INDUCTION OF CYTOCHROME P450 3A BY PACLITAXEL IN MICE: PIVOTAL ROLE OF THE NUCLEAR XENOBIOTIC RECEPTOR, PREGNANE X RECEPTOR

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ABSTRACT:

Paclitaxel, a taxane anti-microtubule agent, is known to induce CYP3A in rat and human hepatocytes. Recent studies suggest that a member of the nuclear receptor family, pregnane X Receptor (PXR), is a key regulator of the expression of CYP3A in different species. We investigated the role of PXR activation, in vitro and in vivo, in mediating Cyp3a induction by paclitaxel. Pregnenolone 16α-carbonitrile (PCN), an antiglucocorticoid, was employed as a positive control for mouse PXR (mPXR) activation in vitro, and Cyp3a induction in vivo. In cell based reporter gene assays paclitaxel and PCN activated mPXR with an EC50 of 5.6 and 0.27 μM, respectively. Employing PXR wild-type and transgenic mice lacking functional PXR (−/−), we evaluated the expression and activity of CYP3A following treatment with paclitaxel and PCN. Paclitaxel significantly induced CYP3A11 mRNA and immunoreactive CYP3A protein in PXR wild-type mice. Consistent with kinetics of CYP3A induction, the Vmax of testosterone 6β-hydroxylation in microsomal fraction increased 15- and 30-fold in paclitaxel- and PCN-treated mice, respectively. The Cyp3a induction response was completely abolished in paclitaxel- and PCN-treated PXR-null mice. This suggests that paclitaxel-mediated CYP3A induction in vivo requires an intact PXR-signaling mechanism. Our study validates the use of PXR activation assays in screening newer taxanes for potential drug interactions that may be related to PXR-target gene induction.

Paclitaxel, a member of the taxane family of anti-microtubule agents, is widely used in the treatment of several types of cancer, such as, ovarian, breast, and lung carcinomas. Cytochrome P450 3A4-mediated phase I metabolism represents a major route for inactivation and elimination of paclitaxel (Harris et al., 1994; Rahman et al., 1994). Members of the CYP3A subfamily are highly expressed in the liver and intestine and play a central role in the biotransformation of numerous endogenous substances and xenobiotics, including paclitaxel (Harris et al., 1994). In humans, CYP3A enzymes (CYP3A4, 3A5, and 3A7) collectively contribute to the metabolism of approximately two-thirds of all marketed drugs (Wrighton et al., 2000). A variety of structurally diverse compounds induce CYP3A genes, which provides a molecular basis for many clinically observed drug-drug interactions (Kocarek et al., 1995). Recent studies have shown that the pregnane X receptor (PXR1 NR1I2), a member of the nuclear receptor family, mediates induction of CYP3A genes by a wide array of xenobiotics (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). Many structurally dissimilar lipophilic compounds bind and activate PXR. In the presence of a ligand, PXR binds to xenobiotic-response elements present within the promoter region of CYP3A genes as a heterodimer with retinoid X receptor α to induce gene transcription. Recently, it was shown that paclitaxel is an effective inducer of CYP3A expression in primary cultures of rat and human hepatocytes (Kostrubsky et al., 1997; Kostrubsky et al., 1998; Nallani et al., 2001a). Furthermore, employing cell based reporter assays, Synold et al. (2001) and our group showed that paclitaxel activates the human PXR (Nallani et al., 2001b; Synold et al., 2001). In the present study, we employed PXR-null mice to investigate whether targeted disruption of PXR influences the ability of paclitaxel to induce the hepatic CYP3A expression in mice. The results indicate that the induction of CYP3A by paclitaxel is mediated by PXR in vivo.

Materials and Methods

Cell Culture, Chemicals, and Reagents. Paclitaxel, pregnenolone 16α-carbonitrile (PCN), testosterone, and 6β-hydroxytestosterone were obtained from Sigma-Aldrich (St. Louis, MO). African green monkey kidney fibroblasts (CV-1 cells) were obtained from American Type Culture Collection (Manassas, VA). Cell culture media and supplements were purchased from CellGro (Herndon, VA). Polyclonal antibody for rat CYP3A was obtained from BD Gentest Corporation (Woburn, MA). The horseradish-peroxidase conjugated
anti-goat secondary antibody was obtained from Oxford Biomedical Research Inc. (Oxford, MI). CYP3A11 oligonucleotide probes were custom made from Invitrogen (Carlsbad, CA). Enhanced chemiluminescence detection reagent was obtained from Amersham Biosciences Inc. (Piscataway, NJ).

**Mouse PXR (mPXR) Activation Studies.** Transient transfection of CV-1 cells was performed as described earlier (Jones et al., 2000). The luciferase reporter gene construct p3A4−362 (7836/7208ins) was used to examine activation of mPXR by paclitaxel and PCN, employing a concentration range of 1 nM to 10 µM. This construct contains the promoter proximal region of the CYP3A4 gene (bases −362 to +53) linked to the distal xenobiotic-response element module region (Goodwin et al., 1999). Transfection mixtures contained 8 ng luciferase reporter gene construct, 5 ng PXR expression vector (pSG5mPXR) (Lehmann et al., 1998), 8 ng pβ-actin-SPAP (an expression vector containing the secreted placent al alkaline phosphatase cDNA under control of the β-actin promoter), and 52 ng pBluescript (Stratagene, La Jolla, CA). Following overnight transfection, the cells were replenished with drug-containing medium and incubated for an additional 24 h. At the end of drug treatment, an aliquot of medium was withdrawn for SPAP assay and the cells lysed prior to luciferase determination. Luciferase activity was normalized to SPAP expression. Dose-response curves were generated and analyzed employing nonlinear data analysis (WinNonlin Standard, version 1.5; Pharsight Corporation, Mountain View, CA).

**Drug Treatment of PXR Wild-type and PXR-null Mice.** PXR-null (−/−) mice were generated and bred as previously described (Staudinger et al., 2001a). Adult male PXR wild-type (+/+) and PXR-null (−/−) mice weighing 25 to 35 g were employed in this study. The mice were provided with water and chow ad libitum during the experiment. Mice (n = 4) were randomly assigned to treatment groups receiving i.p. injection of paclitaxel (40 mg/kg) or PCN (100 mg/kg) in Cremophor solvent for 4 consecutive days. The Cremophor solvent, which comprised of Cremophor EL (5%) and ethanol (5%) in 5% dextrose for injection solution, is the clinically used paclitaxel formulation. Cremophor Solvent treated mice were maintained as controls. Twenty-four hours after the drug treatment, the mice were sacrificed employing carbon dioxide asphyxiation, and the livers were isolated and snap frozen in liquid nitrogen. Livers were processed for total RNA isolation and microsome preparation.

**Hepatic Microsomal Metabolism of Testosterone.** The CYP3A activity in liver microsomes isolated from control and treated mice was assessed employing testosterone 6β-hydroxylation assay. The methods employed for the isolation of microsomal fraction and microsomal testosterone metabolism have been described previously (Genter et al., 2002). Based on the initial assessment of the linear range of testosterone 6β-hydroxylation with respect to protein amount and incubation period, we employed 50 µg of microsomal protein and 15-min incubation period. Testosterone (1–100 µM) was incubated with liver microsomes in the presence of an NADPH-regenerating system. The reaction was initiated by the addition of glucose-6-phosphate dehydrogenase. At the end of the incubation period the reaction was terminated by the addition of the reconstituted sample (150 µl) was analyzed employing a previously described high performance liquid chromatography method (Nallani et al., 2001a). To confirm the role of CYP3A in testosterone 6β-hydroxylation, microsomal testosterone metabolism was also examined in the presence of ketoconazole, a known inhibitor of CYP3A. For this purpose, testosterone (10 µM) metabolism was carried out as described in the presence of ketoconazole (10 µM).

**Northern blot Analysis.** Total RNA from control and drug-treated mouse livers was isolated using TRIzol reagent (Invitrogen) and quantitated spectroscopically. Total RNA (10 µg) was then fractionated by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde, followed by overnight transfer onto a nylon membrane (Millipore, Bedford, MA). Equal loading per lane was verified by ethidium bromide staining of 18S and 28S ribosomal RNA, which was visualized and photographed under UV illumination. For the detection of CYP3A11 mRNA, we used the previously described 30 base oligonucleotide probe (5′-TGTCCAGTGTCTCCTAGACATCGCCTTCTG-3′) (Sewer et al., 1998). The oligonucleotide probe was 5′-end labeled with γ32P-ATP using T4 polynucleotide kinase. Hybridization was carried out in the presence of 100 µg/ml of ssDNA (Sigma-Aldrich) at 45°C. Northern membranes were washed at 45°C as described previously (Church and Gilbert, 1984). An autoradiogram was developed using Kodak X-OMAT X-ray film following 2- to 3-day exposure (Eastman Kodak, Rochester, NY).

**Western Blot Analysis.** Western blot analysis of the microsomal protein was performed exactly as described previously (Nallani et al., 2001a). Immunoreactive CYP3A was detected employing a polyclonal anti-rat CYP3A antibody (1:1500) for 1 h. This polyclonal antibody nonspecifically detects various CYP3A isoforms in mouse liver (Yamada et al., 2002). The antibody binding was visualized using a horseradish peroxidase-conjugated anti-goat secondary antibody for polyclonal CYP3A antibody (1:15000 dilution), followed by enhanced chemiluminescence detection (ECL; Amersham Biosciences Inc.). The immunoblots were quantitated employing NucleoVision image analyzer with Gel Expert photodensitometry software (NucleoTech, San Carlos, CA).

**Data Analysis.** Statistical analysis of 6β-hydroxytestosterone formation and -fold increase in immunoreactive CYP3A protein and CYP3A11 mRNA among different treatment groups was performed employing one-way analysis of variance, followed by Tukey’s test. A p < 0.05 was interpreted as the level of statistical significance. The kinetics of microsomal metabolism were analyzed employing WinNonlin Standard (version 1.5) (Pharsight Corporation). The velocity of 6β-hydroxytestosterone formation was plotted as a function of substrate (testosterone) concentration. Data were fitted to a sigmoidal equation

\[
V = V_{\text{max}} \frac{[S]^n}{K_m + [S]^n} + \text{error}
\]

where \(V\) = reaction velocity (nmol/min/mg of microsomal protein), \(V_{\text{max}}\) = maximum velocity, \(S\) = substrate concentration (µM), and \(K_m\) = substrate concentration at half maximal velocity. \(n\) = hill coefficient or coefficient of sigmoidicity. Goodness of fit was assessed based on visual inspection, residual analysis, Akaike and Schwartz criteria, and percent coefficient of variation for \(V_{\text{max}}\) and \(K_m\) values.

**Results and Discussion**

**mPXR Activation.** Activation of PXR and CYP3A induction by different xenobiotics is known to exhibit interspecies variability (Jones et al., 2000). Although it is recognized that paclitaxel activates human PXR, whether it activates PXR from other species is not known. Therefore, we first examined the effect of paclitaxel on mPXR activity employing transient transfection of a reporter gene construct harboring PXR-9-cis retinoic acid responsive elements of CYP3A gene in CV-1 cells. The mPXR activation profiles of PCN, a known CYP3A inducer in rodents and an mPXR ligand, and paclitaxel at a concentration range of 1 nM to 10 µM were compared (Fig. 1). The concentration response for each compound was fit to a Sigmoidal equation employing WinNonlin Standard (version 1.5; Pharsight Corporation) and the maximal effect (E max) and half-maximal effective concentration (EC50) were determined from the model. The EC50 values for PXR activation by paclitaxel and PCN were 5.6 and 0.27 µM, respectively. This indicates that paclitaxel is a strong activator of mPXR, albeit weaker than PCN.

**Paclitaxel-mediated CYP3A Induction in Vivo.** Previous studies have shown that paclitaxel induces CYP3A in rat and human hepatocytes in vitro (Kostrubsky et al., 1998; Nallani et al., 2001a; Synold et al., 2001). However, to date the ability of paclitaxel to induce Cyp3a expression in vivo has not been characterized. The ability of paclitaxel to activate mouse PXR in cell-based assays suggested that the induction of Cyp3a may be mediated by this receptor. Therefore, we compared the induction of Cyp3a in wild-type mice and mice lacking functional PXR. In these experiments, PCN was employed as a positive control for PXR activation. Figure 2A shows the expression of CYP3A11 mRNA in wild-type and PXR-null mice determined using Northern blot analysis. As shown, PCN increased CYP3A11 mRNA levels by 5-fold (range 4- to 7-fold) in PXR wild-type mice. The observed PCN-mediated increase in CYP3A11 mRNA is in agreement with previously published reports (Xie et al., 2000a; Staudinger et al., 2001a,b). Paclitaxel caused a 2.5-fold (range 1.9- to
2.7-fold) induction of CYP3A11 mRNA in PXR wild-type mice. The induction of Cyp3a11 expression by paclitaxel and PCN was completely absent in PXR-null mice; the mRNA levels in paclitaxel- and PCN-treated PXR-null mice were not significantly different from controls (p > 0.05). Interestingly, we observed an approximately 2-fold increase in the basal CYP3A11 mRNA levels in PXR (−/−) mice compared to those in PXR wild type (+/+), which is consistent with previously reported observations (Staudinger et al., 2001a).

Western blot analysis of hepatic microsomal protein fractions isolated from PXR (+/+) and PXR (−/−) revealed that the pattern of increase in the expression of CYP3A immunoreactive protein levels mirrored the increase in the observed CYP3A11 mRNA levels (Fig. 2B). Accordingly, the expression of CYP3A immunoreactive protein levels were 3.5-fold (range 2.6- to 4.5-fold) and 4.6-fold (range 4- to 6.5-fold) higher in PXR (+/+) mice treated with paclitaxel and PCN, respectively, compared with the vehicle-treated PXR (−/−) mice (Fig. 2B). It is noteworthy that the representative Western blot depicting immunoreactive CYP3A protein levels in mouse liver microsomes prepared from PXR wild-type and PXR-null mice treated with vehicle, paclitaxel, or PCN.

To confirm the involvement of the CYP3A enzyme in mediating testosterone 6β-hydroxylation, we evaluated the effect of ketoconazole on the microsomal testosterone metabolism. Ketoconazole is an anti-fungal agent, which is a known inhibitor of CYP3A. As such, it is widely used as one of the tools in identifying the role of CYP3A enzyme in drug metabolism. In the absence of ketoconazole, the rates of metabolic conversion of testosterone 6β-hydroxylation were determined by measuring testosterone 6β-hydroxylation activity in microsomal preparations from PXR wild-type and PXR-null mice treated with vehicle, paclitaxel, or PCN.

The hepatic CYP3A activity of microsomal fractions prepared from PCN and paclitaxel-treated mice was estimated by measuring testosterone 6β-hydroxylation. The rate of the hydroxylation was plotted as a function of substrate (testosterone) concentration, and the kinetics of the reaction was determined. Table 1 shows the Vmax and Km values for 6β-hydroxylation of testosterone by the various microsomal preparations. The Vmax was significantly (p < 0.01) higher in paclitaxel- (41 nmol/min/mg) and PCN-treated (93 nmol/min/mg) mice, compared with the vehicle-treated PXR (+/+) mice (3.1 nmol/min/mg). On the other hand, the Km values remained unchanged (p > 0.05). The observed increase in the Vmax with no change in Km is suggestive of induction of drug-metabolizing enzyme. In contrast, in the PXR-null (−/−) mice, neither the Vmax nor the Km values were different in PCN- or paclitaxel-treated mice compared with the vehicle-treated mice (p > 0.05). Thus, targeted disruption of PXR in mice completely abolished the increase in CYP3A activity of hepatic microsomes prepared from PCN- and paclitaxel-treated animals.

### Table 1

**Kinetics of hepatic microsomal testosterone 6β-hydroxylation**

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<thead>
<tr>
<th></th>
<th>Control</th>
<th>PCN</th>
<th>Paclitaxel</th>
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<tbody>
<tr>
<td><strong>PXR Wild Type</strong></td>
<td></td>
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<tr>
<td>Km (μM)</td>
<td>24.6 ± 2.9</td>
<td>20.4 ± 3.5</td>
<td>17.6 ± 5.4</td>
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<tr>
<td>Vmax (nmol/min/mg protein)</td>
<td>3.1 ± 0.2</td>
<td>93.4 ± 13.1</td>
<td>41.4 ± 8.4</td>
</tr>
<tr>
<td>k</td>
<td>1.7 ± 0.23</td>
<td>1.9 ± 0.1</td>
<td>1.6 ± 0.16</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PCN</th>
<th>Paclitaxel</th>
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<tbody>
<tr>
<td><strong>PXR-null</strong></td>
<td></td>
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</tr>
<tr>
<td>Km (μM)</td>
<td>28.2 ± 4.5</td>
<td>26.5 ± 3.9</td>
<td>29.9 ± 10</td>
</tr>
<tr>
<td>Vmax (nmol/min/mg protein)</td>
<td>4.5 ± 0.36</td>
<td>6.06 ± 0.74</td>
<td>3.5 ± 1.3</td>
</tr>
<tr>
<td>k</td>
<td>1.35 ± 0.16</td>
<td>1.4 ± 0.2</td>
<td>1.56 ± 0.6</td>
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* Vmax values indicated for the treatment group are significantly different (p < 0.05) compared to vehicle-treated PXR wild-type mice (n = 4).
inhibited the testosterone 6β-hydroxylation activity in all of these cases (data not shown). Clearly, these observations suggest that the testosterone 6β-hydroxylation reaction in the mouse hepatic liver microsomal incubations was primarily mediated by CYP3A.

To summarize, our studies indicate that paclitaxel activates the mPXR and induces the transcription and increases the activity of CYP3A in mice with an intact PXR-signaling mechanism. In mice subjected to targeted disruption of PXR, the ability of paclitaxel to induce the enzyme was completely eliminated. It is well established that in addition to PXR, another orphan nuclear receptor referred to as the constitutive androstane receptor (CAR) is capable of activating expression of CYP3A genes. Like PXR, CAR is activated by a structurally diverse set of compounds (Moore et al., 2000; Xie et al., 2000b). Since paclitaxel-mediated induction of CYP3A is completely abolished in PXR null mice, it appears that paclitaxel induction also occurs in a PXR-specific manner. The data presented in this paper suggest that in rodents, induction of CYP3A expression by paclitaxel may not be mediated by CAR since mice lacking functional PXR fail to induce CYP3A expression when exposed to this compound. Moreover, in humans, the role of CAR relative to PXR appears to be restricted. A selective ligand for human CAR has not been identified thus far, and the overall contribution of CAR in CYP3A regulation in humans awaits further investigation (Moore et al., 2000; Tzameli et al., 2000). Since P450 enzymes exhibit marked qualitative and quantitative differences among species, P450 induction studies of investigational agents rely mainly on the use of primary cultures of human hepatocytes. Given the numerous complexities involved in the availability and use of these tissues, availability of alternative approaches may considerably help in accelerating drug development. In this context, the use of in vitro PXR activation and/or binding studies are rapidly gaining acceptance for preliminary screening of compounds that may induce CYP3A and other genes. Our findings validate the use of PXR activation assays as an important tool in the development of taxane anti-cancer drugs. These approaches may reduce our dependence on the use of primary cultures of human hepatocytes and, as such, they may be important tools in drug development.

References


