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A PXR reporter gene assay in a stable cell culture system: CYP3A4 and CYP2B6 induction by pesticides

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Abstract

A stable hepatoma cell line expressing the human pregnane X receptor (hPXR) and the cytochrome P4503A4 (CYP3A4) distal and proximal promoters plus the luciferase reporter gene was developed to assess the ability of several xenobiotic agents to induce CYP3A4 and CYP2B6. After selection for neomycin resistance, one clone, displaying high luciferase activity in response to rifampicin (RIF), was isolated and the stable expression of hPXR was confirmed by reverse transcription polymerase chain reaction (RT-PCR). Dose-response curves were generated by treating these cells with increasing concentrations of RIF, phenobarbital (PB), clotrimazole (CLOT) or 5β-pregnane-3,20-dione (5β-PREGN). The effective concentrations for half maximal response (EC_{50}) were determined for each of these compounds. RIF was the most effective compound, with maximal luciferase activity induced at 10 μM. The agonist activities of PXR-specific inducers measured using our stable model were consistent with those measured in transient transfectants. The abilities of organochlorine (OC), organophosphate (OP) and pyrethroid pesticides (PY) to activate hPXR were also assessed and found to be consistent with the abilities of these compounds to induce CYP3A4 and CYP2B6 in primary culture of human hepatocytes. These results suggest that CYP3A4 and CYP2B6 regulation through PXR activation by persistent pesticides may have an impact on the metabolism of xenobiotic agents and endogenous steroid hormones. Our model provides a useful tool for studying hPXR activation and for identifying agents capable of inducing CYP3A4 and CYP2B6.

Keywords: Pregnane X recepteur; Stable reporter assay; Human hepatocytes; Pesticides; Cytochrome P450; Endocrine disruptors

1. Introduction

The liver and the intestine are sites of major metabolic activity for both endogenous and exogenous chemicals. Members of the CYP monooxygenase family catalyze the oxidative metabolism of a wide variety of endogenous substances and xenobiotic agents [1]. CYP3A4 monooxygenase plays a major role in drug biotransformation in humans and is present at high concentrations in the liver and intestine. This enzyme has a broad substrate specificity and is able to metabolize more than 60% of the drugs that are clinically available [2]. The induction of CYP3A transcription forms the basis of a number of common drug–drug interactions, making it important to identify the agents responsible for this up regulation. However, these agents are difficult to predict because this CYP expression is enhanced by a wide variety of structurally different compounds. This problem can be resolved by developing inexpensive and reproducible tests using in vitro systems for assessing the ability of new drug candidates to enhance the expression of drug-metabolizing enzymes. CYP2B6 was thought to play a minor role in human drug metabolism and xenobiotic biotransformation. However, recent studies have shown that up to 25% of pharmaceutical drugs are metabolized by CYP2B6 [3]. Induction of CYP3A4 and CYP2B6 transcription results

Abbreviations: CAR, constitutive androstane receptor; CLOT, clotrimazole; CYP, cytochrome P450; DMEM, Dulbeccos modified Eagles medium; DMSO, dimethyl sulfoxide; EC_{50}, effective concentration for half maximal response; FBS, fetal bovine serum; GADPH, glyceraldehyde-3-phosphate dehydrogenase; hPXR, human pregnane X receptor; PB, phenobarbital; 5β-PREGN, 5β-pregnane-3,20-dione; PY, pyrethroid pesticides; OC, organochlorine pesticides; OP, organophosphate pesticides; RIF, rifampicin; RT-PCR, reverse transcription polymerase chain reaction; Wy-14,643, [4-chloro-6-(2,3-xylidino)-2-pyrimidylthio]acetic acid; XREM, xenobiotic-responsive enhancer module

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from the activation of hPXR (NR1I2) [4–7]. This receptor forms a heterodimer with the retinoid X receptor (NR2B1), and it has been proposed that this complex constitutes a xenobiotic-responsive transcription factor that regulates multiple drug-metabolizing enzymes [8]. Although, PXR was originally identified as a regulator of CYP3A, many of the agents that induce CYP3A transcription also induce transcription of CYP2B [9].

The hPXR is activated by a wide variety of lipophilic compounds, including agents that induce CYP3A4 and CYP2B6 [7]. Although several compounds can activate both mouse PXR and hPXR, these receptors exhibit divergent activation profiles [10]. This difference between mouse PXR and hPXR activation profiles correlates well with the fact that the CYP3A induction profile is also species–specific [10]. After activation, PXR binds to specific DNA sequence motifs in CYP3A genes and functions as a transcription factor for target gene regulation. The proximal promoter of the CYP3A4 gene contains two copies of an AG(G/T)TCA hexamer, organized to form an ER6 (everted repeat separated by six nucleotides) motif. Barwick et al. [11] demonstrated using rabbit hepatocytes that these half-sites were sufficient to confer rifampicin-responsiveness on a reporter gene construct. However, in comparison to hepatocytes, the extent of this rifampicin response was limited, even when the reporter gene construct contained multiple copies of the CYP3A4 proximal ER-6 element. Additional regulatory regions of the CYP3A4 gene, such as those involved in transcriptional activation by xenobiotics, were defined by Goodwin et al. [12]. These authors isolated and characterized a distal xenobiotic-responsive enhancer module (XREM), which regulates transactivation of the *CYP3A4* gene in response to agents that are also able to induce PXR activation. In addition, these authors showed that the activation mediated by the response element in the distal XREM and that mediated by the proximal promoter region of *CYP3A4* were a cooperation.

Goodwin et al. [9] showed that transcription of human *CYP2B6* is regulated directly by PXR. Transactivation of CYP2B6 by PXR is mediated by the phenobarbital-responsive element enhancer module (PBREM) region of the gene. This 51-bp enhancer module regulates constitutive androstane receptor (CAR)-mediated induction of CYP2B6 [13]. The PBREM contains two DR4 (direct repeat with four base pairs) elements that are capable of binding PXR-RXRα. In addition, a distal region of the *CYP2B6* promoter, together with the PBREM, mediates drug-induced transcription of *CYP2B6*. Wang et al. [14] showed that this distal response region could be activated by PXR.

In rats, OCs, such as DDT and methoxychlor, strongly induce the transcription of CYP2B isoforms and, to a lesser extent, that of the CYP3A subfamily members [15]. Chlordane, dieldrin and endosulfan activate hPXR and subsequently CYP3A4 mRNA expression in human hepatocytes [16]. PY have a toxicity profile similar to OCs and also induce the CYP2B protein and mRNA in rat hepatocytes [17]. CYP3A1 and CYP2B1 catalyze the hydroxylation of testosterone in the rat liver and thus, agents inducing the transcription of these enzymes lower the levels of testosterone in the circulation. Phenobarbital (PB), a well known CYP2B and CYP3A inducing agent, increases androgen hepatic metabolism [18] and as a result leads to developmental abnormalities reflecting androgenic deficiency. OCs have an antiandrogenic effect and may therefore repress androgen-mediated gene activation. In addition, the level of testosterone breakdown mediated by CYP2B and CYP3A may increase as a result of PXR activation by OCs. These synergetic effects may induce reproductive abnormalities and cause demasculinization.

We have developed a stable cellular model to study the activation of hPXR by persistent pesticides and to determine whether this activation was consistent with these compounds inducing CYP3A4 transcription. In addition, we examined the induction of CYP2B6 transcription by these persistent pesticides. For the stable in vitro system, human hepatoma cells were transfected with the entire hPXR gene, the XREM region (bases −7836 to −7208) and the CYP3A4 proximal promoter (bases −362 to +53) plus the luciferase reporter gene. The ability of this cell line to respond to human PXR-specific ligands was then investigated by analyzing the level firefly luciferase activity.

2. Materials and methods

2.1. Materials

All cell culture materials were purchased from Life technologies. Carbaryl (99.5%), 2,4′-DDT (98.0%), diflubenzuron (98.5%), lindane (99.4%), mancozeb (74.0%), 2,4,5-T (97.2%) were obtained from Cluzeau Info Labo. Alachlor (99%), aldrin (98.5%), atrazine (98%), chlordane (mix of isomers), chlorpyrifos (99.5%), cypermethrin (39% trans and 59% cis), 2,4-D (98%), DDT mixture (18% o,p′-DDT; 75% p,p′-DDT), dieldrin (98.8%), endosulfan (99.9%), endrin (99%), pentachlorophenol (99%) were obtained from ChemService. Trans-nonachlor (99.3%) was obtained from AccuStandard Inc. Chlordecone (99.2%), fenvalerate (99.9%), methoxychlor (98.7%), RIF, CLOT, PB were purchased from Sigma–Aldrich. Steady-Glo was purchased from Promega. WY 16,643 ([4-chloro-6-(2,3-xylidino)-2-pyrimidylthio]acetic acid) was obtained from Alexis. 5β-PREGN was purchased from Steraloids. HotStar Taq DNA polymerase, Rneasy, Dnase and the plasmid purification kits were obtained from Qiagen.

2.2. Cell culture

The human hepatocellular carcinoma cell line, HepG2, was obtained from the American Type Culture Collection

and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Biowest). The HepG2-derived cell line (hPXR/HepG2) was cultured as described above, with the exception that the culture medium was also supplemented with 800 μg/mL geneticin (G418, Invitrogen).

Hepatocytes from human liver surgical biopsies (resected from secondary tumors) were isolated by a reverse, two-step, collagenase perfusion as previously described [19]. The isolated cells were resuspended in Williams' medium E containing 10% FBS, penicillin (50 UI/mL), streptomycin (50 μg/mL) and insulin (0.1 UI/mL). Hepatocyte viability was determined using the Erythrosin B exclusion test and was at least 80%. Hepatocytes were seeded on collagen type I-coated dishes and incubated for 4 h at 37 °C in a humidified atmosphere consisting of 95% air and 5% CO_2. The existing medium was then discarded and replaced by medium identical to that described above with the exception that no FBS was added and this medium was supplemented with 1 μM hydrocortisone hemisuccinate and 0.024% (m/v) bovine serum albumin. Hepatocytes were treated over a 2-day period (one treatment every 24 h) with 10 μM OC, OP, PY, 50 μM dexamethasone, 25 μM RIF, 0.5 mM PB, 1 μM 3-MC. These molecules were solubilized in dimethyl sulfoxide (DMSO, final concentration 0.5%, v/v) and then added directly to the cultures.

2.3. Plasmids

The pCDNA 3.1*neo*-hPXR expression plasmid was constructed using pSG5-hPXRΔATG (a kind gift from Dr. Steve Kliewer [7]) as follows: pCDNA 3.1-hPXR was generated by PCR amplification of pSG5-hPXRΔATG, using oligonucleotides (5′-GGG AAT TCA CCA CCA TGG AGG TGA GAC CCA AAG AAA GC-3′ and 5′-GGT CTA GAC TCA GCT ACC TGT GAT GCC G-3′), which introduced an *Eco*RI and an *Xba*I restriction site. This fragment was then cloned into the *Eco*RI/*Xba*I site of a pCDNA 3.1 vector carrying a G418 resistance gene as a selectable marker. This construction was verified by enzyme digestion and confirmed, along with verification of the correct reading frame, by sequencing. The p3A4-362(7836/7208ins) reporter construct (carrying the XREM region, the CYP3A4 proximal promoter and the luciferase reporter gene [12]) was generously provided by Dr. Chris Liddle.

2.4. Transient transfection

Transfection was carried out using plasmid cDNA prepared by purification on Qiagen columns. HepG2 cells, in 100 mm culture plates at a density of 2.4×10^6 cells, were cotransfected with 2 μg of the pCDNA 3.1*neo*-hPXR expression plasmid, 8 μg of p3A4-362 (7836/7208ins) reporter construct, 0.7 μg of pRL-CMV expression plasmid as an internal control, and 9 μg of pCDNA 3.1 carrier plasmid using Lipofectamine 2000 and the procedure recommended by the manufacturer (GIBCO BRL). After 24 h, the cells were trypsinized and seeded in 96-well plates at a density of 20,000 cells/well. Twenty-four hours later, RIF, CLOT, 5β-PREGN, pesticides (10 μM) or DMSO (0.1%) were added to the medium. Luciferase activity was determined 24 h later using the Dual-Glo™ Luciferase assay System (Promega). Firefly luciferase activities were determined on three independent transfections and were normalized by comparison with the *Renilla* luciferase activities of the internal control pRL-CMV vector from the same culture.

2.5. Stable transfection

The HepG2 cells were seeded in 10-cm culture dishes at a density of 1.2×10^6 cells in DMEM containing 10% FBS. After a 48-h recovery period, the cells were transfected overnight with a mixture containing the p3A4-362(7836/7208ins) reporter construct and pCDNA 3.1*neo*-hPXR expression plasmid at a ratio of 10:1 (22 μg DNA in total) using Lipofectamine 2000 and the procedure recommended by the manufacturer (Gibco BRL). When the cells had been exposed to the precipitated DNA for 24 h, the culture medium was removed and replaced with fresh DMEM containing 10% FBS. After a further 24 h, this medium was then replaced by DMEM containing 800 μg/mL G418. The medium was renewed every 3 days for 1 month until small colonies were visible. One month after the initiation of G418 selection, clones that expressed luciferase were identified as follows: the culture medium was replaced with fresh DMEM containing 0.3 mM sterile luciferin. Plates were then placed in the dark and luciferase activity was measured (in arbitrary luminescence values) using a molecular light camera (Night-Owl) linked to the Win light program. After analysis, the luciferin-containing medium was removed and replaced by fresh DMEM containing 10 μM RIF. After 24 h, luminescent and inducible clones were identified with the camera as described above. They were isolated in culture using sterile Teflon cloning rings and grown in DMEM supplemented with 10% FBS and G418 (800 μg/mL).

2.6. PXR assay: stable gene expression assay

The HepG2-derived cell line (hPXR/HepG2) was seeded in triplicate in white 96-well plates, with a density of 30,000 cells/100 μL of DMEM supplemented with 10% serum stripped of steroids by charcoal/dextran treatment. After a 24-h recovery period, the cells were left to incubate for 24 h in DMEM supplemented with the various compounds being tested. Chemicals were stored as DMSO stock solutions and the final DMSO concentration in the culture medium never exceeded 0.1%. At the end of this

incubation period, the culture medium was replaced by the contents of the Steady-Glo® Luciferase Assay System (Promega). Luciferase activity was measured using a MicroBeta Wallac luminometer (EG&G Wallac) and luminescence was measured following cellular lysis for 2 s per well. Luminescence was stable for at least 2 h.

2.7. RNA preparation, RT-PCR and PCR

Total RNA from HepG2 was isolated according to the manufacturer's instructions using the Rneasy kit (Qiagen) with on-column Dnase treatment. RNA was quantified using the Ribogreen RNA Quantitation Kit (Molecular Probes). First-strand cDNA was synthesized from 1 μg of total RNA in a 20 μL reaction mixture using the Taqman PCR Core Reagent kit and random hexanucleotide primers. The primers 5′-GAC CCA AAG AAA GCT GGA-3′ (forward) and 5′-AGC ACA TAC TCC TCC TCA-3′ (reverse) for hPXR and, 5′-AAT CCC ATC ACC ATC TTC CA-3′ (forward) and 5′-GTC ATC ATA TTT GGC AGG TT-3′ (reverse) for human glyceraldehyde-3-phosphate dehydrogenase (hGADPH) were used to amplify the 1014 and 557 bp fragments, respectively. The cDNA obtained was diluted twenty times and 5 μL was amplified in a 20 μL reaction volume containing 0.5 U HotStar *Taq* DNA polymerase, 1X PCR buffer, 50 pmol of both primers and 0.2 mM dNTP mix. Amplifications were performed using a thermocycler with the following PCR profile: 95 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s. Thirty cycles were used for amplification of both hGADPH and hPXR.

2.8. Western blot

Hepatocytes were lysed in 100 mM phosphate buffer (pH 7.4), scraped and disrupted. Protein concentration was estimated using the Pierce bicinchoninic acid Protein Assay Kit and BSA as the standard. Ten micrograms of the cell protein extracts were loaded onto a 10% SDS-polyacrylamide gel and subjected to electrophoresis. Proteins were then transferred onto a PVDF membrane by electroblotting. After blocking with 5% non-fat, skimmed milk suspended in TTBS (10 mmol/L Tris–HCl [pH 7.5], 140 mmol/L NaCl, 0.1% Tween 20) overnight at 4 °C, the membranes were incubated with anti-human CYP3A4 monoclonal antibody (Oxford) or anti-human CYP2B6 monoclonal antibody (Gentest) in TTBS containing 3% BSA. The membranes were then washed with TTBS and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody. Peroxidase-labeled proteins were revealed using the ECL® detection kit (Amersham Life Sciences) and Kodak ML-Biomax films (Eastman Kodak). The films were scanned and densitometric quantification (Scion imaging Software) was used to asses the intensity of the CYP3A4 and CYP2B6 immunoreactive bands.

3. Results

3.1. PXR expression in the stable HepG2 transfectant

A one-step transfection procedure was used to obtain a stable cell line expressing functional PXR and the p3A4-362 (7836/7208ins) reporter construct. Colonial luciferase activity was monitored and one G418-resistant clone was selected on the basis of its luminescence after incubation with 10 μM RIF (Fig. 1A).

The stable integration of PXR was verified by RT-PCR. RNA from stable hPXR/HepG2 cells and HepG2 cells was subjected to reverse transcription and hPXR mRNA was assessed after PCR amplification. After 30 cycles, a 1014-nt fragment corresponding to hPXR cDNA was detected from the stable hPXR/HepG2 cells, whereas no fragment of this size was detected from the HepG2 cells (Fig. 1B), confirming that the lower expression of the hPXR in the HepG2 cells. In addition, Fig. 1C shows that none of the typical PXR inducers, such as CLOT (10 μM) and 5β-PREGN (10 μM), were able to induce the expression of the reporter gene in cells transfected with the XREM reporter plasmid but not the hPXR expression plasmid. RIF induced expression of the reporter construct to a limited extent (<2-fold) in the absence of cotransfected PXR.

To validate our model system, we compared RIF-, CLOT-, 5β-PREGN-regulated expression of luciferase in cells transiently transfected with the luciferase reporter construct with that in the stably transfected HepG2 cells. The results of the transactivation studies were similar for both the stable hPXR/HepG2 and the transiently transfected HepG2 cells. There was no significant difference in the levels of luciferase expression between transiently transfected HepG2 cells and stable hPXR/HepG2 cells incubated in the presence 10 μM concentrations of RIF, CLOT and 5β-PREGN for 24 h. RIF (10 μM) induced the highest level of luciferase expression in both the stable and transient assays, followed by CLOT (10 μM) and then 5β-PREGN (10 μM). The extent of the response (fold induction) to the three compounds was slightly lower in the stable cell line compared to the transient cell line.

hPXR/HepG2 cell sensitivity and the reproducibility of the assay were assessed by measuring the response to known hPXR activators, such as RIF, CLOT, PB and 5β-PREGN (Fig. 2). Twenty-four hours after seeding, the cells were treated with increasing concentrations of RIF (1.5×10^{-9} to 3×10^{-5} M), CLOT (1.5×10^{-9} to 1×10^{-5} M), 5β-PREGN (1.5×10^{-9} to 5.0×10^{-4} M) and PB (7.6×10^{-8} to 1.5×10^{-3} M) for 24 h and the concentrations required to obtain the EC_{50} value were determined by non-linear sigmoidal analysis of the dose-response of hPXR to these compounds. Values are expressed as percentage luciferase activity and were calculated by using the value obtained in the presence of 10 μM RIF as the standard for 100% luciferase activity.

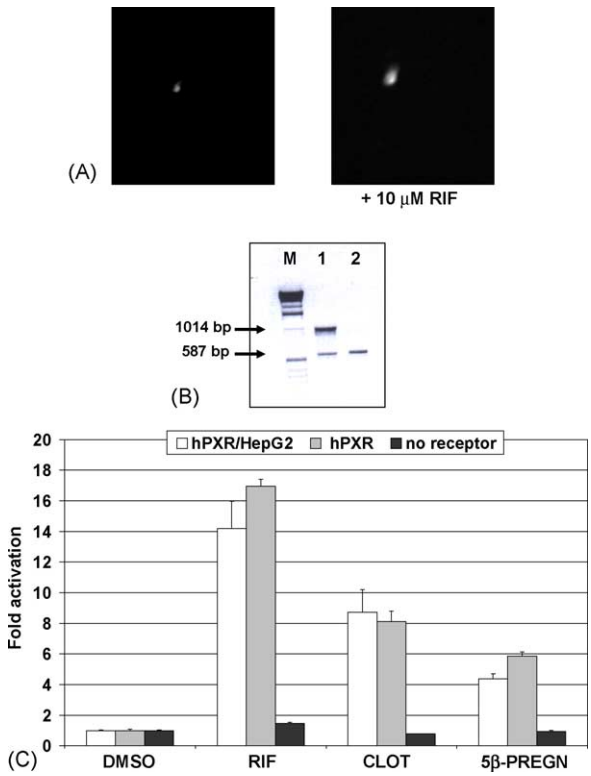

Fig. 1. Comparison between hPXR/HepG2 cells and HepG2 cells. (A) Detection of stably transfected clones expressing the XREM-regulated luciferase gene. The expression of luciferase was determined using a photon-counting camera and a nondestructive assay. Measurements were taken before and 24 h after addition of 10 μM RIF to the medium. (B) cDNA was derived from 1 μg the total RNA extracted from the hPXR/HepG2 or HepG2 cells. For both cell types this cDNA was then subjected to 30 cycles of PCR amplification using gene-specific oligos for PXR and GADPH. Reaction products were visualized after separation on 2% agarose gels containing ethidium bromide. Product size was estimated by comparison with a 1-kb ladder: 1014-nt for PXR and 557-nt for GADPH. *Lane M*, 1-kb ladder; *lane 1*, PXR/HepG2 cells; *lane 2*, HepG2 cells. (C) Comparison of the transient HepG2 transfectants and the stable hPXR/HepG2 cell line. HepG2 cells were cotransfected with the XREM reporter plasmid, either in the absence (closed bars) or in the presence (open bars) of the pCDNA 3.1-hPXRΔATG expression plasmid. The cells were incubated with 10 μM rifampicin (RIF), 10 μM clotrimazole (CLOT) or 10 μM 5β-pregnan-3,20-dione (5β-PREGN) for 24 h. Data represent the mean of assays performed in triplicate ± S.D.

Measurements were taken for each well (with triplicate wells for each dose) and the values are the mean ± S.D. of three individual experiments.

For the stable hPXR/HepG2 cell line, the EC_{50} values for RIF-, CLOT-, 5β-PREGN and PB-mediated induction of the p3A4-362 (7836/7208ins) reporter gene (Table 1) were highly consistent with those reported in the literature for hPXR activation by these drugs.

3.2. Stable transfectant response to various pesticides

The abilities of pesticides belonging to different families, such as OC, OP and PY, to induce luciferase activity were tested in stable hPXR/HepG2 cells using 3-methylcholanthrene (3-MC, 1 μM), clofibrate (10 μM), WY-16,643 (10 μM) and dexamethasone (10 μM) as negative controls and 5β-PREGN (10 μM), CLOT (10 μM), PB (0.5 mM) and RIF (10 μM) as positive controls (Fig. 3). Cells were exposed to each chemical for 24 h and the concentration of all the pesticides tested was 10 μM. RIF (10 μM) and PB (0.5 mM) produced the largest increase in firefly luciferase activity (Fig. 4). Treatment with 10 μM RIF resulted in a 14.2 ± 1.8-fold increase in luciferase activity whereas treatment with 10 μM CLOT or 10 μM 5β-PREGN was less effective, leading to increases in luciferase activity of 7.3 ± 0.7 and 4.4 ± 0.3-fold, respectively. Treatment of the stable hPXR/HepG2 cell line with 3-MC (1 μM), clofibrate (10 μM), WY 16,643 (10 μM) and dexamethasone (10 μM) resulted in only negligible induction (<1.7-fold).

The pesticides ranked according to their ability to induce luciferase activity as follows (listed in order of agonistic activity): cypermethrin ≈ fenvalerate > lindane > endosulfan > methoxychlor ≈ chlordane > dieldrin > transnonachlor ≈ DDT ≈ o,p′-DTT ≈ chlordecone > aldrin ≈ chlorpyrifos > pentachlorophenol (Fig. 4). Pesticides from the PY family were the most effective compounds; cypermethrin and fenvalerate induced 12.8 ± 1.0 and 12.2 ± 0.8-fold increases in luciferase activity, respectively. These values are similar to that obtained for the increase in firefly luciferase in response to 10 μM RIF.

Among the OCs, lindane and endosulfan were the most potent inducers of hPXR transactivation in the stable transfectants. The response to the other OCs tested ranged from a 4.3 ± 0.6-fold increase in luciferase activity in response to aldrin to a 8.4 ± 0.7-fold increase in response to chlordane. The fungicide pentachlorophenol did not induce any increase in luciferase activity. The only OP tested, chlorpyrifos, produced a 4.4 ± 0.3-fold increase in luciferase activity.

3.3. CYP3A4 and CYP2B6 protein levels in cultured human hepatocytes

We investigated CYP3A4 and CYP2B6 protein levels in cultured hepatocytes treated with pesticides (10 μM), dexamethasone (50 μM), RIF (25 μM), PB (0.5 mM) by Western immunoblotting (Figs. 5A and 6A). The hepatocyte cultures used were derived from liver biopsies of three liver donors. Protein levels in the treated cultures were determined by comparing the intensities of the blots obtained for the treated cultures with those obtained for the DMSO-treated hepatocytes. Values represent the fold increases over the vehicle controls and are plotted in Figs. 5B and 6B. Treatment with RIF and PB resulted in an increase in CYP3A4 and CYP2B6 immunoreactive protein levels whereas 3-MC and WY 16,643 did not induce either protein. CYP3A4 and CYP2B6 protein levels were respectively, 2.3 ± 0.2- and 2.8 ± 0.3-fold higher than the controls for cells treated with PB and 3.3 ± 0.1- and 4.7 ± 0.2-fold higher for than controls for cells treated with RIF.

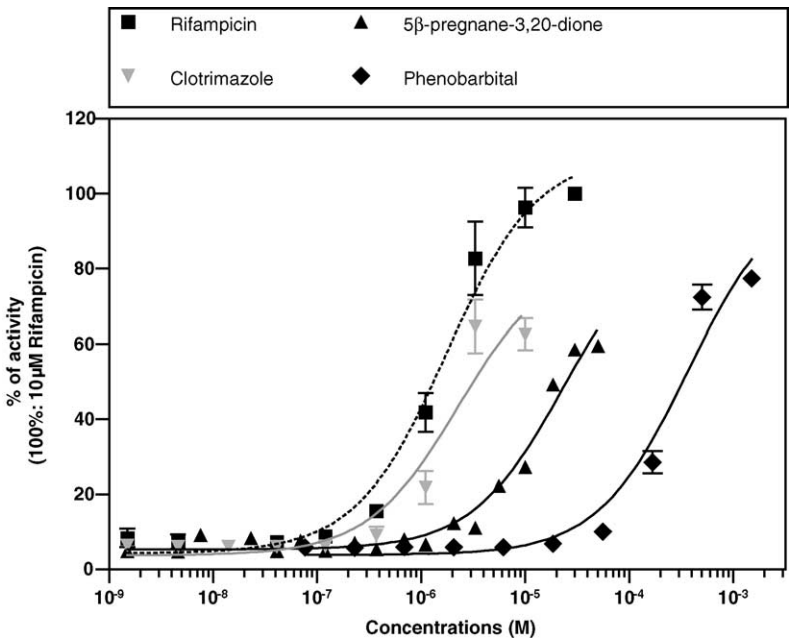

Fig. 2. Dose-response curves of the PXR/HepG2 cells to human PXR-specific ligands. Cells were incubated for 24 h with various concentrations of RIF (1.5×10^{-9} to 3.0×10^{-5}), CLOT (1.5×10^{-9} to 1.0×10^{-5}), PB (7.6×10^{-8} to 1.5×10^{-3}), and 5β-PREGN (1.5×10^{-9} to 5.0×10^{-5}). Results are expressed as the percentage of luciferase activity measured in each well (with triplicate wells for each dose). Percentages were calculated using the value obtained in the presence of 10 μM RIF as the 100% value for luciferase activity. The values represent the means ± S.D. of three separate experiments.

The abilities of the pesticides (10 μM) to induce CYP3A4 protein were ranked as follows (listed in order of agonistic activity): chlorpyrifos > lindane ≈ o,p′-DDT > chlordane ≈ cypermethrin ≈ DDT > chlordecone ≈ dieldrin ≈ pentachlorophenol > aldrin ≈ endosulfan ≈ fenvalerate > endrin ≈ methoxychlor ≈ trans-nonachlor (Fig. 5).

Among the PYs, cypermethrin was more effective than fenvalerate in inducing CYP3A4 protein. Among the OCs, lindane and o,p′-DDT were the most potent inducers of CYP3A4 protein in the human hepatocytes. The other OC pesticides tested (methoxychlor and chlordane) resulted in 1.5 ± 0.3 and 2.2 ± 0.3-fold increases in CYP3A4 protein level (respectively). The fungicide pentachlorophenol induced a 1.8 ± 0.3-fold increase in the level of CYP3A4 protein. Chlorpyrifos, the only OP tested, was an effective pesticide and induced a 2.6 ± 0.5-fold increase in the level of CYP3A4 protein.

With regard to the increases in CYP2B6 protein levels, the pesticides were ranked as follows (listed in order of agonistic activity): trans-nonachlor > lindane ≈ o,p′-DDT > endosulfan ≈ chlordane > dieldrin > aldrin ≈ chlorpyrifos ≈ cypermethrin ≈ DDT ≈ endrin ≈ fenvalerate > methoxychlor ≈ pentachlorophenol ≈ chlordecone (Fig. 6).

Among the PYs, cypermethrin and fenvalerate were moderate CYP2B6 protein level inducers. Pesticides from the OC family were the most effective compounds in inducing CYP2B6 protein level: 3.2 ± 0.7, 3.0 ± 0.2, 2.9 ± 0.6, 2.8 ± 0.2, 2.7 ± 0.3-fold increases were found in response to treatment with trans-nonachlor, lindane, o,p′-DDT, chlordane and endosulfan, respectively. CYP2B6 protein level was moderately induced by dieldrin, endrin and DDT but only weakly induced by methoxychlor, pentachlorophenol and chlordecone. Chlorpyrifos was also a moderate inducer (2.2 ± 0.3-fold).

Table 1
EC_{50} values (μM) for activation of hPXR in stable hPXR/HepG2 cells

Chemicals	EC_{50} (μM)
Rifampicin	1.8 ± 0.2
Clotrimazole	2.5 ± 0.3
5β-Pregnane-3,20-dione	20
Phenobarbital	370 ± 11

EC_{50} values (μM) are given for rifampicin, clotrimazole, 5β-pregnagne-3,20-dione and phenobarbital. Stable hPXR/HepG2 cells were treated with several concentrations (from 1.5×10^{-9} to 1.5×10^{-3} M) of each compound.

4. Discussion

We have used a one-step transfection procedure to generate a stable HepG2-derived cell line that expresses a functional human PXR gene product and the distal (bases −7836 to −7208) and proximal (bases −362 to +53) enhancer elements of the CYP3A4 promoter coupled to the luciferase reporter gene. Analysis and selection of transfectants expressing the bioluminescent reporter gene was simplified by the use of a low-light imaging system.

Another model cell system expressing a functional PXR gene product and carrying a plasmid containing the CYP3A4 proximal promoter has been described in the

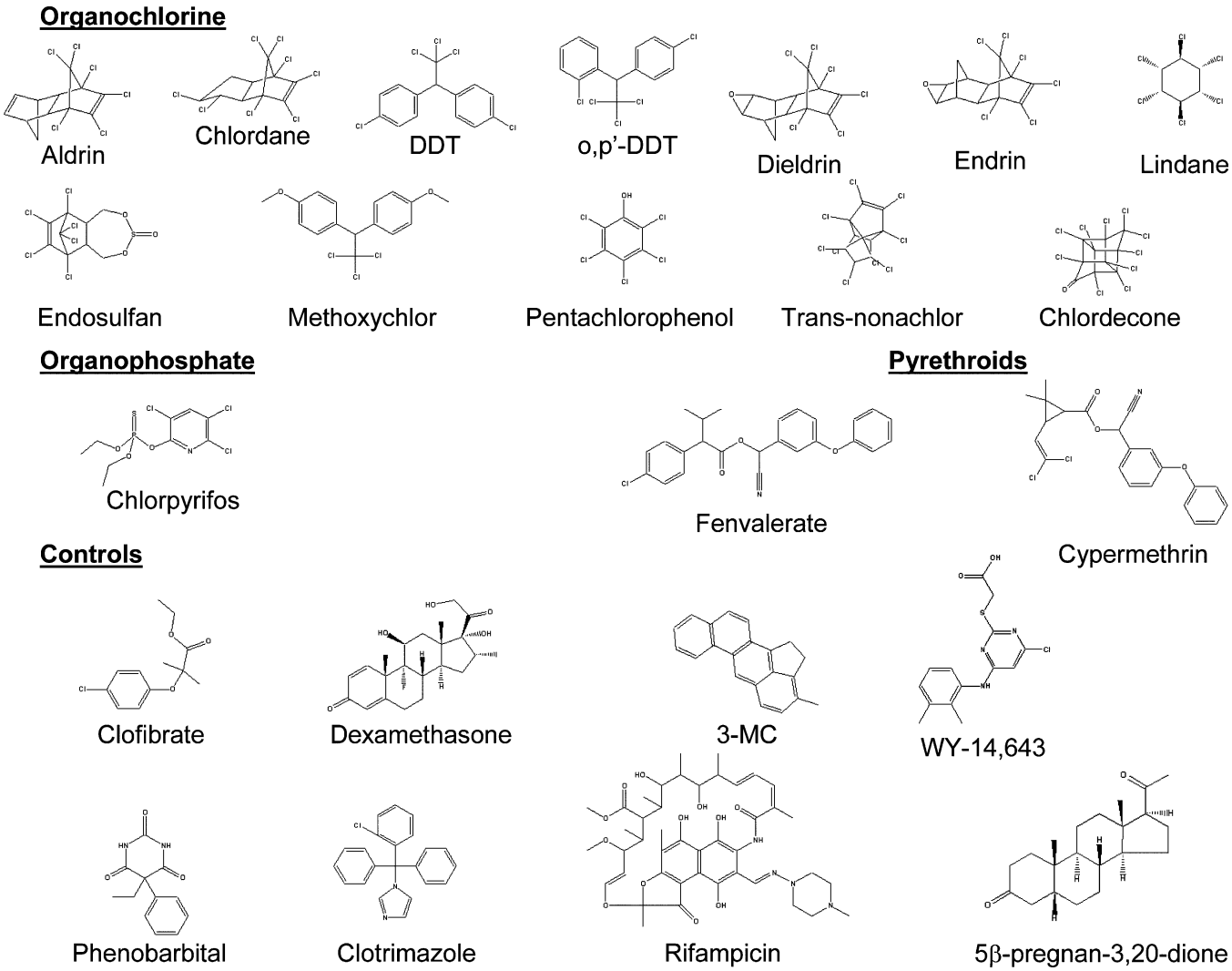

Fig. 3. Chemical structures of the organochlorine pesticides (Ocs: aldrin, chlordane, DDT, dieldrin, endrin, lindane, endosulfan, methoxychlor, o,p′-DDT, pentachlorophenol and trans-nonachlor, chlordecone), the organophosphorus pesticide (OP: chlorpyrifos) pyrethroids (PY: cypermethrin, fenvalerate), negative controls (3-MC, clofibrate, WY-16,643 and dexamethasone) and positive controls (RIF, clotrimazole, PB and 5β-pregnane-3, 20-dione).

literature. This report [20] described a 5–6-fold induction in response to RIF in the C1F1 cell stable model. In our model system, the distal enhancer element previously described in [12] and the proximal element were both incorporated into our reporter construct. In response to treatment with RIF, we observed a 14–15-fold increase in luciferase activity in our stable hPXR/HepG2 cell line (Fig. 1). This increase in the magnitude of the response allowed us to discriminate more clearly between potent, moderate and low activators of PXR.

As in Raucy [20], we compared the luciferase activities obtained with DMSO directly with those obtained with RIF and the background luciferase activity induced by the empty control vector was not deducted from values obtained for DMSO- and RIF-treated cells. Only a negligible increase in luciferase activity was observed in HepG2 cells transiently transfected with the CYP3A4 promoters (distal and proximal enhancer construct) but not with hPXR when they were treated with RIF, CLOT and 5β-PREGN (Fig. 1). In addition, we confirmed that hPXR was expressed at an undetectable level in HepG2 cells by RT-PCR: we were able amplify a 1014-nt region of hPXR cDNA from the stable hPXR/HepG2 cell line but not from the HepG2 cells.

To test our model system, the hPXR/HepG2 cell lines (stable and transient) were exposed several known PXR activators and CYP3A4 inducers, including RIF, CLOT, PB and 5β-PREGN. As a negative control, the response of the cell lines to several other compounds, including 3-MC, clofibrate, WY, and dexamethasone, were also tested. For our stable cell line model, we generated dose-response curves for RIF, PB, CLOT and 5β-PREGN and the EC_{50} values for these compounds (Table 1) were highly consistent with those found in the literature for activation of hPXR with these drugs [21].

We used our stable model cell line to evaluate the abilities of persistent pesticides to induce CYP3A4 and CYP2B6 in human hepatocytes and investigate the role

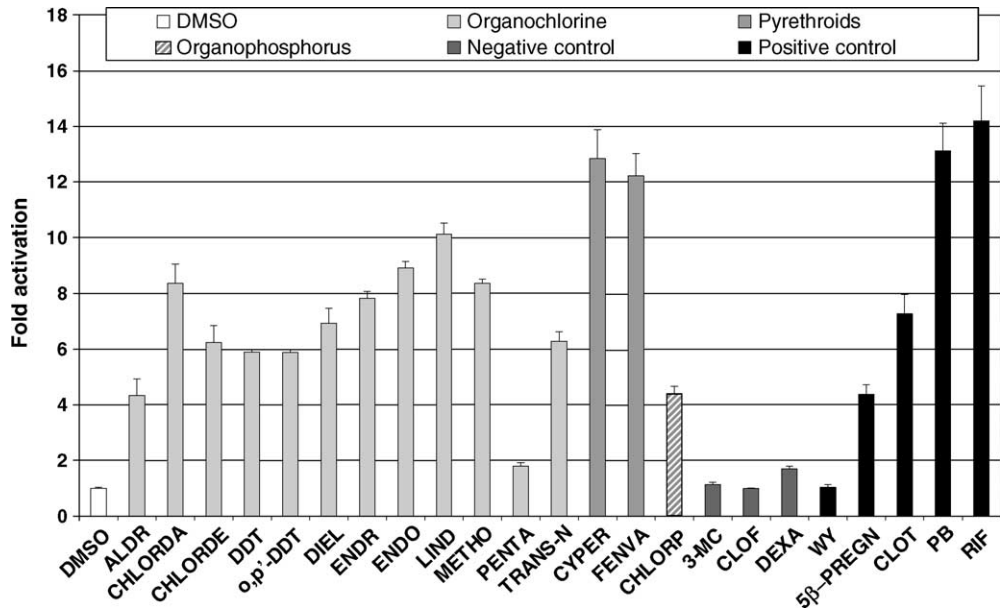

Fig. 4. Agonist activity of pesticides in stable hPXR/HepG2 cells. Cells were incubated for 24 h in the presence of aldrin (ALDR, 10 μM), chlordane (CHLORDA, 10 μM), chlordecone (CHLORDE, 10 μM), DDT (10 μM), o,p′-DTT (10 μM), dieldrin (DIEL, 10 μM), endrin (END, 10 μM), endosulfan (ENDO, 10 μM), lindane (LIND, 10 μM), methoxychlor (MET, 10 μM), pentachlorophenol (PENTA, 10 μM), trans-nonachlor (TRANS-N, 10 μM), cypermethrin (CYPER, 10 μM), fenvalerate (FENVA, 10 μM), chlorpyrifos (CHLORP, 10 μM), 3-MC (1 μM), clofibrate (CLOF, 10 μM), dexamethasone (DEXA, 10 μM), WY16,643 (WY, 10 μM), 5β-PREGN (10 μM), CLOT (10 μM), PB (0.5 mM) and RIF (10 μM). The values represent the means ± S.D. of three separate experiments.

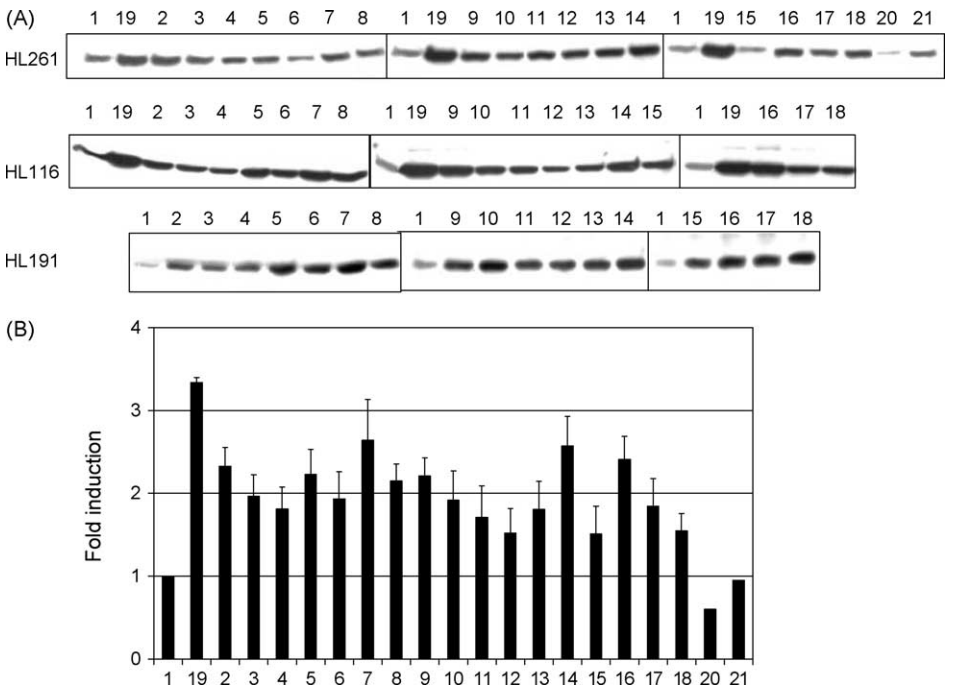

Fig. 5. Immunoblot analysis of CYP3A4 protein levels in human hepatocytes. Human hepatocytes were incubated for 48 h with (1) DMSO 0.5%, (2) PB 0.5 mM, (3) dexamethasone 50 μM, (4) aldrin 10 μM, (5) chlordane 10 μM, (6) chlordecone 10 μM, (7) chlorpyrifos 10 μM, (8) cypermethrin 10 μM, (9) DDT 10 μM, (10) dieldrin 10 μM, (11) endosulfan 10 μM, (12) endrin 10 μM, (13) fenvelarate 10 μM, (14) lindane 10 μM, (15) methoxychlor 10 μM, (16) o,p′-DDT 10 μM, (17) pentachlotophenol 10 μM, (18) trans-nonachlor 10 μM, (19) RIF 25 μM, (20) 3-MC 1 μM; (21) WY 10 μM. Ten micrograms of total cell protein extract was applied per lane. Proteins were separated on a 10% SDS-polyacrylamide gel. (A) a typical western blot using the CYP3A4 antisera. The blot shown is representative of those obtained for three different cultures. (B) The levels of CYP3A4 were determined by densitometric scanning of autoradiograms obtained using the ECL detection system. The values were calculated by comparing the densities of the bands from treated cultures with those from DMSO-treated hepatocytes (control). The results are expressed as fold increases in band densities above those found for the control and values are the mean ± S.D. of three separate samples.

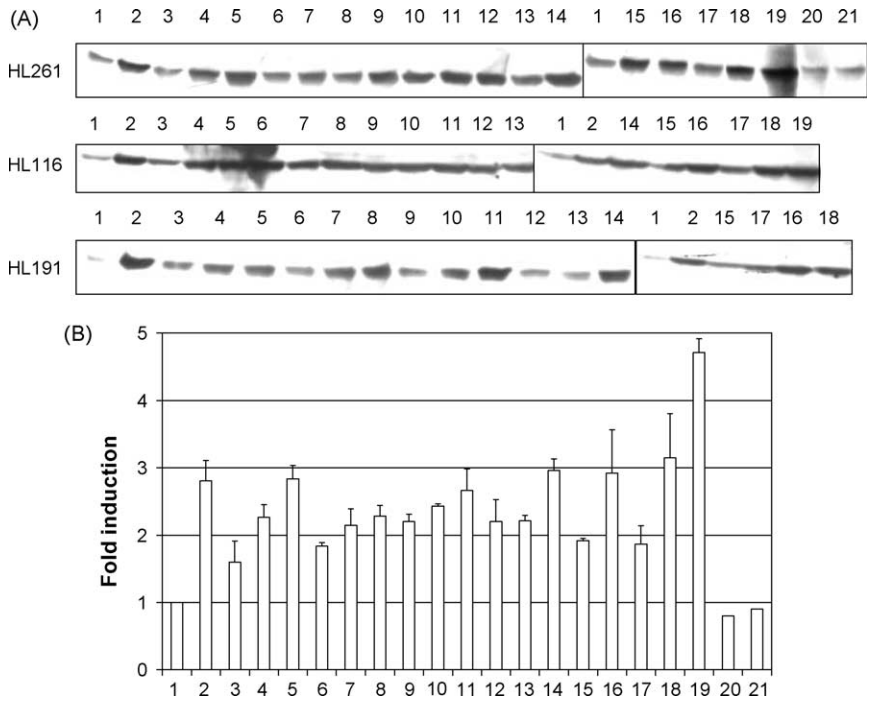

Fig. 6. Immunoblot analysis of CYP2B6 protein levels in human hepatocytes. Human hepatocytes were incubated for 48 h with (1) DMSO 0.5%, (2) PB 0.5 mM, (3) dexamethasone 50 μM, (4) aldrin 10 μM, (5) chlordane 10 μM, (6) chlordecone 10 μM, (7) chlorpyrifos 10 μM, (8) cypermethrin 10 μM, (9) DDT 10 μM, (10) dieldrin 10 μM, (11) endosulfan 10 μM, (12) endrin 10 μM, (13) fenvelarate 10 μM, (14) lindane 10 μM, (15) methoxychlor 10 μM, (16) o,p'-DDT 10 μM, (17) pentachlotophenol 10 μM, (18) trans-nonachlor 10 μM, (19) RIF 25 μM, (20) 3-MC 1 μM; (21) WY 10 μM. Thirty-five micrograms of total cell protein extract per lane was applied. Proteins were separated on a 10% SDS-polyacrylamide gel. (A) a typical western blot using CYP2B6 antisera. The blot shown is representative of those obtained for three different cultures. (B) The levels of CYP3A4 were determined by densitometric scanning of autoradiograms obtained using the ECL detection system. The values were calculated by comparing the densities of the bands from treated cultures with those from DMSO-treated hepatocytes (control). The results are expressed as fold increases in band densities above those found for the control and values are the mean ± S.D. of three separate samples.

played by hPXR in this induction. Previous studies have found that CYP3A4 and CYP2B6 are induced by PXR ligands [22,23]. Therefore, we also investigated whether compounds that activated hPXR in our stable cellular model also induced CYP3A4 and CYP2B6 proteins in human hepatocytes.

According to the literature, pesticides such as PY, OC and OP induce CYP3A and CYP2B in rat hepatocytes [15,17,24]. In addition, Wyde [25] showed that p,p′-DDE, the main derivative of DDT, induces hepatic CYP3A1 and CYP2B1 via a mechanism that involves activation of rat PXR.

Our study demonstrates, for the first time, that pesticides induce CYP3A4 and CYP2B6 proteins in human hepatocytes. In addition, we showed that hPXR was also activated by these pesticides. PXR was involved in CYP3A4 and CYP2B6 induction in response to all the PY, OP and OC pesticides tested except pentachlorophenol. We found that our stable PXR reporter gene assay cell line and our primary culture of human hepatocytes produced similar but not identical CYP3A4 and CYP2B6 induction responses. PB, RIF, lindane, endosulfan and chlordane strongly activated hPXR and resulted in large increases in the level of CYP3A4 protein. Dieldrin, DDT, chlordecone, aldrin, endrin, and trans-nonachlor moderately activated PXR and produced intermediate increases in the levels of the CYP3A4 and CYP2B6 proteins. Finally, 3-MC and WY did not induce CYP3A4 and CYP2B6 in either system. Cypermethrin and fenvalerate strongly activated PXR in the stable hPXR/HepG2 cells (Fig. 4) but only induced moderate increases in the levels of the CYP3A4 and CYP2B6 proteins (Figs. 5 and 6) in the hepatocytes. In contrast, RIF treatment resulted in a large increase in CYP2B6 and CYP3A4 protein levels in human hepatocytes (Figs. 5 and 6) and a large increase in luciferase activity in the stable hPXR/HepG2 cells (Fig. 1). The discrepancy between the extent of the hPXR activation response and that of the CYP3A4 and CYP2B6 induction response after exposure to pyrethroids may be due to the fact that these pesticides are rapidly metabolized in human hepatocytes via a mechanism involving hydroxylation and cleavage of cypermethrin and fenvalerate. This mechanism may not exist in the HepG2 cell line. Indeed, Ray [26] found that in humans, urinary excretion of cypermethrin metabolites was complete 48 h after the final dose of a five-dose 1.5 mg/kg/day regime was administered. In addition, cypermethrin is metabolized by hydroxylation and cleavage in rats, more than 99% being eliminated within hours of administration. Chlorpyrifos moderately activated PXR in the reporter gene assay but resulted in a large increase in

the level of CYP3A4 but not CYP2B6 protein. The results obtained in the reporter gene assay for methoxychlor and pentachlorophenol were inconsistent with those obtained in the hepatocyte cultures. Methoxychlor induced a marked increase in luciferase activity (Fig. 4) but only induced a moderate increase in the levels of the CYP3A4 and CYP2B6 proteins in hepatocytes (Figs. 5 and 6). In contrast, pentachlorophenol did not result in an increase in PXR and XREM promoter-mediated luciferase activity, suggesting that pentachlorophenol does not induce CYP3A4 and CYP2B6 protein level via a process involving the PXR. Alternatively, this absence of an increase in luciferase activity but induction of CYP3A4 and CYP2B6 protein by pentachlorophenol in hepatocytes may be due to an effect of the metabolites of this compound rather than pentachlorophenol itself. In HepG2 cells, the activity of many enzymes is minimal. The reason why methoxychlor resulted in such a potent increase in luciferase activity but such a small increase in CYP3A4 and CYP2B6 protein in human hepatocytes is unclear but may also be related to the metabolism of this compound. However, Blizard [27] found that the major metabolites of methoxychlor were able to induce CYP2B in rat liver microsomes. Trans-nonachlor markedly induced the CYP2B6 protein but only moderately activated PXR in the reporter assay system. This discrepancy between PXR activation and CYP2B6 induction may be due to the involvement of CAR in trans-nonachlor-mediated CYP2B6 induction.

In cultured hepatocytes and in vivo, PXR is the major transcription factor involved in the CYP3A4induction. However, other signaling pathways such as CAR may also participate in CYP3A4 and CYP2B6 regulation. Indeed, CAR may directly transactivate CYP3A4 promoters by binding to the ER6 or DR3 motifs in the XREM region. This XREM region also serves as a PXR binding site [21,28,29]. In addition to PB, pesticides also induce CYP3A4 and CYP2B6 via several pathways. Indeed, methoxychlor and DDT derivatives have been shown to activate CAR in rat hepatocytes [25,27].

In summary, the results from the stable PXR reporter gene system and those from the primary hepatocyte culture were generally consistent. Some discrepancy between the extent of the PXR activation response and that of the CYP3A4 and CYP2B6 protein response was observed with cypermethrin, fenvalerate, pentachlorophenol and methoxychlor. This discrepancy was possibly due to the metabolization of these compounds by a functional xenobiotic metabolizing enzyme in the primary human hepatocyte cultures. Furthermore, the stable PXR reporter gene assay could not show the capacity of the compounds to induce CYP3A4 and CYP2B6 via the CAR pathway. A previous study has shown that in HepG2 cells, CAR expression was too weak to induce CYP2B6 in response to treatment with PB [28].

In addition to differences in metabolic activity between the stable cell line and the hepatocytes, the discrepancy in our results may also be related to competition between PXR and other receptors, such as CAR, for ligands and DNA binding sites. Hepatocyte nuclear factor-3 (HNF-3), HNF-4 and CCAAT/enhancer-binding protein (C/EBP) can all modulate the expression of the CYP3A4 and CYP2B6 gene products in human hepatocytes. These hepatocyte-specific transcription factors are capable of physically interacting with elements within the CYP3A4 distal and proximal promoter [30,31].

In our stable cell culture model system, the p3A4-362(7836/7208ins) reporter gene construct contained two *cis*-acting elements: a functional HNF4α-binding site (bases −7783 to −7771) within the XREM region [12,30], and functional C/EBPα (bases −132 to −121) and HNF-3 (base −188) binding sites in the proximal CYP3A4 promoter [31,32]. Two additional C/EBPα binding sites were found at the distal position in the promoter (−1393/−1402 and −1659/−1668). In contrast to those at the proximal site, these distal sites are functional in hepatic cells but not in extrahepatic cells.

The HN4Fα response element is an important regulatory region of CYP3A4 as it confers basal and maximal PXR-mediated transcriptional activation [30]. In addition, the level of HNF-4 expression in HepG2 cells was comparable to that found in human hepatocytes [33]. Thus, the difference between the results obtained with the stable model and those obtained with hepatocytes was not due to the HNF-4 transcription factor. In contrast to HNF-4, the level of C/EBPα and HNF-3 mRNAs were lower in HepG2 cells than in human hepatocytes, particularly in the case of C/EBPα. Thus, the discrepancy in our results may be related to low levels of both C/EBPα and HNF-3 mRNA [33], as well as the absence of the distal C/EBPα binding site in the reporter construct [32].

Alternatively, the difference between the extent of PXR activation and that of the CYP induction in response to certain compounds may also be due to the presence of different transport mechanisms in the two cell types. The HepG2 cells may lack, or produce only low levels of, one of the transporters present in the hepatocytes. This deficiency may have led to the accumulation of pesticides in HepG2 cells. PXR activation response resulting from this accumulation of pesticides would be expected to be stronger than CYP3A4 and CYP2B6 induction response in human hepatocytes. Inversely, if the pesticide efflux is increased in HepG2 cells compared to hepatocytes, these compounds would be expected to be less potent PXR activators in HepG2 cells than they are inducers of CYP in hepatocytes. The presence of additional transporters in HepG2 cells may diminish the importance of extensive PXR activation in response to pesticides.

Both HepG2 cells and hepatocytes express detectable levels of the MDR1, MRP2, MRP3 transporters [34,35]. However, no comparative studies of mRNA and protein have been performed for MRP transporters in these cell types. In addition, other transporters may also contribute to

pesticide efflux and therefore maybe responsible for the differences between PXR activation and CYP induction.

Our results demonstrate that potent inducers of human CYP3A4 and CYP2B6 can be identified using a stable transfectant cell system containing the appropriate CYP-enhancer elements. This system can be used to rapidly, reliably and reproducibly assess the potential of candidate drugs to induce human CYP. However, tests should be carried out in parallel using primary cultures of human hepatocytes to assess the inductive potential of the metabolic intermediates of these new drugs.

Our results indicate that PXR is a xeno-sensor that protect against the presence of significant concentrations of harmful environmental compounds. However, pesticides that induce CYP2B and CYP3A in human hepatocytes may lead to procarcinogen activation and thus disrupt the metabolism of drugs and endogenous substances such as steroid hormones. CYP3A4 metabolizes more than 60% of all the drugs that are clinically available and CYP2B6 metabolizes up to 25%. The impact that induction of these enzymes may have on the activity of these drugs may cause serious health problems. In addition, normal endocrine function may be affected by the by steroid hormone (e.g., testosterone) metabolizing activity of these CYP. As an example, PB increases androgen hepatic metabolism and induces developmental disorders indicative of androgen deficiency [36]. In addition, a link has been identified between the effects of pesticides on enzyme induction and reproductive disorders.

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