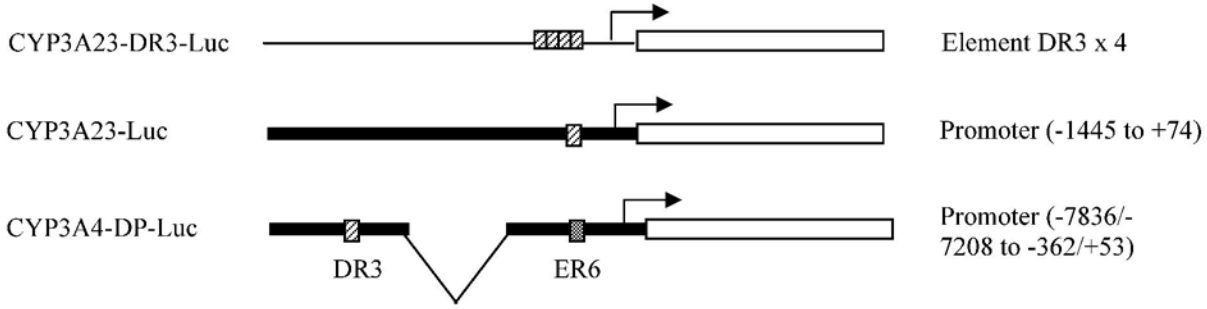

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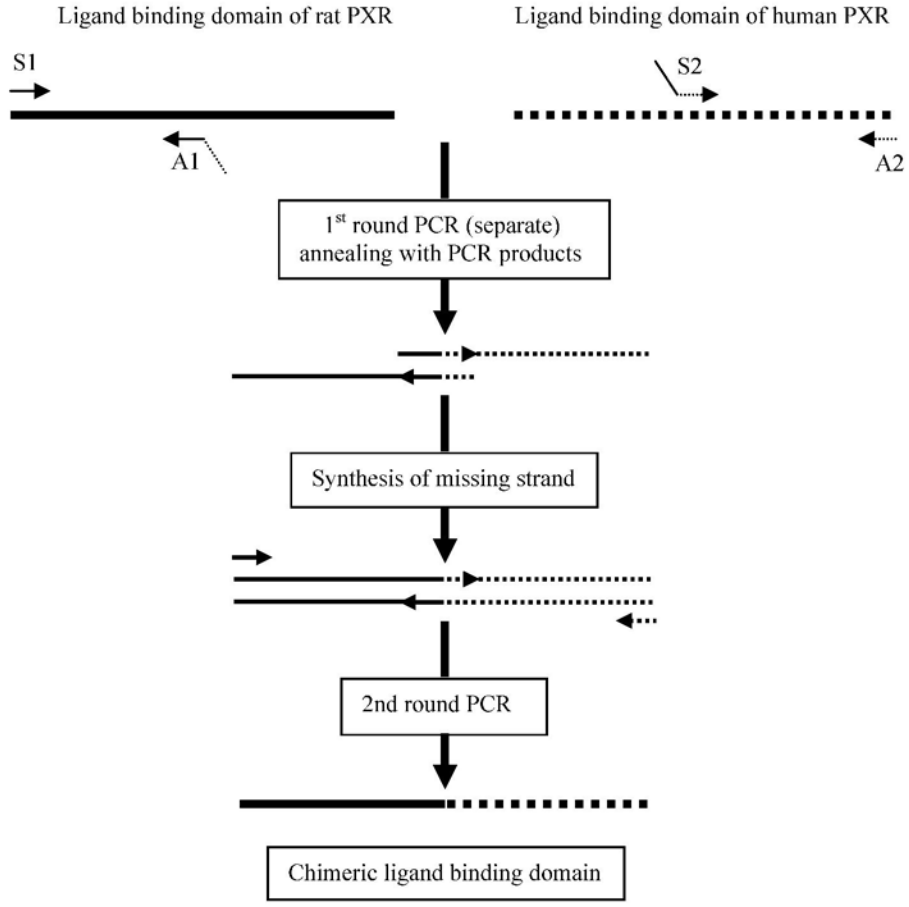

Fig. 1 JPET/2004/074971

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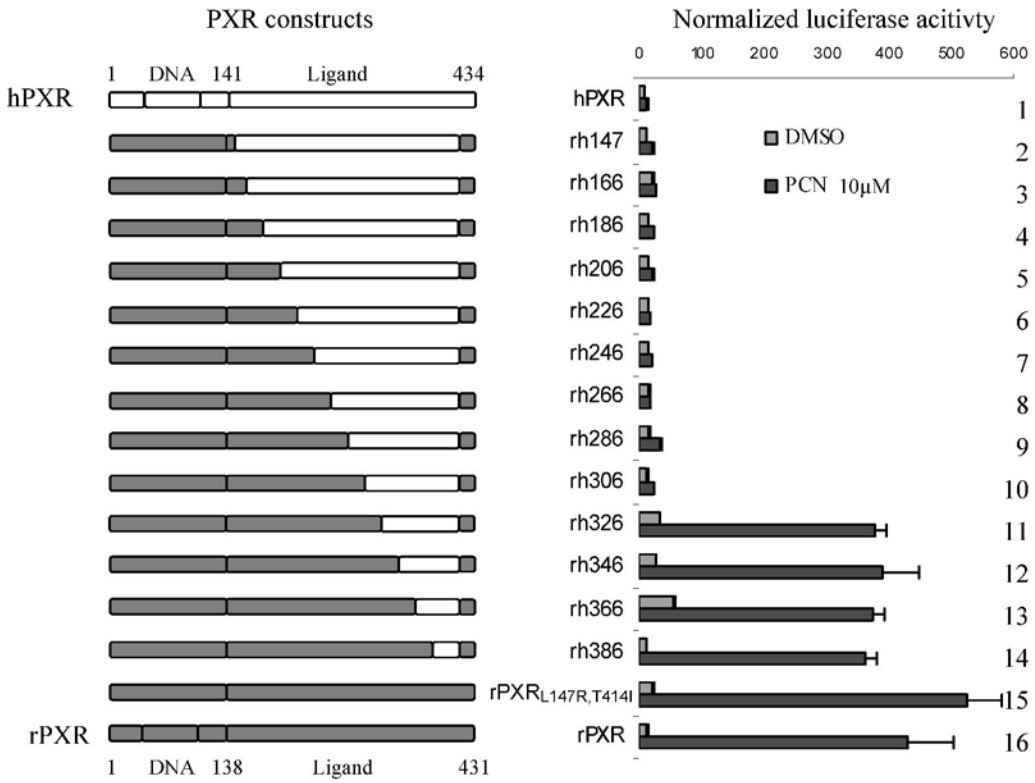

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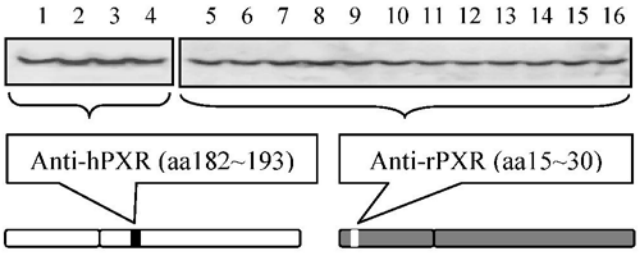

Fig. 2 JPET/2004/074971

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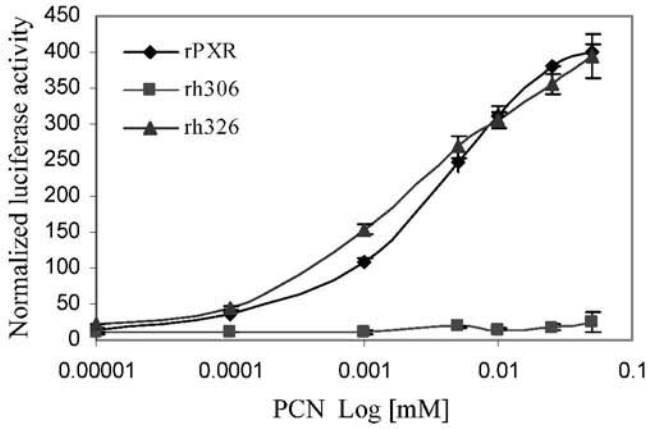

B

Rat PXR 301-L**A**YC**F**ED**PN**GGFQ**K**LLL**D**P**LM**KFH**C**M-326

Human PXR 304-L**S**YC**L**ED**TA**GGFQ**Q**LLL**E**P**ML**KFH**Y**M-330

C

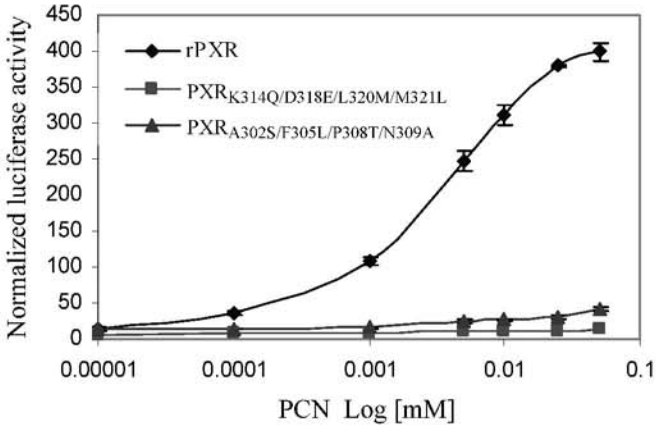

Fig. 3 JPET/2004/074971

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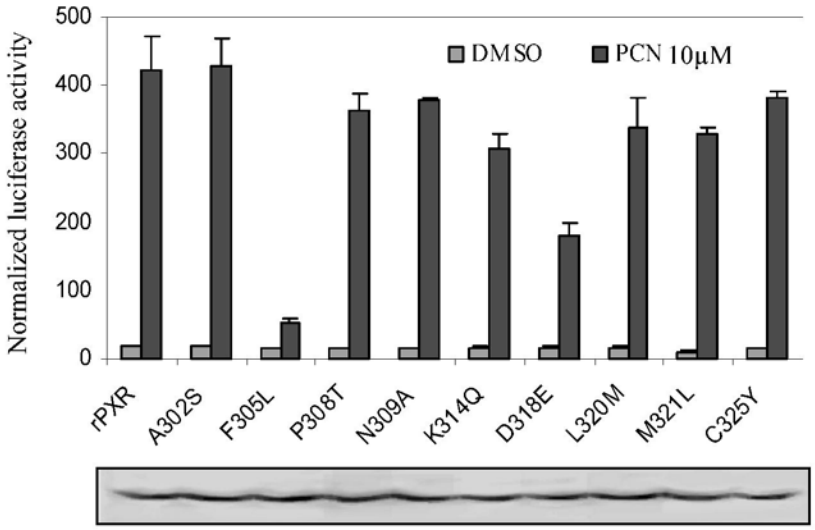

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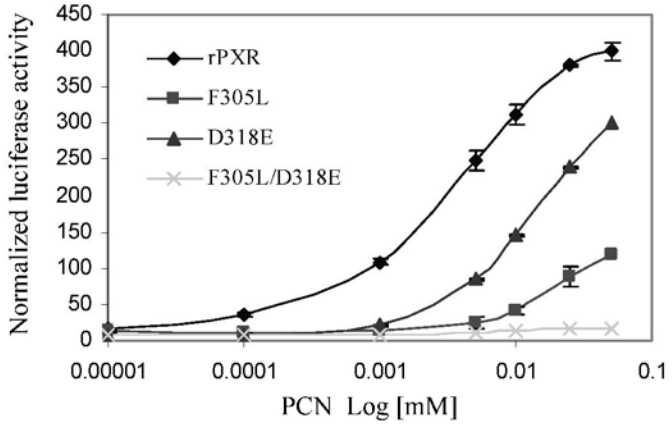

Fig. 4 JPET/2004/074971

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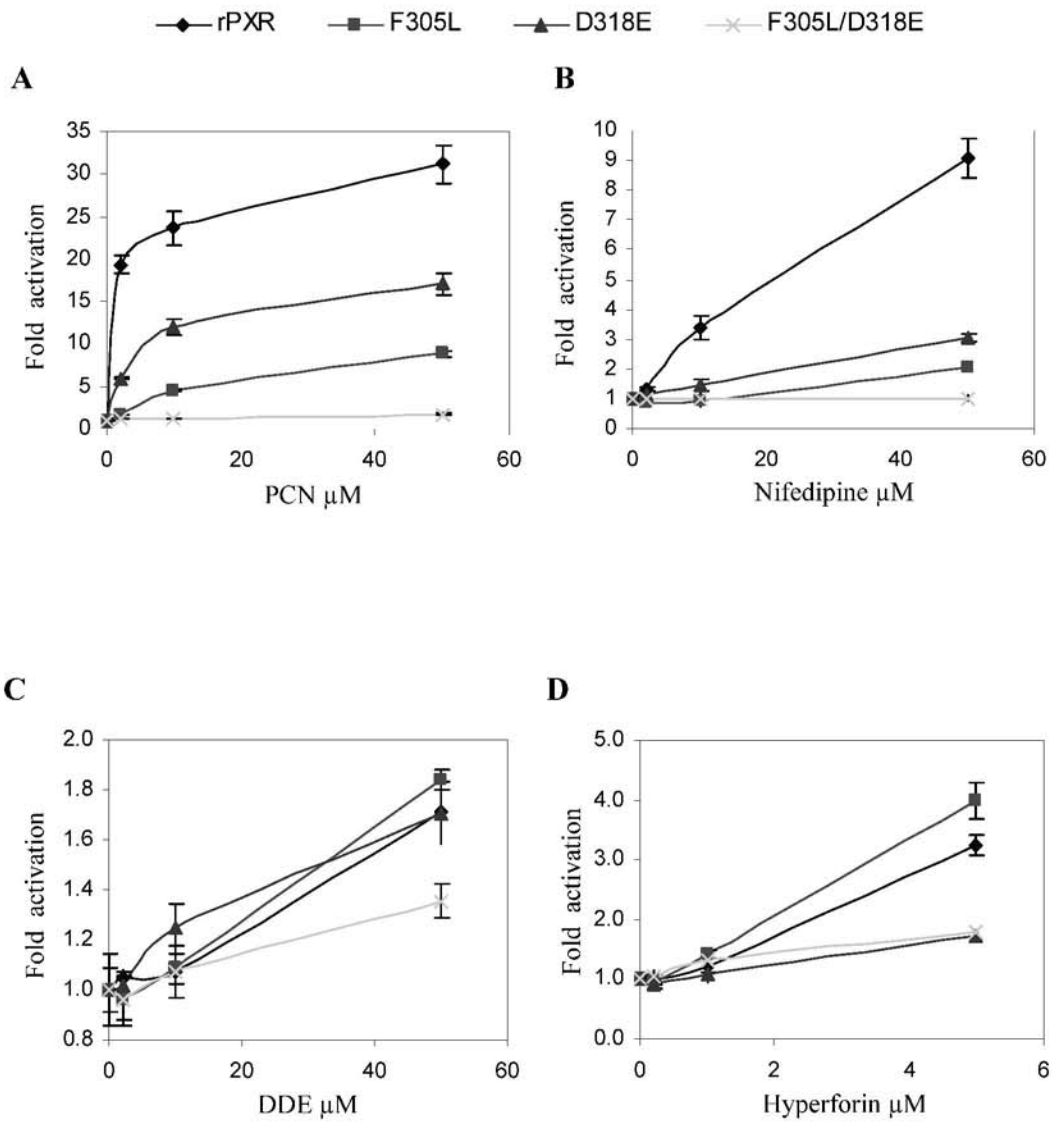

Fig. 5 JPET/2004/074971

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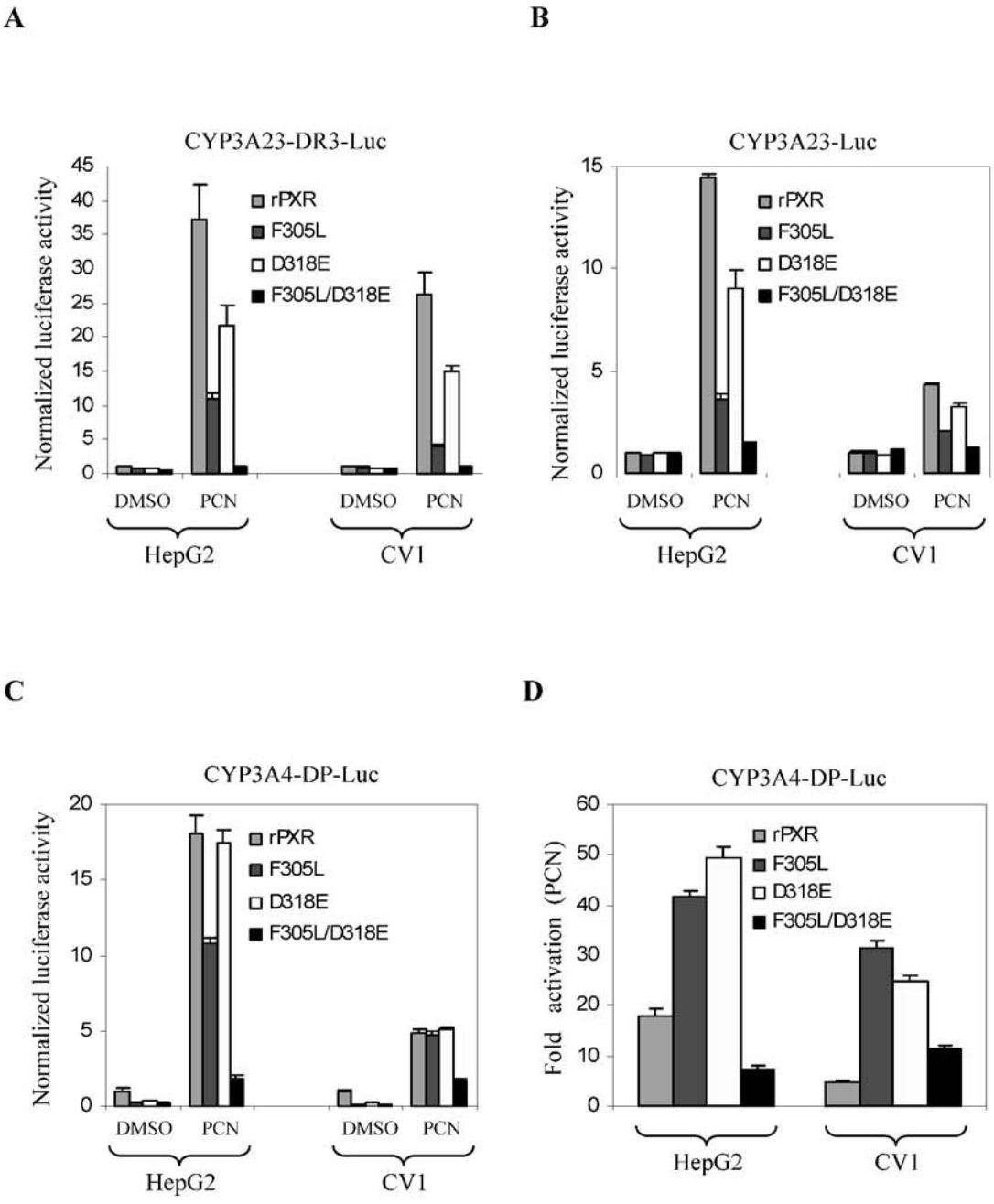

Fig. 6 JPET/2004/074971

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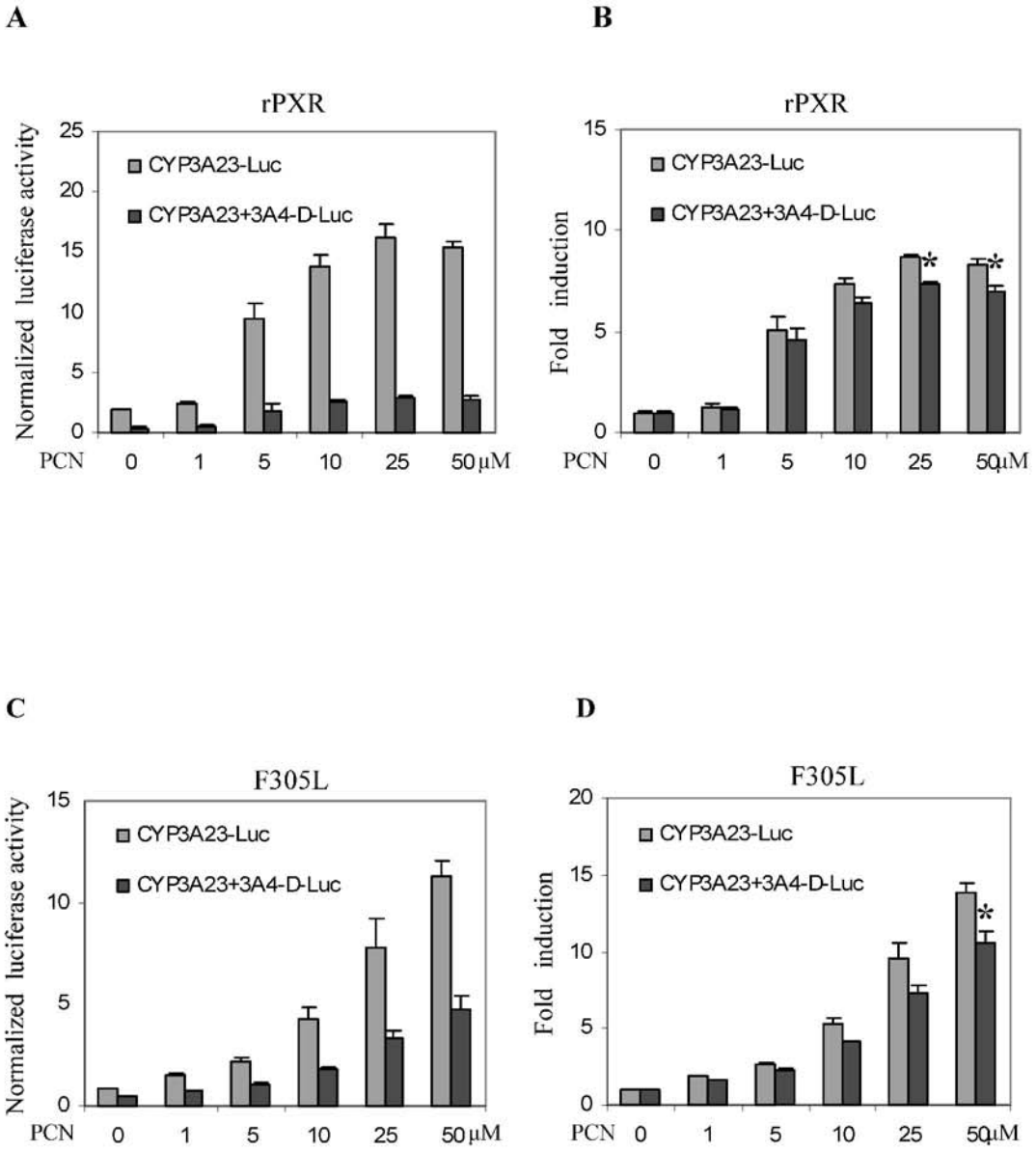

Fig. 7 JPET/2004/074971

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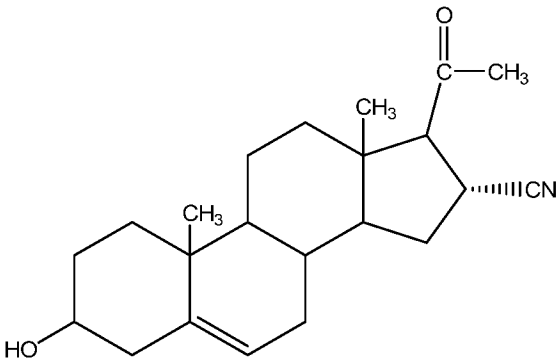
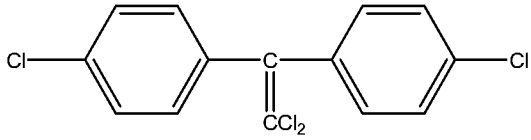
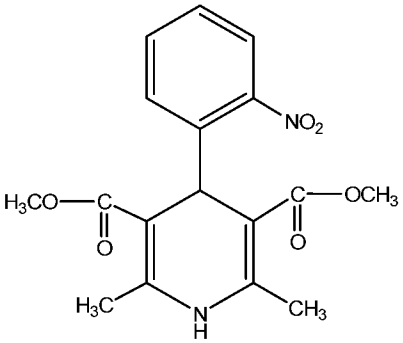


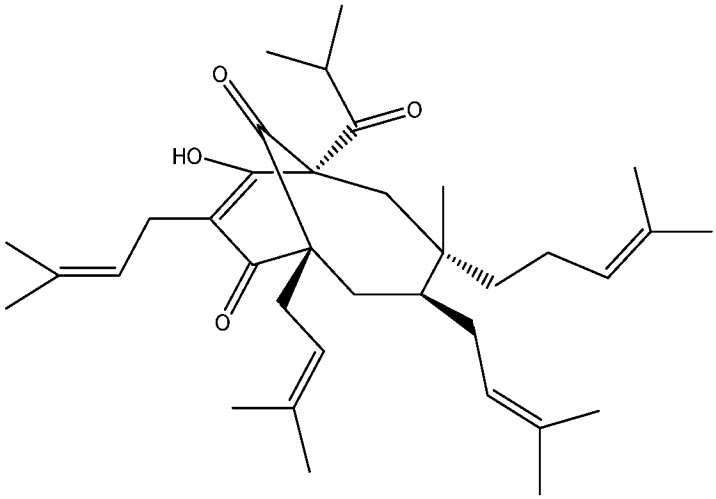
PCN (MW 342)



DDE (MW 310)



Nifedipine (MW 346)



Hyperforin (MW 537)

Fig. 8 JPET/2004/074971