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Identification of New Human Pregnane X Receptor Ligands among Pesticides Using a Stable Reporter Cell System

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Pregnane X receptor (PXR, NR1I2) is activated by various chemically unrelated compounds, including environmental pollutants and drugs. We proceeded here to *in vitro* screening of 28 pesticides with a new reporter system that detects human pregnane X receptor (hPXR) activators. The cell line was obtained by a two-step stable transfection of cervical cancer HeLa cells. The first transfected cell line, HG_5LN, contained an integrated luciferase reporter gene under the control of a GAL4 yeast transcription factor–binding site. The second cell line HGPXR was derived from HG_5LN and stably expressed hPXR ligand-binding domain fused to GAL4 DNA-binding domain (DBD). The HG_5LN cells were used as a control to detect nonspecific activities. Pesticides from various chemical classes were demonstrated, for the first time, to be hPXR activators: (1) herbicides: pretilachlor, metolachlor, and alachlor chloracetanilides, oxadiazon oxiconazole, and isoproturon urea; (2) fungicides: bupirimate and fenarimol pyrimidines, propiconazole, fenbuconazole, prochloraz conazoles, and imazalil triazole; and (3) insecticides: toxaphene organochlorine, permethrin pyrethroid, fipronil pyrazole, and diflubenzuron urea. Pretilachlor, metolachlor, bupirimate, and oxadiazon had an affinity for hPXR equal to or greater than the positive control rifampicin. Some of the newly identified hPXR activators were also checked for their ability to induce cytochrome P450 3A4 expression in a primary culture of human hepatocytes. HGPXR, with HG_5LN as a reference, was grafted onto nude mice to assess compound bioavailability through *in vivo* quantification of hPXR activation. Altogether, our data indicate that HGPXR cells are an efficient tool for identifying hPXR ligands and establishing pesticides as hPXR activators.

Key Words: **pesticide; pregnane X receptor; luciferase reporter; HeLa cell; nude mouse; cytochrome P450 3A4.**

The human pregnane X receptor (hPXR), also called steroid and xenobiotic receptor, was originally shown to mediate a pathway-regulating gene expression of cytochrome P450 3A (CYP3A) (Bertilsson *et al.*, 1998). It is now known that pregnane X receptor (PXR) is a key factor in the oxidative metabolism of xenobiotics, through CYP3A and CYP2B induction, and also in their conjugative metabolism and transport (Maglich *et al.*, 2002). Its action might even extend to several new human genes (Vyhlidal *et al.*, 2004). Cytochrome P450 (CYP) induction may interfere with endocrine function through the metabolism of endogenous substances like steroid hormones, which are substrates for CYP3A and 2B (Wyde *et al.*, 2003). PXR may also induce unwanted drug metabolism, given that approximately 50% of all clinically used drugs are metabolized by the CYP3A family (Thummel and Wilkinson, 1998).

Because the hPXR ligand-binding domain (LBD) is highly flexible (Watkins *et al.*, 2003), the potential exists for promiscuous binding of ligands that belong to completely structurally unrelated groups of molecules. These comprise numerous natural and xenobiotic molecules (Lehmann *et al.*, 1998), such as organochlorine (Coumoul *et al.*, 2002; Lemaire *et al.*, 2004), pyrethroid, and organophosphorus (Lemaire *et al.*, 2004) insecticides, which were recently shown to induce CYP3A4 expression through hPXR activation. Pesticides are widely used throughout the world—in 2004 alone, 1.4 t were used on crops in the top 22 agriculturally productive countries (Dr Antony Goulds, Kynetec Limited, personal communication)—and are therefore ubiquitous environmental contaminants. Our laboratory has designed a series of bioluminescent reporter cell lines to detect nuclear receptor ligands (Balaguer *et al.*, 1999, 2000, 2001; Seimandi *et al.*, 2005; Terouanne *et al.*, 2000). Some were developed from the HG_5LN cell line, which is a HeLa-derived cell line containing a luciferase reporter gene driven by a pentamer of the yeast transactivator GAL4 recognition sequence in front of the β-globin promoter. These HG_5LN cells were then transfected with different receptor genes to obtain cells expressing the GAL4 DNA-binding domain (DBD) fused

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to nuclear receptor LBD chimeric proteins (Balaguer *et al.*, 2000; Seimandi *et al.*, 2005). Because they are built in the same reporter cell line, these biosensors allow easy comparison of nuclear receptors for ligand binding and coregulator interaction. The present work describes the establishment of HGPXR, the cells designed to detect compounds that activate hPXR. They were obtained by stable transfection of HG_5LN cells with a vector expressing the hPXR(LBD) fused to GAL4(DBD).

Twenty-eight widely used pesticides, in particular preemergence herbicides, were screened with our model, and 15 new activators of hPXR were identified. In parallel, the results were validated by testing some of the positive hPXR activators as inducers of CYP3A4 expression in a primary culture of human hepatocytes. Moreover, the HGPXR reporter cells, with HG_5LN cells as reference, were also implanted into nude mice, allowing *in vivo* detection of the xenobiotic response. The HGPXR cell line should therefore be an efficient tool for identifying new hPXR ligands and endogenous hPXR activators, designing specific PXR agonists, and conceiving drug compounds with no unwanted endocrine interference.

MATERIALS AND METHODS

Materials. The materials for cell culture, the RNA extraction TRIzol reagent, and the SuperScript-II First-Strand Synthesis System for RT-PCR were from Invitrogen (Cergy-Pontoise, France). Restriction enzymes were from Ozyme (Saint-Quentin Yvelines, France). Luciferin, sodium salt, and G418 were purchased from Promega (Charbonnières-les-Bains, France). The rifampicin and puromycin antibiotics, aminotriazole, bupirimate, clotrimazole, cypermethrin, diuron, fenarimol, fenbuconazole, fenvalerate, heptachlor, imazalil, isoproturon, mecoprop, methyl parathion, nicotine, permethrin, prochloraz, propiconazole, and vinclozolin, were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) together with Ham F-12 and Williams' E culture media for primary culture human hepatocytes. Azimsulfuron was from DuPont de Nemours (Nambsheim, France). Pretilachlor was from Dr. Ehrenstorfer Laboratories (Augsburg, Germany); fipronil and oxadiazon from Rhône Poulenc (Lyon, France); hyperforin from Alexis Biochemicals (San Diego, CA); and SR12813 from Tebu-bio (Le Perray en Yvelines, France). Alachlor, atrazine, *o,p′*-DDT, metolachlor, and toxaphene (mix of isomers) were obtained from ChemService (West Chester, PA). 2,4-D, 2,4,5-T, carbaryl, diflubenzuron, mancozeb, and thiabendazole were purchased from Cluzeau Info Labo (Sainte Foy-la-Grande, France). Dexamethasone and RU486 were gifts from Aventis (Romainville, France). All effectors were dissolved in DMSO at 10mM, and successive dilutions were performed in culture medium.

Plasmids. pSG5-GAL4(DBD)-hPXR(LBD)-puro was built in two steps. First, pPM-hPXR expressing the DBD of GAL4 (met 1 to ser 147) yeast activator fused to hPXR(LBD) was obtained by PCR cloning of hPXR(LBD) (met 107 to ser 434) and insertion in the *Eco*RI and *Xba*I sites of pPM (BD Biosciences, from Ozyme). We then obtained pSG5-GAL4(DBD)-hPXR(LBD)-puro by PCR cloning of the GAL4-hPXR part of pPM-hPXR and inserted it in the *Bam*HI site of pSG5-puro, a gift from Hinrich Gronemeyer (Strasbourg, France). Plasmid preparation was carried out using the Nucleobond PC500 Kit (Macherey-Nagel, Hoerdt, France). Correct cloning was checked by restriction enzyme digestion and sequencing.

Generation of stable reporter cell lines. HG_5LN cells, obtained by integration of $GAL4RE_5$-βGlob-Luc-SVNeo in HeLa cells and containing a stably integrated GAL4-responsive gene, have already been described (Seimandi *et al.*, 2005). hPXR(LBD)–expressing cells were obtained by transfecting HG_5LN cells with pSG5-GAL4(DBD)-hPXR(LBD)-puro, which enables the expression of chimeric hPXR(LBD) and confers resistance to puromycin. Selection of resistant clones by puromycin was performed at 0.5 μg/ml. The most rifampicin-responsive clone was named HGPXR. For the strain culture, cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing phenol red and 1 g/l glucose and supplemented with 5% fetal calf serum in a 5% CO_2 humidified atmosphere at 37°C. HG_5LN cell medium was supplemented with 1 mg/ml geneticin and HGPXR cell medium, with 1 mg/ml geneticin and 0.5 μg/ml puromycin. To test compounds for their hPXR-activating capacity, cells were grown in DMEM without phenol red and supplemented with 6% dextran-coated, charcoal-treated fetal calf serum (DCC-FCS).

Living cell luciferase assay. HG_5LN and HGPXR cells were seeded at a density of 5×10^4 cells per well in 96-well white, opaque tissue culture plates (Greiner CellStar, D. Dutscher, Brumath, France) and grown in 200 μl DCC-FCS. The compounds to be tested were added 24 h later at concentrations varying between 10nM and 10μM, and the cells were then incubated for 16 h with the compounds. The experiments were performed in triplicate. At the end of incubation, effector-containing medium was removed and replaced by 0.3mM luciferin-containing culture medium. At this concentration, luciferin diffuses into the cell and produces a luminescent signal that is approximately 10-fold less intense than the signal obtained after cell lysis but stable for several hours. The 96-well plates were then introduced into a Microbeta Wallac luminometer, and intact living cell luminescence was measured for 2 s and expressed as relative luminescence units (RLU).

In experiments aimed at determining the hPXR-activating potency of pesticides, the RLU of HGPXR cells in the presence of varying pesticide concentrations was expressed relative to the value obtained with 3μM rifampicin taken as 100. Means ± SDs were calculated from three independent experiments in triplicate. For some of the tested compounds, the efficient concentration (EC_{50}, concentration giving 50% maximum activity) and the 95% confidence interval were evaluated with statistical software (GraphPad Prism, version 4.0, 2003, GraphPad Software Incorporated, San Diego, CA) if the curves were classic sigmoid curves. When this was not the case, EC_{50} ± SD was evaluated graphically.

For comparison of the luminescence of HGPXR and the parent HG_5LN cells, the RLU in the presence of various pesticide concentrations was expressed relative to the basal value, referred to as DMSO control and taken as 100. Means ± SDs were calculated from three independent experiments in triplicate.

Liver samples, primary hepatocyte cultures, and treatment. Human hepatocytes were prepared and cultured as previously described (Pascussi *et al.*, 2000). Cells were plated into collagen-coated P12 dishes at 0.17×10^6 cells/cm^2 in a hormonally and chemically defined medium consisting of a mixture of Williams' E and Ham F-12 (1:1 vol/vol). Treatment with 1 or 10μM effector solutions started 48 h after plating and lasted for 36 h.

Total RNA purification and CYP3A4 quantitative PCR. Total RNA was isolated with TRIzol reagent. cDNA was synthesized from 1 μg of total RNA using the SuperScript-II First-Strand Synthesis System for RT-PCR at 42°C for 60 min in the presence of random hexamers. One-tenth was used for quantitative PCR amplification of CYP3A4 and β-actin, as internal control, using the LightCycler apparatus (Roche Diagnostic Corporation, Meylan, France). The following program was used: denaturation at 95°C for 10 min and 45 cycles of PCR (denaturation at 95°C for 10 s, annealing at 65°C for 8 s, and elongation at 65°C for 15 s). The sense and reverse primers, respectively, were as follows: β-actin: 5′-tgggcatggtgcagaaggat and 5′-tccatcacgatgccagtggt; CYP3A4: 5′-cacaaaccggaggccttttg and 5′-atccatgctgtagggccccaa. The results are expressed as fold induction versus untreated cells.

In vivo bioluminescent imaging of HGPXR-implanted cells. The technique of implanting luminescent reporter cells was described earlier (Pillon *et al.*, 2005). Briefly, female athymic nude mice, about 50 days old and weighing 18–20 g, were obtained from Harlan France (Gannat, France) and acclimatized for a week before the experiment started. All experiments were

performed in compliance with the French guidelines for experimental animal studies (agreement no. B-34-172-27).

Approximately 1×10^6 cells, prepared in serum-free DMEM, were sc grafted onto the mouse right dorsal flank (HGPXR cells) and left dorsal flank (HG_5LN cells, as an internal control), and a week later tumor size was considered sufficient for performing the *in vivo* experiments. The mice were first sedated using the isoflurane gas anesthesia system from T.E.M. (Bordeaux, France), with 4% isoflurane in air in an anesthesia induction box and then with 1.5% isoflurane in air continuously delivered in the dark box via a nose cone system. Luciferase activity was then measured after the mice had been ip injected with luciferin saline solution, 125 mg/kg body weight, which gave rise to a luminescent signal that was maximal 10 min later and remained stable for 20 min. A gray-scale, body-surface reference image was collected with a NightOWL LB 981 CCD camera (Berthold Technologies, Bad Wildbad, Germany). The photons emitted from luciferase were integrated for 2 min, and the pseudocolor luminescent image was generated using WinLight software (Berthold Technologies). The overlay of the body image and the luminescence representation allowed the localization of the xenografts. The luminescent signal intensities from the regions of interest (ROIs) were obtained, and the data were expressed as photon flux (photons/s). Background photon flux was defined from an ROI of the same size placed in a nonluminescent area near the animal and then subtracted from the measured luminescent signal intensity.

The PXR activities of different compounds were measured as follows. Constitutive HG_5LN cell level and HGPXR cell responses were determined before and 8 h after ip administration of compound in DMSO. HGPXR luminescence was evaluated relative to that of the HG_5LN cells in the same mouse, and an induction factor was determined in treated versus untreated animals. Mean values ± SDs were calculated from six independent experiments. Eight mice were necessary to accomplish the present work.

RESULTS

Establishment of HGPXR Cells and Their Activation by Known hPXR Ligands

HGPXR cells, obtained as described in "Materials and Methods," were designed to detect hPXR agonists, and the intermediary HG_5LN cell line allowed us to evaluate non-PXR–mediated luciferase gene expression.

We first tested the HGPXR cells for their response to known hPXR activators: the drugs were rifampicin, hyperforin, SR12813, clotrimazole, and RU486 (Fig. 1A) (Lehmann *et al.*, 1998; Watkins *et al.*, 2003), and the insecticides were fenvalerate, cypermethrin, and *o,p′*-DDT (Fig. 1B) (Lemaire *et al.*, 2004). The antibiotic rifampicin, the Saint-John's-wort constituent hyperforin, and the cholesterol-lowering drug SR12813 activated HGPXR cell luciferase expression in a dose-dependent and saturable manner. Rifampicin, being the most widespread hPXR ligand, was chosen as our reference molecule, and the activation values obtained with all the tested compounds were expressed as luciferase activity relative to the activity in the presence of 3μM rifampicin and taken as 100. The baseline activity of the HGPXR cells was 21.3 ± 3.1% (SD).

The EC_{50} values for hyperforin and SR12813 were very close, 0.11 and 0.14μM, respectively, and that of rifampicin was 0.72μM (Table 1). The anti-progestin RU486 and the fungicide clotrimazole produced noticeable transactivation of the hPXR-dependent reporter gene, but even at high concentration, luciferase expression did not reach 100% (Fig. 1A). As

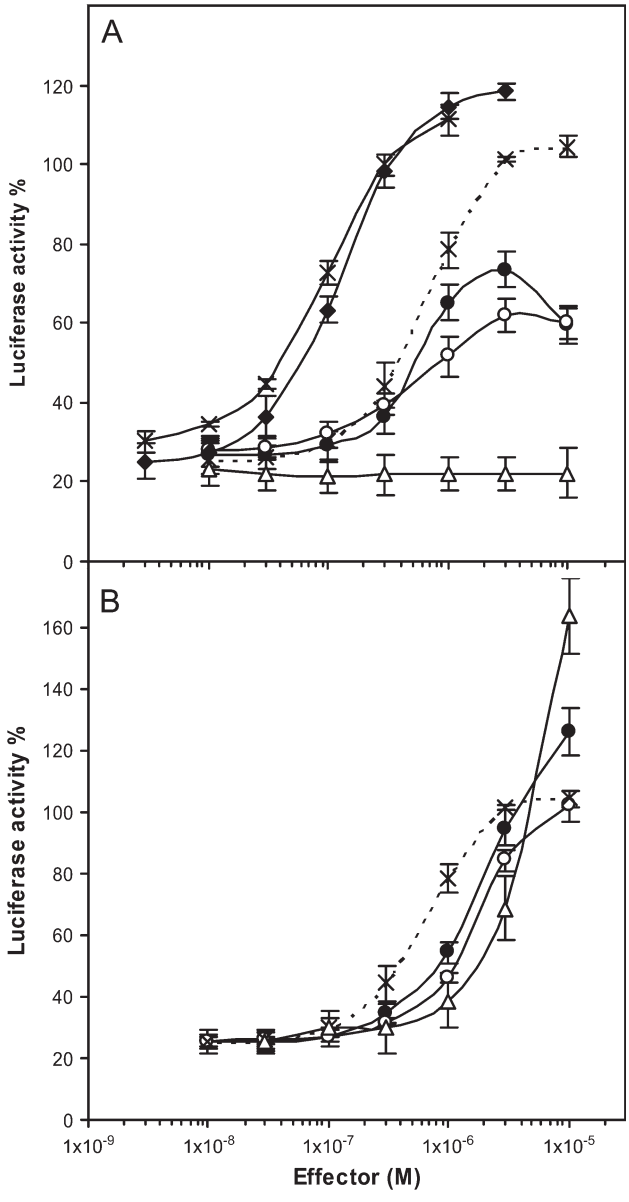

FIG. 1. Luciferase activity induced by known hPXR ligands. The activity of HGPXR cells was measured as a function of the concentration of (A) the reference ligands: rifampicin (dotted line), clotrimazole (●), RU486 (○), hyperforin (×), and SR12813 (♦), and dexamethasone as negative control (Δ); and (B) the known pesticide ligands: fenvalerate (●), cypermethrin (○), and *o,p′*-DDT (Δ) as well as rifampicin (dotted line). Activity was expressed as a percentage of 3μM rifampicin-induced activity, and means ± SDs were calculated from three independent experiments performed in triplicate.

expected, dexamethasone, a known specific activator of rabbit (Jones *et al.*, 2000) and mouse (Moore *et al.*, 2000) PXR (mPXR), did not activate HGPXR cell luciferase expression. Furthermore, it did not antagonize the luciferase gene expression induced by rifampicin (data not shown). Two insecticides, fenvalerate and cypermethrin pyrethroids, were strong inducers of luciferase expression (Fig. 1B), and their EC_{50} values were 2.9 and 2.6μM, respectively (Table 1). The organochlorine

TABLE 1
EC_{50} Values Obtained with HGPXR Cells

Compound	EC_{50} (μM)	95% Confidence interval
Hyperforin	0.11	0.09–0.14
SR12813	0.14	0.12–0.16
Pretilachlor	0.18	0.10–0.34
Rifampicin	0.72	0.4–1.4
Bupirimate	0.95	0.7–1.3
Metolachlor	1.2	0.7–2.0
Cypermethrin	2.6	1.1–6.0
Fenvalerate	2.9	2.0–4.3
Compound	**EC_{50} (μM)**	**Graphic SD**
Oxadiazon	0.64	0.13

o,p′-DDT was less active at low concentrations but produced a sharp rise at 10μM. Altogether, these results obtained with known hPXR ligands showed that our model is a reliable tool to screen new hPXR ligands.

Detection of Nonspecific Activities Using HG_5LN Cells

One of the advantages of our PXR reporter cell system is that the parent HG_5LN can be used to check the specificity of a tested compound. Indeed, it contains the same reporter gene as the HGPXR cells, and this gene is integrated at the same site in the genome. Moreover, its expression is not controlled by PXR. In the absence of tested compound, a basal luciferase is expressed by the cells, and modification of the baseline activity by a tested compound would demonstrate a nonspecific activity. As shown in Figure 1, we observed HGPXR luciferase superactivation by 10μM *o,p′*-DDT (164%) and fenvalerate (138%). To determine whether these superactivations were PXR specific, the effect of 10μM *o,p′*-DDT and fenvalerate on parent HG_5LN cell luciferase was measured. We noted a nearly twofold increase in HG_5LN luciferase expression with *o,p′*-DDT and a slight increase with fenvalerate (Fig. 2). No effect was detected at the 1μM compound concentration (results not shown). The superactivation of HGPXR luciferase observed with 10μM *o,p′*-DDT and fenvalerate was therefore at least partly nonspecific, whereas luciferase activation at lower compound concentration was fully PXR specific. The reference compounds, like rifampicin and SR12813, did not superactivate HG_5LN luciferase expression (Fig. 2), and HGPXR luciferase activation with rifampicin and SR12813 was thus entirely specific. Nevertheless, the minimal inhibition of rifampicin on HG_5LN luciferase expression (Fig. 2) and its submaximal HGPXR activation (Fig. 1A) suggested that rifampicin would have a weak toxicity. With 10μM clotrimazole and RU486 (Fig. 1A), a plateau was reached at 60% maximal HGPXR activity, and a corresponding decrease in HG_5LN luciferase expression was observed with both compounds (Fig. 2). Consequently, at high clotrimazole and RU486

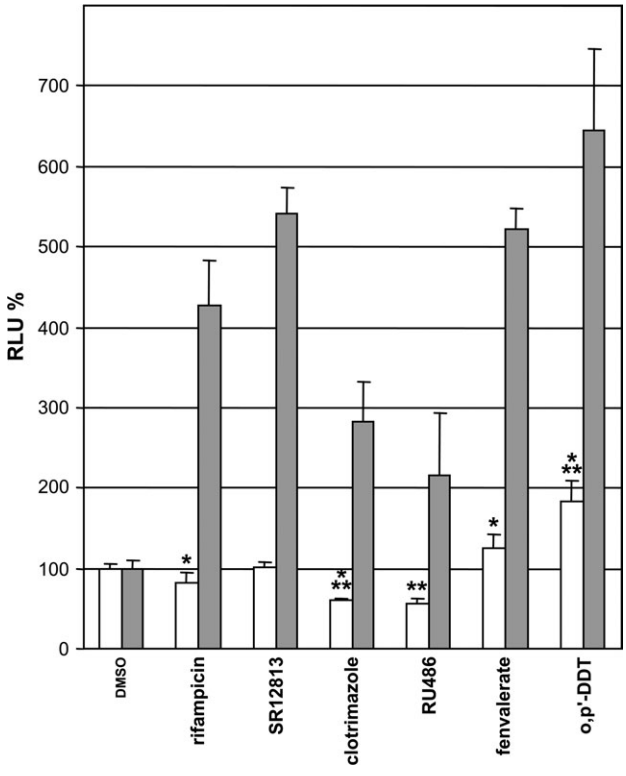

FIG. 2. Detection of nonspecific activities with the parent HG_5LN cells. Activities were expressed as RLU relative to DMSO control in the presence of 10μM effector, except for SR12813 (3μM). Filled columns: HGPXR cells. Empty columns: HG_5LN cells. Means ± SDs were calculated from three independent experiments performed in triplicate. HG_5LN values in the presence of DMSO solvent alone and in the presence of hPXR ligands were compared with Student's *t*-test: $*p \leq 0.05$; $**p < 0.01$, $***p < 0.001$.

concentrations, the plateau was at least partly due to a nonspecific effect, which might have been caused by toxicity, rather than to partial agonistic activity. In our screening study, all compounds were thus systematically checked at 10 and 1μM for their activation of parent HG_5LN cell luciferase.

Activation of HGPXR Cells by New hPXR Ligands

Most PXR ligands are xenobiotics, and among them, pesticides are major environmental contaminants that threaten human and animal health. The goal of the present study was to screen pesticides from various chemical classes for their induction of hPXR. We therefore selected a group of widely used pesticides never before described as hPXR ligands or activators and assayed them at 10μM on HGPXR cells (Table 2). Alachlor, bupirimate, fenarimol, metolachlor, oxadiazon, permethrin, pretilachlor, and propiconazole exhibited over 70% luciferase activation, and fenbuconazole, fipronil, imazalil, prochloraz, and isoproturon exhibited about 50% luciferase activation.

Dose-response curves were obtained for all the active compounds, and the curves that reached a plateau in the concentration range tested (10nM–10μM) are presented (Figs. 3A

TABLE 2
Effect of Pesticides on hPXR Transactivation in HGPXR Cells

Type[a]	Pesticide	Relative activity[b]	SD	Class[c]	Chemical Abstract Service registry number
f	Bupirimate	93.5	5.4	P	41483-43-6
f	Fenarimol	89.6	5.7	P	60168-88-9
f	Propiconazole	85.1	2.7	C	60207-90-1
f	Fenbuconazole	56.1	6.8	C	114369-43-6
f	Prochloraz	50.5	0.1	C	67747-09-5
f	Imazalil	46.5	5.9	C	35554-44-0
f	Mancozeb	29.3	4.9	Dithiocarbamate	7/01/18
f	Vinclozolin	19.4	1.5	Oxazole	50471-44-8
h	Pretilachlor	129.5	5.5	CA	51218-49-6
h	Metolachlor	107.2	10.3	CA	51218-45-2
h	Oxadiazon	94.2	7.0	Oxiconazole	19666-30-9
h	Alachlor	71.3	4.3	CA	15972-60-8
h	Isoproturon	50.1	5.0	U	34123-59-6
h	2,4-D	26.1	2.1	PH	94-75-7
h	Diuron	24.5	0.7	U	330-54-1
h	Azimsulfuron	23.8	1.9	U	120162-55-2
h	2,4,5-T	23.5	3.5	PH	93-76-5
h	Aminotriazole	21.6	0.2	Triazole	61-82-5
h	Mecoprop	18.0	0.1	PH	93-65-2
h	Atrazine	16.9	2.9	Triazine	1912-24-9
i	Toxaphene	114.2	3.8	OC	8001-35-2
i	Permethrin	88.4	6.5	Pyrethroid	52645-53-1
i	Fipronil	58.7	6.9	Pyrazole	120068-37-3
i	Diflubenzuron	33.0	1.0	U	35367-38-5
i/f	Thiabendazole	31.5	2.1	Thiazole	148-79-8
i	Carbaryl	25.0	3.5	Carbamate	63-25-2
i	Methyl parathion	23.1	1.0	Organophosphorus	298-00-0
i	Nicotine	18.8	0.8	Botanical	54-11-5
i	Heptachlor	18.7	0.6	OC	76-44-8

Note. The dashed lines separate the agonists from the chemicals that failed to activate PXR within each class.

[a]Type: f, fungicide; h, herbicide; i, insecticide.

[b]Induction of HGPXR luciferase activity by 10μM pesticides expressed as a percent of 3μM rifampicin-induced activity. Background value without pesticide was 21.3 ± 3.1%.

[c]Class from Compendium of Pesticide Common Names (www.hclrss.demon.co.uk): P, pyrimidine; C, conazole; CA, chloroacetanilide; U, urea; PH, phenoxy; OC, organochlorine.

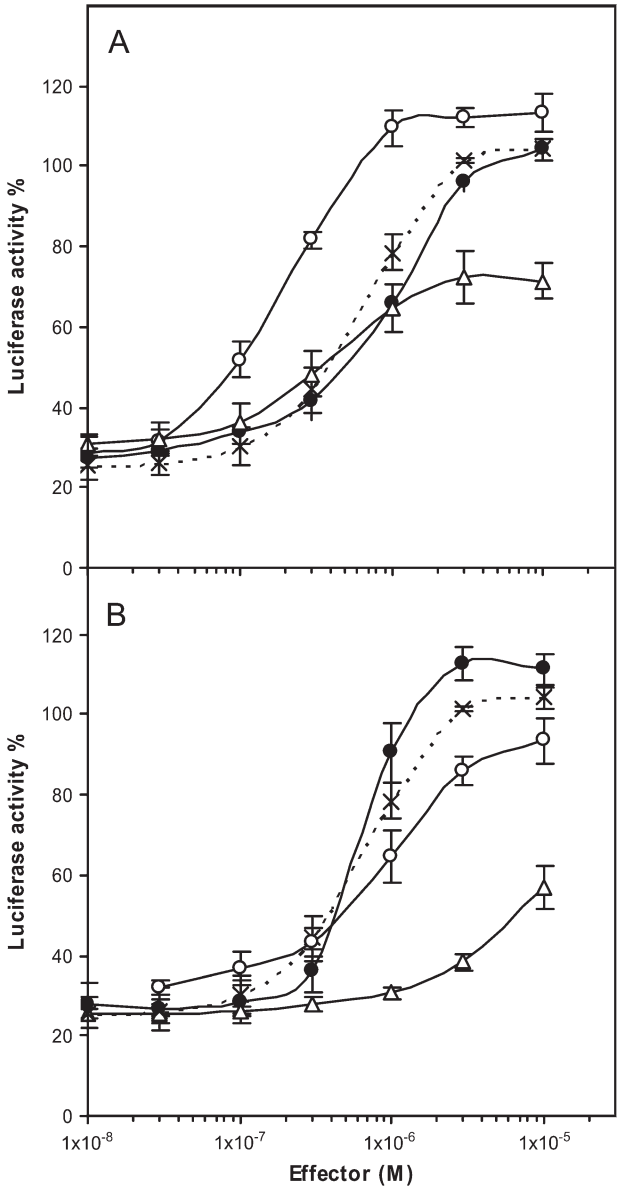

FIG. 3. Induction of luciferase activity by new ligands of hPXR. The activity of HGPXR cells was measured as a function of the concentration of (A) herbicides: pretilachlor (○), metolachlor (●), and alachlor (Δ) as well as rifampicin (dotted line); and (B) various pesticides: oxadiazon (●), bupirimate (○), and fipronil (Δ) as well as rifampicin (dotted line). The activity was expressed as a percentage of 3μM rifampicin-induced activity. Means were calculated from three independent experiments performed in triplicate and expressed ± SDs.

and 3B). Pretilachlor, metolachlor, and oxadiazon were full agonists of hPXR. Bupirimate and alachlor activation reached 93 and 70% of maximal rifampicin induction, respectively. As suggested by their EC_{50} values (Table 1), metolachlor (1.2μM) and bupirimate (0.95μM) had an affinity for hPXR that was close to that of rifampicin (EC_{50} of 0.72μM, see above). Pretilachlor was found to be a better agonist than rifampicin with an EC_{50} value of 0.18μM. Oxadiazon behaved differently from the other ligands. The S-shaped curve obtained when luciferase activity was plotted as a function of ligand concentration (Fig. 3B) was steeper than expected from a classic ligand. We therefore had to calculate its EC_{50} graphically. It was 0.64μM, similar to that of rifampicin. The fipronil curve (Fig. 3B) was chosen as an example of curves that did not reach a plateau in the range of studied concentration, as was observed for fenbuconazole, prochloraz, imazalil, isoproturon, permethrin, and diflubenzuron (not shown). All the pesticides described in Table 2 were also tested for their activation of parent HG_5LN

cell luciferase. Only toxaphene at 10μM exhibited nonspecific activity (not shown).

The following compounds, with no or weak ability to activate hPXR-mediated transcription, were tested for their ability to antagonize hPXR in our cell model: 2,4,5-T, 2,4-D, aminotriazole, atrazine, azimsulfuron, carbaryl, diuron, heptachlor, mancozeb, mecoprop, methyl parathion, nicotine, thiabendazole, and vinclozolin (Table 2), together with dexamethasone. HGPXR cells were incubated with 1μM rifampicin (80% maximal activity) in the presence of 1–10μM effector. No antagonistic activity was detected (results not shown).

Our model therefore allowed us to identify several efficient hPXR ligands: pretilachlor, with an affinity close to that of SR12813, and oxadiazon, bupirimate, and metolachlor, with affinities comparable to that of rifampicin.

Regulation of CYP3A4 Expression in Primary Culture of Human Hepatocytes

Because CYP3A4 expression is controlled by PXR, the effect of some of the newly detected PXR ligands—alachlor, metolachlor, oxadiazon, and pretilachlor—was checked by measuring CYP3A4 mRNA expression in independent primary cultures of human hepatocytes. Cell treatment with the reference rifampicin was performed on four specimens, and treatments with the four pesticides and SR12813 were carried out on two specimens. The greatest induction versus absence of treatment was obtained with rifampicin and SR12813 (up to 287-fold). Despite the high batch-to-batch variation inherent to a primary culture model (Roymans *et al.*, 2004), we observed that CYP3A4 mRNA levels were efficiently induced by oxadiazon (12.4- and 26.8-fold), pretilachlor (3.9- and 11.1-fold), metolachlor (3.3- and 17.0-fold), and alachlor (176.0- and 39.0-fold) (Table 3), confirming our observations with HGPXR cells.

TABLE 3
CYP3A4 mRNA Expression in Four Primary Culture Specimens of Human Hepatocytes

Specimen number	FT257	FT258
No treatment	1.0	1.0
Rifampicin	241.3	287.2
SR12813	259.0	216.0
Alachlor	176.0	39.0
Specimen number	**FT243**	**FT245**
No treatment	1.0	1.0
Rifampicin	44.7	25.9
Metolachlor	17.0	3.3
Pretilachlor	11.1	3.9
Oxadiazon	26.8	12.4

In Vivo *Response of Xenografts to hPXR Ligands*

The above results led to the identification of several pesticides as PXR ligands able to increase *in vitro* receptor activity. However, these molecules might display different *in vivo* efficiencies due to their metabolism and/or bioavailability.

To better characterize the pesticides identified as PXR ligands, we implanted HGPXR cells in nude mice and studied receptor-mediated *in vivo* luciferase response (Fig. 4), as previously described (Pillon *et al.*, 2005). Mice were grafted with HG_5LN cells onto the left dorsal flank and with HGPXR cells onto the right dorsal flank, as described in "Materials and Methods." The luminescent signal was recorded before and 8 h after ip injection of 25 mg/kg rifampicin. A strong signal was observed on the right HGPXR-grafted flank (Fig. 4). After correcting the specific response in the HGPXR tumor by accounting for the basal response in the HG_5LN tumor, an induction factor was evaluated with a given compound. When 5, 15, and 25 mg/kg rifampicin were injected, the induction factor was concentration dependent (Fig. 5). At 25 mg/kg of rifampicin or SR12813, the induction factors were comparable—about 4—whereas the oxadiazon induction factor was weaker. No significant effect of alachlor or pretilachlor was detected, although the *in vitro* experiments indicated that these compounds were good hPXR ligands. These preliminary data thus demonstrated, for the first time, that *in vivo* activation of hPXR could be achieved with only some of the pesticides identified *in vitro*.

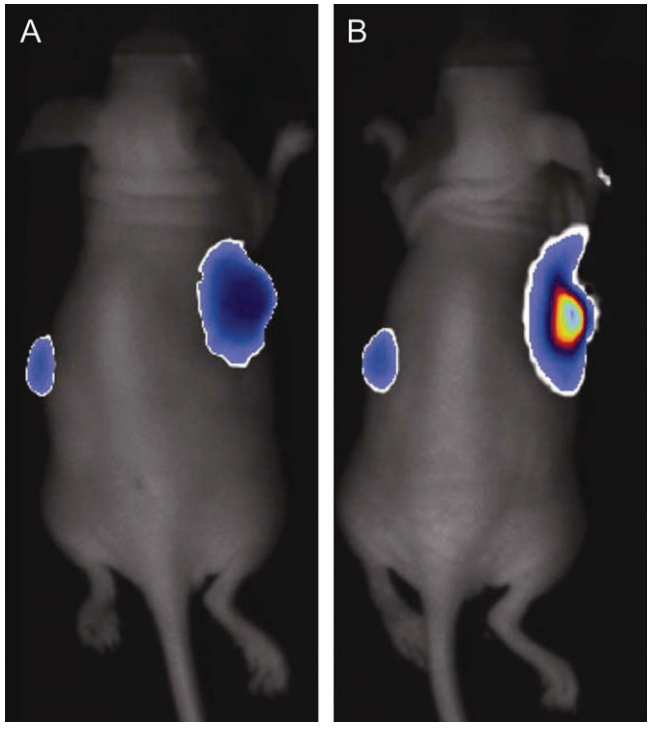

FIG. 4. Bioluminescence imaging of nude mouse xenografts. HG_5LN (left flank) and HGPXR (right flank) xenografts were imaged before (A) and 8 h after (B) 25 mg/kg rifampicin ip injection.

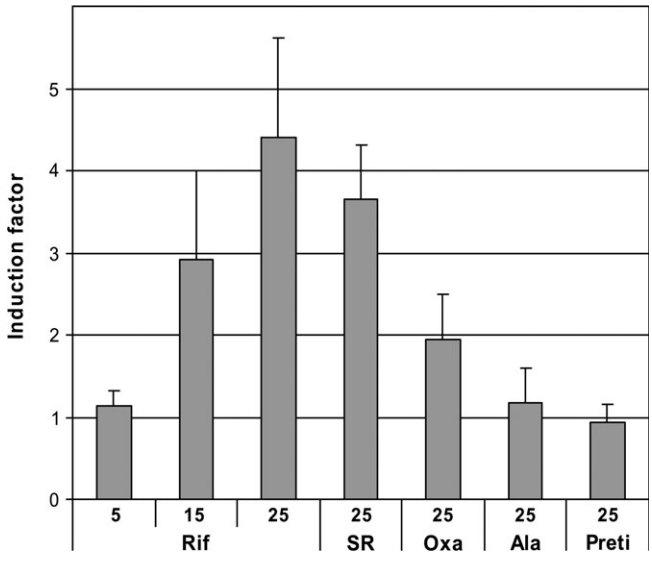

FIG. 5. *In vivo* induction of hPXR activity. The compounds Rif (rifampicin), SR (SR12813), Oxa (oxadiazon), Ala (alachlor), and Preti (pretilachlor) were ip injected in nude mice at the indicated concentrations (mg/kg). Mean values ± SDs were calculated from six independent experiments.

DISCUSSION

This work describes a new high-throughput system for evaluating hPXR-dependent gene expression. It is based on a stable reporter cell line which derives from HeLa cells through a two-step procedure. The first step led to HG_5LN cells by stable transfection of a reporter plasmid expressing luciferase under the control of the GAL4 yeast transcription factor (Seimandi *et al.*, 2005). A large series of reporter cell lines are being built in our laboratory from HG_5LN cells. These cell lines are being created to compare different nuclear receptors for ligand binding and coregulator interaction. In these cell models, the reporter system is insensitive to endogenous receptors, which cannot recognize the GAL4-binding site, and the chimeric nuclear receptor cannot activate endogenous genes. Moreover, HG_5LN cells can be used in a control test to detect nonreceptor-mediated luciferase expression. In the present work, one of these cell models, HGPXR cells—expressing the GAL4(DBD) fused to hPXR(LBD)—and parent HG_5LN cells, were used to identify hPXR ligands among pesticides. The HGPXR and HG_5LN cells were also grafted onto nude mice for *in vivo* quantification of nuclear receptor activation.

HGPXR cells were first shown to respond to known hPXR ligands among drugs, such as rifampicin, hyperforin, SR12813, clotrimazole, and RU486, and pesticides, such as cypermethrin, fenvalerate, and *o,p′*-DDT. The rifampicin, SR12813 and hyperforin EC_{50} values were identical to or slightly greater than the published values (Lemaire *et al.*, 2004; Moore *et al.*, 2000; Watkins *et al.*, 2003). The RU486 and clotrimazole activations did not reach 100%, corresponding to what was already described for RU486 (Lehmann *et al.*, 1998) and clotrimazole (Lemaire *et al.*, 2004). However, an additional study showed that clotrimazole reached a greater activation level than did rifampicin (Lehmann *et al.*, 1998).

We then performed a large screening of 28 functionally different pesticides belonging to various chemical classes of molecules. Our data revealed 15 new activators: (1) herbicides: alachlor, isoproturon, metolachlor, oxadiazon, and pretilachlor; (2) fungicides: bupirimate, fenarimol, fenbuconazole, imazalil, prochloraz, and propiconazole; and (3) insecticides: diflubenzuron, fipronil, and permethrin. Note that 13 compounds, belonging to all three pesticide types and various chemical classes, exhibited no induction of hPXR activity.

Some of the tested pesticides could have been expected to be hPXR agonists because studies performed in rodents showed that they are inducers of hepatic metabolism or CYP2B and CYP3A enzymes, or even PXR activators. Alachlor was indeed shown to be a rodent PXR activator (Mikamo *et al.*, 2003). Pretilachlor is capable of inducing phase I and II xenobiotic-metabolizing enzyme activities in rat liver (Ishizuka *et al.*, 1998), and fipronil enhances rodent hepatic metabolism (Hurley, 1998). Diflubenzuron, which only slightly activated HGPXR luciferase expression, was reported to produce a small induction of mouse CYP2B and CYP3A (Sapone *et al.*, 2005). Fenarimol (Paolini *et al.*, 1996), metolachlor (Dalton *et al.*, 2003), permethrin (Heder *et al.*, 2001), and propiconazole (Sun *et al.*, 2005) were described as inducers of rodent CYP2B and CYP3A. According to Sun *et al.* (2005), N-substituted azoles are able to induce rat hepatic cytochrome P450 enzymes. We indeed observed that propiconazole and three other N-substituted azoles, fenbuconazole, imazalil, and prochloraz, were ligands of hPXR, although fenbuconazole, imazalil, and prochloraz were only weak PXR activators. Note that clotrimazole is also an N-substituted azole and a reference hPXR ligand. Lastly, among the newly detected hPXR activators, bupirimate and oxadiazon have not been described as either CYP or hepatic metabolism inducers. A group of pesticides was found to be unable to induce HGPXR luciferase expression: 2,4-D, 2,4,5-T, aminotriazole, atrazine, azimsulfuron, carbaryl, diuron, heptachlor, mancozeb, mecoprop, methyl parathion, nicotine, and vinclozolin. Among them were again compounds containing an azole group—thiabendazole, azimsulfuron, and aminotriazole—but interestingly, these compounds are not N-substituted. Confirming their lack of activity on HGPXR cells, these pesticides have never even been shown to be CYP2B or CYP3A inducers. A concordance was observed between activation of HGPXR cell luciferase expression and hepatocyte CYP3A expression by rifampicin, SR12813, alachlor, metolachlor, pretilachlor, and oxadiazon. This work therefore demonstrates that alachlor, oxadiazon, and pretilachlor are inducers of human cytochrome P450 3A4 (hCYP3A4).

Importantly, the HGPXR reporter cells implanted in nude mice allowed an *in vivo* detection of the xenobiotic response.

A transgenic model expressing luciferase under the control of hCYP3A4 promoter was described to test mPXR ligands (Zhang *et al.*, 2003, 2004). This model allows the study of hCYP3A4 gene regulation but lacks specificity since the target gene is not strictly regulated by PXR. In addition, the PXR in these studies was from mouse origin. The advantage of our model is that we measured hPXR activation, and it was normalized with the constitutive HG_5LN cells implanted in parallel. Our *in vivo* data showed that rifampicin and SR12813 similarly activated PXR and that, among the tested pesticides, oxadiazon was also able to induce a luciferase response. The lack of effect of pretilachlor and alachlor in grafted nude mice reflects a reduced bioavailability, which might have resulted from their weaker polarity compared with that of rifampicin, SR12813, and oxadiazon or from a different metabolism. To address the involvement of PXR activation in mouse metabolism of pesticides, we are currently establishing a mPXR cell line in HG_5LN cells. Although preliminary, these data are the first report of an *in vivo* activation of hPXR by pesticides identified *in vitro*.

Considering the broad range of chemicals to which humans are exposed, a robust strategy for detecting nuclear receptor ligands is very useful. PXR ligands are quite likely to interfere with endocrine function, as exemplified by their ability to: (1) induce metabolism of CYP3A and 2B substrates, such as steroid hormones (Wyde *et al.*, 2003); (2) modulate vitamin D–dependent gene expression (Pascussi *et al.*, 2005); (3) activate constitutive androstane receptor (NR1I3) target genes (Moore *et al.*, 2000), thereby affecting reproductive function (Dessens *et al.*, 2001); and (4) alter thyroid hormone metabolism (Qatanani *et al.*, 2005). The HGPXR cell system is a new tool to detect hPXR activators in *in vitro* and *in vivo* procedures. It allowed the *in vitro* detection of 15 new hPXR activators among common pesticides. Grafted HGPXR cells could moreover shed light on the bioavailability of tested compounds and detect compounds that become active once metabolized. The combined *in vitro*/*in vivo* HGPXR cell system will be beneficial in preclinical tests when designing efficient drugs without toxicity through unwanted endocrine interference and drug-drug interaction. It will also be useful in developing PXR agonists to enhance hepatic detoxification of toxic metabolites (Sonoda *et al.*, 2005) or antagonists. PXR is furthermore known to respond to a wide array of endogenous chemicals and is becoming established as an ''endobiotic receptor'' (Xie *et al.*, 2004). Therefore, our HGPXR system will also be an advantageous tool in fundamental research to identify endogenous hPXR activators.

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