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**Evaluation of an hPXR reporter gene assay for the detection of aquatic emerging pollutants: screening of chemicals and application to water samples**

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## ABSTRACT

Many environmental endocrine disrupting compounds act as ligands for nuclear receptors. Among these receptors, the human pregnane X receptor (hPXR) is well described as a xenobiotic sensor to various classes of chemicals including pharmaceuticals, pesticides and steroids. To assess the potential use of PXR as a sensor for aquatic emerging pollutants, we employed an *in vitro* reporter gene assay (HG5LN-hPXR cells) to screen a panel of environmental chemicals and to assess PXR active chemicals in (waste) water samples. Of the 57 compounds tested, 37 were active in the bioassay and 10 were identified as new PXR agonists: triazin pesticides (promethryn, terbuthryn, terbutylazine), pharmaceuticals (fenofibrate, bezafibrate, clonazepam, medazepam) and non co-planar PCBs (PCB101, 138, 180). Furthermore, we detected potent PXR activity in two types of water samples: passive polar organic compounds integrative sampler (POCIS) extracts from a river moderately impacted by agricultural and urban inputs and three effluents from sewage treatment works (STW). Fractionation of POCIS samples showed the highest PXR activity in the less polar fraction, while in the effluents PXR activity was mainly associated with the dissolved water phase. Chemical analyses quantified several PXR active substances (i.e. alkylphenols, hormones, pharmaceuticals, pesticides, PCBs, bisphenol A) in POCIS fractions and effluent extracts. However, mass-balance calculation showed that the analysed compounds explained only 0.03 % and 1.4 % of biological activity measured in POCIS and STW samples, respectively. In effluents, bisphenol A and 4-tert-octylphenol were identified as main contributors of instrumentally-derived PXR activities. Finally, the PXR bioassay provided complementary information as compared to estrogenic, androgenic and dioxin-like activity measured in these samples. This study shows the usefulness of HG5LN-hPXR cells to detect PXR active compounds in water samples, and further investigation will be necessary to

identify the detected active compounds. *Keywords: Reporter bioassays, endocrine disrupters, passive sampling, wastewater.*

## **INTRODUCTION**

Anthropogenic activities lead to a continuous contamination of aquatic environment by a wide variety of chemicals. Among them, the so-called endocrine disrupting compounds (EDCs) can mimic or alter the action of endogenous hormones, through multiple mechanisms of action as they can interfere with the synthesis, transport, action, metabolism and excretion of natural hormones, which control homeostasis, development and reproduction functions [1]. Abnormalities linked to EDCs exposure has been several times observed in aquatic environment, such as **disrupted** vitellogenin synthesis or development of ovo-testis in male fish [2]. In this context, characterization and identification of aquatic contamination by EDCs has become a major issue for the last decade. To tackle this challenge, the recent development of integrated bio-analytical approaches using *in vitro* bioassays combined to chemical analyses has proven powerful methodology to detect and identify bioactive compounds within complex environmental samples [3]. Particularly, *in vitro* reporter gene bioassays based on the mechanisms of action of chemicals (e.g. hormone receptor activation) serve as screening tools for sensitive and specific detection of hormone-like activities in complex samples.

To date, *in vitro* bio-analytical assessment of EDCs has mainly concerned estrogen receptor (ER) and androgen receptor (AR) mediated activities [4-6]. However, these bioassays do not take into account large diversity of EDCs since several emerging contaminants, such as some pharmaceutical compounds, are not ligands of these receptors. Apart from ER and AR, other key nuclear receptors (NRs) such as pregnane X receptor (PXR) [7], peroxisome proliferator-activated receptor (PPAR) [8] or glucocorticoid receptor (GR) [9], are known to be directly activated by environmental contaminants, and thus can be potentially used as xenobiotic

sensors for environmental bio-analytical purpose. Among them, the PXR, also called steroid and xenobiotic receptor (SXR) or pregnane activated receptor (PAR), is of high interest for such purpose since it has been shown to be activated by a diversity of environmental ligands such as steroids, pharmaceuticals, pesticides, alkylphenols, polychlorobiphenyls (PCBs) and polybromodiethylethers (PBDEs) [10-17].

The PXR is an orphan NR because no endogenous ligand with high affinity has been identified so far [18]. As a nuclear receptor, PXR acts as a transcription factor. After ligand binding, it functions as a heterodimer with the retinoid X receptor (RXR), in a non permissive way [19]. It is mainly expressed in the liver and the intestine, the main organs involved on the metabolism of endogenous and exogenous compounds, where it plays a crucial role in the regulation of genes involved in xenobiotic detoxification. PXR controls the transcription of phase I cytochrome P450 (CYP) genes (e.g. CYP3A4, -2B6, -2C8, -2C9 and -2C19), phase II conjugating enzymes (e.g. UDP-glucuronosyltransferases and glutathion-S-transferase), as well as phase III transporter genes (e.g. MDR-1 and MRP-2 multidrug resistance proteins) [20]. In addition, PXR is also activated by endogenous ligands, notably biliary acids, pregnanes and hormones and thereby regulates the transcription of CYP7A and oatp2 transporter involved in cholesterol homeostasis and tissue protection from potent toxic endogenous compounds [21]. Overall, the essential biological role in both xenobiotic and endogenous metabolism regulation together with its ability to directly interact with various environmental chemicals make the PXR a toxicologically and environmentally relevant target for EDCs.

The aim of this study was to explore the potential use of a recently described human PXR (hPXR) reporter gene bioassay [12] for the detection of EDCs in the aquatic environment. This *in vitro* bioassay is based on cultured HeLa cells that permanently express the luciferase

reporter gene under the control of a chimeric hPXR (HG5LN-hPXR cells), and has been shown to sensitively respond to several pesticides [12], as well as antiestrogens, mycoestrogens and phthalates [14]. In the present study, in order to characterize the activity of a broader range of environmental contaminants, more than 60 environmentally occurring chemicals including pharmaceuticals, pesticides, plasticizers and persistent organic pollutants (POPs) were tested in this *in vitro* system. In addition, the detection of hPXR ligands in different effluent samples and polar organic compound integrative samplers (POCIS) extracts from freshwater sites has been investigated. The detected biological responses were compared to targeted chemical analyses by using mass balance calculation in order to identify candidate hPXR ligands in active samples.

## **MATERIALS AND METHODS**

### *Chemicals and reagents*

All standard chemicals listed in Tables 1-3 were purchased from Sigma Aldrich (St Quentin-Fallavier, France), except PCB congeners that were from LGC standards (Molsheim, France). Luciferin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide tetrazolium (MTT), methanol (MeOH) and dimethylsulfoxide (DMSO) were also purchased from Sigma-Aldrich (St Quentin Fallavier, France).

### *In vitro bioassays*

#### *Cell culture*

The HG5LN-hPXR cell line results from a two-step stable transfection [12]. As a first step, HeLa cell were stably transfected with a GAL4RE5-BGlob-Luc-SVNeo plasmid, leading to

the HG5LN cell line which expresses constitutively luciferase activity. Then, HG5LN cells were stably transfected, with the pSG5-GAL4(DBD)-hPXR(LBD)-puro plasmid to obtain the HG5LN-hPXR cell line. The HG5LN cell line was used to assess toxic or unspecific effects on luciferase in the bioassay, hence providing information on the specificity of hPXR activation in HG5LN-hPXR cells.

Both cell lines were routinely cultured in a 5 % CO<sub>2</sub> humidified atmosphere at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) containing phenol red (Sigma-Aldrich, St Quentin Fallavier, France) and supplemented with 5% fetal calf serum (FCS), 1% nonessential amino acids, penicillin/streptomycin (50 U/mL each) and 1 mg/mL G418 (all purchased from Invitrogen, Cergy Pontoise, France). Additionally, 0.5 µg/mL puromycin was added in HG5LN-hPXR cell medium.

#### *Luciferase assay*

The cells were seeded in 96-well white opaque culture plates (Greiner cellStar ; D. Dutscher, Brumath, France) at a density of  $1 \times 10^5$  cells per well in 100 µl of DMEM without phenol red, supplemented with 6% dextran-coated charcoal-treated fetal calf serum (DCC-FCS). After 24 h, a range of concentration of compounds to be tested was added to the culture medium in triplicates and cells were then incubated for 16 h. For environmental samples, cells were exposed to serial dilution of extracts. At the end of exposure, the medium was removed and 50 µl per well of medium containing 0.3 mM luciferin were added. Five minutes later, necessary for luciferin to diffuse into the cell and to produce a stable luminescence signal, the intact living cells luminescence was measured in for 2 seconds per well with a microtiter plate luminometer (MicroBeta, [PerkinElmer SAS, Courtaboeuf, France](#)).

#### *Cytotoxicity assay*



In order to assess the effect of test compounds on cell viability in HG5LN-hPXR cells, the metabolism of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), was measured. After luciferase assay, culture medium containing the luciferin was removed and replaced by 100 µl of DCC-FCS with 0.5 mg/mL of MTT. Cells were incubated for 3h. In metabolically active cells, MTT is reduced by the mitochondria onto a blue formazan precipitate, which was solubilised by adding 80 µl of isopropanol and agitation for 30 min. Plates were then read at 570 nm against a 640 nm reference wavelength on a microplate reader (KC-4, BioTek Instruments, France). Cell viability was expressed as a percentage of the control value. By using the MTT assay, no significant cytotoxic effects of chemicals were noted at the concentrations tested in luciferase assays.

#### *Other in vitro bioassays*

The estrogenic, (anti-)androgenic and dioxin-like activities of the extracts were assessed by using three *in vitro* bioassays based on marker gene activation in the MELN, MDA-kb2 and PLHC-1 cell lines, respectively. The MELN cells consist of the human MCF-7 cells that were stably transfected by the luciferase reporter gene controlled by endogenous estrogen receptor alpha (ER $\alpha$ ) [22]. The MDA-kb2 cell line (ATCC, #CRL-2713) was derived from the MDA-MB-453 human breast cancer cells. They were stably transfected by a MMTV promoter-luciferase plasmid construct, which is under the control of endogenous androgen receptor (AR) and glucocorticoid receptor (GR) [23]. The fish hepatic PLHC-1 cell line (ATCC, #CRL-2406) was described by Ryan and Hightower [24]. Protocols for routine cell culture and environmental sample assessment has been reported in details previously [25]. Briefly, in the MELN and MDA-kb2 bioassays, cells were exposed for 16 h and processed for luciferase activity assay as described above. In the PLHC-1 bioassay, cells were exposed for 4 h (PAH-

like activity) and 24 h (dioxin-like activity) and were then processed for 7-ethoxyresorufin-O-deethylase (EROD) activity assessment in intact cells.

### Environmental samples

#### *Wastewater effluents*

Three sewage water effluents (SWE), called A, B and C, were sampled in the South West of France at three different stations: effluent A was a paper mill effluent; effluent B was sampled at the outlet of an urban wastewater treatment plant which serves a catchment of about 150,000 equivalent/inhabitant population equivalent, and ensured the treatment of mainly domestic sewage; effluent C was a mix of sewage effluents A and B.

Effluent extracts were prepared as summarized in figure 1. Briefly, a part of each effluent was filtered on GFF filters immediately after collection. Then, liquid/liquid extractions were performed on both the filtered fraction and the crude fraction of each effluent (A, B and C). A volume of 250 mL of each fraction was extracted 3 times by shaking with 50 mL of dichloromethane. The total extract was dried with anhydrous sodium sulfate. It was concentrated with a rotary evaporator, and then under a gentle flow of nitrogen and transferred into 200 µL of methanol. Procedural blank using ultra pure water was also prepared in the same conditions in order to test the potential influence of the extraction process on bioassay responses. Each extract was used for bioassay and chemical analyses.

#### *POCIS extracts*

POCIS samplers (version for sampling pharmaceuticals) were deployed in May 2007 in the Baise River (South West of France) during 1 month. This site is under mixed anthropogenic pressures and is classified as good quality water. POCIS were provided by Exposmeter (Tavelsjö, Sweden). They contain 200 mg of Oasis HLB sorbent enclosed between two

polyethersulfone (PES) membranes. The membranes, which confine the sorbent, are compressed between two metal disks (5.1 cm ID, 8.9 cm OD). The total exchanging surface area of the membranes is 41 cm<sup>2</sup>. The ratio surface area to mass of sorbent is about 200 cm<sup>2</sup>/g. After exposure, each POCIS was rinsed with ultra pure water to remove any material present on the outer surface of the membranes (particles and biofilms). The metal disks were disassembled and the membranes were detached from the disk. The sorbent was carefully transferred into an empty glass SPE tube by rinsing it with ultrapure water and then dried by applying vacuum for 1 hour. The organic compounds were eluted in 3 fractions: a first fraction (F1) of 10 ml of dichloromethane, a second fraction (F2) of 10 ml of a dichloromethane - methanol mixture (50:50 v/v) and a third one (F3) of 10 ml of methanol. After elution, extracts were evaporated to complete dryness and dissolved in 200 µl of methanol. Each of the three fractions was assessed by bioassays and chemical analyses.

#### *Chemical analysis*

Pharmaceuticals, alkylphenols, and a part of pesticides (phenylurea herbicides) were analyzed by LC/MS/MS. PAHs, hormones and remaining pesticides (triazines, organophosphate and pyrethrenoid pesticides) were analyzed by GC/MS. PCBs and organochlorinated pesticides were analysed by GC/ECD. The analytical procedures were adapted from Togola and Budzinski [26] for the pharmaceuticals, Labadie and Budzinski [27] for the hormones, Budzinski et al. [28] for the PAHs, Alder et al [29] for the pesticides, Cailleaud et al [30] for the PCBs and Cailleaud et al [31] for the alkylphenols. Hydrophilic compounds (pharmaceuticals, phenylureas, triazines, organophosphate and pyrethrenoid pesticides) have been analyzed only on dissolved phase of effluent samples.

#### *Data analysis*

### *Bioassay data modeling*

The Regtox 7.5 Microsoft Excel<sup>TM</sup> macro, freely available at <http://eric.vindimian.9online.fr>, and using the Hill equation [32], was used to model sigmoid dose-response curves and to calculate efficient concentrations (i.e. EC<sub>20</sub> and EC<sub>50</sub>, corresponding to concentrations of chemical and samples that induced 20 and 50% of maximal luciferase activity, respectively). For compounds that provided incomplete dose responses curves, i.e. that does not reached a plateau, the Hill parameter for maximal effect was fixed at 100% (maximal response given by the positive control SR12813) before modelling of the data.

### *Mass balance calculations*

Toxic equivalent activities derived from chemical analysis (Chem-TEQ) in environmental samples were calculated according to the following equation:  $TEQ = \sum (C_i \times SREF_i)$ , where, for a given chemical  $i$ ,  $C_i$  is the measured concentration in a sample and  $SREF_i$  is the inducing equivalent factor relative to the reference ligand SR12813 ( $IEF_i = EC_{20}$  of reference compound /  $EC_{20}$  of test compound, on mass basis) and expressed as SR12813-equivalent quantities (SREQ).  $SREF_i$  were determined by establishing dose-response curves for individual standard chemicals according to the criteria proposed by Villeneuve et al [33] (i.e. parallel dose response curves and equal efficacy). Thus, for all compounds, including those with incomplete dose response curves, we fixed the minimal and maximal activity and the slope as identical to that of SR12813 before modelling of experimental data (data not shown). The biological toxic equivalent activities (Bio-TEQ) in environmental samples were calculated as the ratio of EC<sub>20</sub> of reference compound (expressed as g/L) to that of the sample expressed as EQ-L water/L (equivalent litre of water sample per litre). For the samples that yielded incomplete dose response curves, we fixed the minimal and maximal activity and the slope as identical to that of SR12813 for experimental data modelling. **Although fixed-effect**

level modelling often assumes an extrapolation of experimental data, it allows standardised treatment of all data and thus EC<sub>20</sub> comparison between samples and reference compounds.

### *Statistics*

Significant luciferase induction in treated cells as compared to solvent treated cells was determined by one way analysis of variance (ANOVA) followed by a Dunnett's unilateral posthoc test. A value of  $p < 0.05$  was considered significant.

## **RESULTS**

### *Activation of hPXR by reference and environmental chemicals*

In order to validate the use of the hPXR bioassay in our experiments, four reference hPXR ligands were first tested (Fig. 2). They induced potent and complete dose-response curves with sensitivities that were very similar than those previously reported in the same cell model [12]. The specific hPXR-mediated activity by these ligands was confirmed by a lack of effect on luciferase expression in the parental HG5LN cell line that does not express hPXR (Fig 2b). These cells were then further used to assess the activity of a panel of about 60 compounds that belong to different classes of contaminants. In each group of compounds, some chemicals, which PXR activity has been previously reported in this or other cell model, were also assessed in order to determine their hPXR potency in our test conditions.

### *Activation of hPXR by pesticides*

Sixteen pesticides from different classes (organochlorine, triazin, urea) were screened (Tab. 1). Oxadiazon, pretilachlore, *o,p'*-DDT and linuron activated the luciferase with the same induction pattern (i.e. in terms of affinity, maximal activity and specificity) as previously reported by Lemaire et al. [12] by using the same cell line (Fig. 3 and Tab. 1). The

organochlorine lindane and endosulfan were strong hPXR activators in HG5LN-hPXR cells, while triclosan, terbutylazine, terbuthryn and promethryn were found as weak to moderate hPXR activators (Fig 3a). Moreover, terbutylazine, terbuthryn and promethryn, could be considered as specific hPXR activators since they did not alter luciferase expression in HG5LN cells, whereas triclosan was slightly toxic in this cell line **as indicated by a decrease of luciferase** (Fig 3b). Finally TBT, chlorotoluron, metoxuron, chlorosulfuron, nicosulfuron and hexachlorobenzene were found as inactive compounds (Tab. 1).

#### *Activation of hPXR by pharmaceuticals*

Studies on *in vitro* hPXR transactivation by environmental pharmaceuticals are scarce. Here, we have tested hPXR activation by 21 compounds that were selected among those commonly found in surface waters (Tab. 2). Ten out of 21 environmental pharmaceuticals were specific hPXR activators in HG5LN-hPXR cells (Fig 4a, Tab. 2), with induction patterns varying according to effective concentration and maximal levels of luciferase induction. Mevastatin and fenofibrate were the most potent hPXR activators as they induced more than 60% of luciferase activity at 10 and 3  $\mu\text{M}$ , respectively. Diclofenac, tamoxifen, medazepam, carbamazepin, triazolam, diazepam, clonazepam, and bezafibrate were weak to moderate hPXR activators as they induced partial dose-response curves at relatively high concentrations (3 to 100  $\mu\text{M}$ ) (Fig. 4a, Tab. 2). Ketoprofen also slightly induced luciferase at 100  $\mu\text{M}$ ; however luciferase induction by this compound is likely to be non specific of the hPXR as it similarly occurred in HG5LN cells (Fig 4b). Finally, loprazolam, bromazepam, alprazolam, naproxen, ibuprofen, aspirin, caffeine, flumazemil, pravastatin and sulfamethoxazole were found inactive in HG5LN-hPXR cells (Tab. 2).

#### *Activation of hPXR by POPs*

Because of the ubiquitous character of PCBs and PAHs as environmental contaminants, we looked at hPXR activation by 10 dioxin-like and non dioxin-like PCBs and 4 toxic PAHs. Strong luciferase induction was observed with the non dioxin-like PCBs congeners #101, 138, 153 and 180, albeit at relatively high concentrations ( $EC_{50}s > 10 \mu M$ ) (Fig. 5a, Tab. 3). In HG5LN cells, PCB101, PCB153 and PCB180 were also able to slightly increase luciferase (i.e. by two-fold for PCB101), thus suggesting that a small part of their induction potency in HG5LN-hPXR was due to non specific activation of luciferase at high concentrations (Fig. 5b). PCB118 was found to weakly increase luciferase in both HG5LN-hPXR and HG5LN cells and thus should not be considered as an hPXR activator (Fig. 5). Finally, the other PCBs #28, 77, 126 and 169 as well as the 4 PAHs tested, i.e. benzo[k]fluoranthene, fluoranthene, naphthalene, and 3OH-benzo(a)pyrene, were unable to activate the hPXR after a 24 h exposure at concentrations up to  $1 \mu M$  (Tab. 3).

#### *Activation of hPXR by other compounds*

Other emerging pollutants, including parabens, antioxidants and plasticizers, were tested (Tab. 3). Among them, triphenyl phosphate (TPP), which is used as plasticizer and fire retardant, induced partially (Fig. 5a) but specifically (Fig 5b) the hPXR. Conversely, no effect was noted with parabens and butylated hydroxytoluene (BHT).

#### *Detection of hPXR activities in environmental samples*

In order to assess the environmental occurrence of hPXR ligands in surface waters, the hPXR bioassay was applied to different water samples issued from a contaminated river (POCIS extracts) and three sewage water effluents.

#### *Activation of hPXR by POCIS extracts*

POCIS samplers were deployed for 1 month in the Baïse River, which is under agricultural and urban pressure, and were then extracted and fractionated onto 3 fractions of increasing polarity (F1, F2, F3), as described in Material and Methods. Biological analysis showed that, among the 3 fractions, F1 and F2 expressed a PXR activity (Fig. 6a), whereas protocol blanks were inactive (data not shown). F1 and F2 gave an incomplete dose response curve with the highest activation for F1 ( $61 \pm 4 \%$ ) and a moderate activation for F2 ( $38 \pm 1 \%$ ) (Fig. 6a). In HG5LN cells, no significant alteration of luciferase activity was noted (Fig. 6b). These results show that POCIS extracts contained specific hPXR ligands that were likely to be semi-polar rather than polar compounds (i.e. hormones).

Targeted chemical analyses of POCIS fractions showed the presence of PXR ligands such as several pharmaceuticals compounds, pesticides, alkylphenols and hormones. The table 4 summarizes those chemicals that were identified as hPXR ligands among all chemicals analysed; a detailed analytical evaluation of these samples has been reported elsewhere ([34] and Tapie et al, in prep). On the basis of SR12813-Equivalent Factors (SREF) for individual compounds determined in the present study, instrumentally derived SR12813-equivalents (Chem-SR-EQ) were calculated and compared to SR-EQ determined by the bioassay (Bio-SR-EQ). The results (Tab. 4.) showed that, on an additive model basis, the targeted compounds weakly contributed ( $<0.1\%$ ) to Bio-SR-EQs in the samples and the detected PXR activities were likely due to other non analysed compounds.

#### *Activation of hPXR by effluent extracts*

All three effluent samples induced significant activation of luciferase activity in a dose-dependent (Fig. 7a) and specific (Fig. 7b) manner. Procedural blanks were inactive in HG5LN-hPXR cells (data not shown). The paper mill effluent (site A) was the most active site, followed by the mixed industrial/urban effluent (site C) and then by the urban effluent



(site B). When comparing the response of crude effluent extract to that of filtered effluent extracts, no significant difference could be noted on hPXR activation, suggesting that the detected compounds were associated with the soluble phase of the samples. Only a toxic effect was noted for the crude extract of site A (sample A-c in Fig 7), which led to a decrease of luciferase activity at higher sample concentrations (not illustrated).

Targeted chemical analyses of the samples showed the occurrence of several compounds that were hPXR ligands such as pharmaceuticals, hormones, pesticides, PCBs, bisphenol A (BPA) and 4-tert-octylphenol (4tOP) (Tab. 5). Mass balance calculation showed that the quantified chemicals explained only a few part of the biological activity determined by the bioassay (0.13-1.42%). Nevertheless, it was noted that BPA, which was among the most abundant contaminants, contributed to more than 95 % of the total Chem-SR-EQs at sites A and C, and to 10 % at site B. In this later site, an urban one, 4tOP contributed to more than 62 % of the calculated Chem-SR-EQs. Pharmaceuticals, hormones and pesticides (i.e. diuron and linuron) were also present at significant concentrations in the urban and mixed effluent (sites B and C), but they were identified as minor contributors to the Chem-SR-EQs. Moreover, effluent filtration had very low influence on Chem-SR-EQ determination, which confirmed the biological assessment and strengthened the hypothesis that the detected PXR ligands were mainly present in the dissolved phase. However, except for BPA and 4tOP, the compounds that mainly contributed to biological activities remain to be identified.

#### *Comparison with ER, AR and AhR activities*

To get further knowledge of the contamination of the samples by EDCs, we measured estrogenic, (anti)androgenic and AhR activities by using *in vitro* bioassays and compared them with hPXR activity (Tab. 6). In POCIS fractions, estrogenic and PAH-like activities were mainly detected in F1 and to a lesser extent in F2, yielding the same elution pattern as

hPXR activities. No (anti)androgenic or TCDD-like activity was detected in the three POCIS fractions. In effluents samples, ER and PAH-like activities were detected in all samples while a significant AR activity was also present at site C (Tab. 6.). Effluent filtration had either no influence at site A or enhanced ER and AR activity at sites B and C, thus showing that the detected active substances were in the soluble phase. Conversely, PAH-like activities were higher in crude than in filtered samples suggesting that the detected chemicals were mainly associated with the particulate fraction. Unlike POCIS fractions, profiles of ER activities in effluent samples differed from patterns of PXR responses, i.e. in a decreasing order, for estrogenic activity: site C > site B > site A, whereas for PXR activity: site A > site C >> site B. These results suggest that different chemicals were detected by the two bioassays, and stress the view that PXR activity provided additional information about chemical contamination.

## **DISCUSSION**

By using a recently developed stable reporter gene bioassay, we report i) the characterization of the hPXR transactivation potency of a large panel of environmental chemicals, including emerging pollutants that were not tested before, and ii) the occurrence of hPXR ligands in effluents and surface water samples.

### *hPXR is activated by various chemicals occurring in aquatic environment*

Of the 53 substances tested on HG5LN-hPXR cells, 37 were found active and 10 were identified as new hPXR agonists. These include three triazin pesticides (prometryn, terbutryn, terbutylazine), four pharmaceuticals (fenofibrate, bezafibrate, clonazepam, medazepam) and three PCB congeners (PCB 101, 138 and 180).

Pesticides and human pharmaceuticals were selected as relevant emerging contaminants that are increasingly detected in surface waters. Several pesticides have been described as potent PXR activators, among which organochlorinated pesticides, chloroacetanilides, pyrimidines and azoles fungicides were the most active ones [12,35]. In complement to these previous studies, we report here three triazin pesticides as hPXR modulators in HG5LN-hPXR cells. These triazin pesticides are widespread aquatic contaminants and among the most frequently detected pesticides in surface waters [36,37]. Accordingly, they were also detected in POCIS extracts from the Bâise River in the present study (Table 4).

A number of human pharmaceutical drugs have been described as CYP3A inducers through their capacity to bind and activate PXR [15]. Ten out of 21 compounds tested were found to partially but specifically activate hPXR in HG5LN-hPXR cells. Some of them such as tamoxifen [10], mevastatin [38] and carbamazepin [17] have previously been described as PXR ligands. Our data confirmed their activity and depicted their potency in our bioassay. Moreover, to our knowledge, the cholesterol-lowering drugs fenofibrate and bezafibrate and the benzodiazepines medazepam and clonazepam were here newly described as hPXR ligands. For fibrates, Prueksaritanont et al [39] reported a lack of hPXR transactivation by fenofibric acid, the metabolite of fenofibrate, in HepG2 cells transfected with a Gal-PXR/luciferase system. To some extent, this partly contrasts with our results with fenofibrate, the parent compound however, which activated the hPXR at micromolar concentrations. Finally, diclofenac, a non-steroidal anti-inflammatory drugs (NSAID) usually found in the aquatic environment [40], was also active on hPXR, in agreement with a recent report [15]. Besides, it is noted that 11 of the tested drugs, including environmentally recurrent ones (e.g. caffeine), were not active in the assay, hence showing some limitation of the bioassay as a biotector for pharmaceuticals. Overall, our data on hPXR activation by various classes of

pesticides and **environmental** pharmaceuticals confirm its potential use as a sensor for **certain** emerging aquatic contaminants.

Previous studies pointed out the ability of nonplanar PCBs to bind and activate or inhibit PXR from different species [11,16,41]. Accordingly, we observed that nonplanar PCBs but not coplanar PCBs were hPXR ligands, although some discrepancies with previous studies were noted. By using a transient Gal-hPXR reporter gene assay, Tabb et al. [16] described several highly chlorinated PCBs as very weak hPXR agonists and as potent hPXR antagonists, especially for congeners #153, 145, 184 and 197. In contrast, we found that nonplanar PCBs with more than five chlorine substituents (PCBs #101, 138, 153 and 180) were potent hPXR activators (Fig. 5), but they were unable to antagonize hPXR in HG5LN-hPXR cells **after co-exposure with 0.1  $\mu$ M SR12813** (data not shown). Our results are however in agreement with other published studies reporting the agonistic effect of non planar PCBs on mouse (i.e. for PCB #47, 184, 188, 196, 200 [41]) and human PXR (i.e. for PCB #118, 153 [11]) in transient hPXR transactivation assays. For PCB118 however, we strongly suspected unspecific luciferase activation in both HG5LN-hPXR and HG5LN cells, hence we ranked this compound as a negative compound. Altogether, the ability of non coplanar PCBs to interfere with hPXR, here reinforced by our new results with PCB101, 138 and 180, stresses the view that such a PXR bioassay could serve as a biodetector for non dioxin-like PCBs in environmental bio-analysis.

Among the other emerging EDCs tested, we notably report TPP as a relatively potent active chemical on HG5LN-hPXR cells, in accord with a previously published work [42]. TPP belongs to high-production-volume organophosphate esters (OPEs) widely used as flame retardant and plasticizers in several applications. OPEs are widespread water and air, sediment and soil contaminants [43]. Recently, TPP was shown to bioaccumulate in aquatic

organisms such as **molluscs** (mussels, oysters, clams) at concentration up to 378 ng/g dry wt [44] and environmental risk assessment for this substance is of current concern [45].

Overall, the chemical screening carried out in the present study complemented previous studies performed with HG5LN-hPXR cells [12,14] in that it further characterized and extended to various structurally different contaminants the range of chemicals to be detected by this bioassay. In a bioanalytical perspective, establishment of dose-response curves for each individual compound allowed determining their relative potency as SR12813 equivalent factors (SREF) essential for mass balance calculation in the bioanalysis of environmental samples. This has been investigated in the second part of the study.

*HG5LN-hPXR cell line as a biosensor for the detection of emerging compounds in environmental matrices?*

To our knowledge, this is one of the first reports of the biological detection of PXR activating substances in surface water (POCIS) and effluent samples. Although we did not identify the nature of major chemicals responsible for the detected activities, mid-polar compounds in the dissolved water phase from both river and effluent samples could be suspected. In addition, the comparison of PXR activity with other receptor-mediated activities suggested that the PXR bioassay provided different information on chemical contamination, likely related to different detected compounds, and was thus complementary to the other assays within such a multi-tests battery (Tab. 6).

By using the same *in vitro* model as in the present study, very recent studies reported the detection of PXR activities in wastewaters [46,47], and river sediments from contaminated areas [48]. In these studies, instrumental analyses of target chemicals, including alkylphenols, steroid estrogens or organochlorinated pesticides, did not permit to identify the active

chemicals responsible for the PXR activity. Moreover, recent studies reported induction of Cyp3A expression by municipal effluents in cultured trout hepatocytes *in vitro* [49] or in zebrafish liver *in vivo* [50]. In the study of Lister et al., PXR mRNA expression in zebrafish was not affected after effluent exposure. In any case, the substances responsible for biological activities were not identified.

Altogether, these results clearly show that chemical agents that can affect the PXR signaling pathway are present in the aquatic environment. Due to the important role of PXR in endocrine and xenobiotic metabolism regulation, identification of these agents is an important issue to be addressed. For such purpose, application of mass balance analysis (MBA) based on targeted chemical analyses coupled to *in vitro* bioassays has proven useful as a first characterization step of the chemicals responsible for biological effects, provided that significant information background on environmental levels of bioactive substance is available. For instance, by targeting steroid estrogens or PAHs, MBA approach has often succeeded in characterizing, at least partly, the chemicals responsible for “classical” activities such as ER or AhR activity, respectively [48,51,52]. For PXR activity however, such an *a priori* based investigation failed in identifying the main substances responsible for PXR activities in our study. Only BPA and 4tOP were shown to weakly contribute to overall PXR activity in wastewater effluent samples, while the other analyzed substances known to activate hPXR, including pharmaceuticals, planar PCBs or pesticides, were present at too low concentrations to significantly account for the measured biological activities.

Several hypotheses can be advanced to explain the lack of concordance between biological and chemical analyses. The most probable cause is that we did not target the “good” chemicals by the chemical analyses. As a matter of fact, PXR can be activated by a wide diversity of known (and likely unknown) environmental compounds [53], and it is technically

impossible to perform exhaustive analyses. For instance, it is noted that some strong PXR activators like mevastatin, clotrimazole, pretilachlor or oxadiazon could not be measured in our samples. Moreover, the serious lack of data on the environmental occurrence of PXR active substances makes difficult *a priori* based investigation, as stated above. On the other hand, the mass balance approach we used assumes a concentration additive model, based on parallel dose-response curves and equal efficacy of chemical agonists [33]. It is noteworthy that some of the individual chemicals that we tested behave as partial PXR agonists (e.g. clotrimazole, mediazepam, oxadiazon, TPP) and it can be expected that other than additive interactions may occur when they are present in complex mixtures, hence impeding PXR activity prediction on the basis of individual potency of measured active compounds. Moreover, hPXR has a large and flexible ligand binding domain, allowing the binding of a wide range of structurally different ligands with molecular weights ranging from less than 250 kDa to more than 800 kDa [19,54]. Thus, its smooth binding pocket could host several ligands at the same time, which might result in synergic response. However, to our knowledge, interactive effects of chemical mixtures on PXR activation has not been reported so far, and would thus be an important issue to be addressed within the context of the present study.

Overall, this study should be considered as an exploratory study and further research will be necessary to identify PXR active chemicals in environmental samples. This will go through the implementation of effect-directed analyses coupled to dedicated sample fractionation protocols [55]. Also, the development of nuclear receptor-affinity columns [56], based on recombinant hPXR, represents a promising way to isolate and purify hPXR ligands from complex mixtures. These approaches are under evaluation in our laboratories and are expected to provide new information on the chemical nature of active PXR ligands present in the environment.

## CONCLUSION

In this work, we report the successful use of the stable HG5LN-hPXR reporter gene assay to detect hPXR activation by a panel of emerging compounds and by surface and waste water samples, albeit the main compounds that contributed to the detected activities remain to be identified. Combined to other in vitro bioassays for EDCs and AhR activating chemicals, the hPXR assay provided complementary information on bioactive contaminants and thereby enhanced the environmental diagnostic. As PXR plays a crucial role in xenobiotic detoxification and hormone metabolism, alteration of PXR signaling pathway may lead to biological effects in exposed organisms, as recently reported in fish [50]. However, marked cross-species differences have been reported for PXR transactivation by xenobiotics [57]. Thus, for a proper risk assessment to aquatic organisms, the use of a dedicated fish PXR bioassay to characterize potential hazard to fish population may also be considered.

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## Figure captions

**Fig. 1** Schematic presentation of the preparation of effluent extracts and *in vitro* testing using bioassays.

**Fig. 2** (a) Dose-response curves of reference compounds in HG5LN-hPXR cells. Results are expressed as percentage of maximal luciferase activity induced by SR12813 at 3  $\mu$ M. (b) Specificity test for luciferase induction in HG5LN cells. Results are expressed as percentage of luciferase activity measured in DMSO treated cells (solvent control). Values are means of triplicates  $\pm$  SD and are representative of at least three independent experiments

**Fig. 3** (a) Dose-response curves of several pesticides in HG5LN-hPXR cells. Results are expressed as percentage of maximal luciferase activity induced by SR12813 at 3  $\mu$ M. (b) Specificity test of luciferase induction by pesticides in HG5LN cells. Results are expressed as percentage activity measured in the presence of DMSO (solvent control). \*: Significantly different from solvent control in HG5LN cells ( $p < 0.05$ ). **In HG5LN-hPXR cells, all values are significantly different from control ( $p < 0.05$ ).** Values are means of triplicates  $\pm$  SD and are representative of at least three independent experiments

**Fig. 4** (a) Dose-response curves of pharmaceuticals compounds in HG5LN-hPXR cells. Results are expressed as percentage of maximal luciferase activity induced by SR12813 at 3  $\mu$ M. (b) Specificity test of luciferase activity induction by pharmaceuticals in HG5LN cells. Results are expressed as percentage activity measured in the presence of DMSO (solvent control). \*: Significantly different from solvent control ( $p < 0.05$ ). **In HG5LN-hPXR cells, all values are significantly different from control ( $p < 0.05$ ).** Values are means of triplicates  $\pm$  SD and are representative of at least three independent experiments

**Fig. 5** (a) Dose-response curves of TPP and non co-planar PCBs in HG5LN-hPXR cells. Results are expressed as percentage of maximal luciferase activity induced by SR12813 at 3  $\mu$ M. (b) Specificity test of luciferase activity induction by TPP and non co-planar PCBs in HG5LN cells. Results are expressed as percentage activity measured in the presence of DMSO (solvent control). \*: Significantly different from solvent control ( $p < 0.05$ ). **In HG5LN-hPXR cells, all values are significantly different from control ( $p < 0.05$ ).** Values are means of triplicates  $\pm$  SD and are representative of at least three independent experiments

**Fig. 6** (a) Dose-response of POCIS extracts in HG5LN-hPXR cells. Results are expressed as percentage of maximal luciferase activity induced by SR12813 at 3  $\mu$ M. (b) Specificity test of luciferase activity induction by POCIS extracts in HG5LN cells. Results are expressed as percentage activity measured in the presence of DMSO (solvent control). \*: Significantly different from solvent control ( $p < 0.05$ ). **In HG5LN-hPXR cells, all values are significantly different from control ( $p < 0.05$ ).** Values are means of triplicates  $\pm$  SD and are representative of at least three independent experiments

**Fig. 7** (a) Dose-response curves by organic extracts of crude (-c) or filtered (-f) effluents A, B and C in HG5LN-hPXR cells. Results are expressed as percentage of maximal luciferase activity induced by SR12813 at 3  $\mu$ M. (b) Specificity test of luciferase activity induction by effluents extracts in HG5LN cells. Results are expressed as percentage activity measured in the presence of DMSO (solvent control). Values are means of triplicates  $\pm$  SD and are representative of at least three independent experiments

Table 1. Summary of hPXR activation by pesticides in HG5LN-hPXR cells.

	Compound	EC <sub>50</sub> (mol/l)	Maximal luciferase induction <sup>a</sup>	Concentration at max. induction (μM)
Active compounds	Oxadiazon <sup>b</sup>	5.31 E-07	68 ± 2	3
	Pretilachlore <sup>b</sup>	1.32 E-07	94 ± 8	3
	<i>o,p'</i> -DDT <sup>b</sup>	3.96 E-06	143 ± 19	10
	Linuron <sup>b</sup>	3.68 E-05	32 ± 3	10
	Lindane	5.45 E-06	71 ± 4	10
	Endosulfan	3.84 E-06	73 ± 3	10
	Triclosan	8.0 E-06	32 ± 2	3
	Prometryn	1.44 E-05	51 ± 2	10
	Terbutryn	1.78 E-05	48 ± 2	10
	Terbutylazine	3.34 E-05	32 ± 1	10
No active compounds	Tributyltin, chlorotoluron, metoxuron, chlorosulfuron, nicosulfuron and hexachlorobenzene			

*a*, maximal luciferase induction is expressed as a percentage of maximal luciferase activity induced by SR12813 at 3 μM (mean value of triplicates ± SD); *b*, compounds already tested by Lemaire et al. (2004, 2006).

Table 2. Summary of hPXR activation by pharmaceuticals in HG5LN-hPXR cells

	Compounds	EC <sub>50</sub> (mol/l)	maximal luciferase induction <sup>a</sup>	Concentration at max. induction (μM)
Active compounds	Rifampicin <sup>b</sup>	3.80 E-07	87 ± 6	3
	Clotrimazole <sup>b</sup>	4.71 E-07	54 ± 2	3
	T0901317 <sup>b</sup>	5.24 E-09	83 ± 2	0.1
	EE2 <sup>b</sup>	3.01 E-06	37 ± 2	10
	SR 12813 <sup>b</sup>	6.90 E-08	100 ± 5	3
	Fenofibrate	1.21 E-06	61 ± 8	3
	Tamoxifen	1.26 E-06	39 ± 6	10
	Mevastatin	2.39 E -06	80 ± 1	30
	Medazepam	4.22 E-06	50 ± 4	10
	Diazepam	8.15 E-05	39 ± 3	30
	Clonazepam	1.11 E-04	27 ± 1	10
	Diclofenac	1.23 E-04	48 ± 1	100
	Bezafibrate	2.02 E-04	39 ± 4	100
	Carbamazepin	2.72 E-04	40 ± 1	100
	Ketoprofene	3.49E-04	30 ± 3	100
Triazolam	3.77E-04	36 ± 1	30	
Inactive compounds	loprazolam, bromazepam, alprazolam, naproxen, ibuprofen, aspirin, caffen, flumazemil, pravastatin and sulfamethoxazole			

*a*, maximal luciferase induction is expressed as a percentage of maximal luciferase activity induced by SR12813 at 3 μM (mean value of triplicates ± SD); *b*, compounds already tested by Lemaire et al. (2004, 2006) and/or Mnif et al., 2007.

Table 3. Summary of hPXR activation by POPs, plasticisers and other compound tested on HG5LN-hPXR cells

	Compound	EC <sub>50</sub> (mol/l)	Maximal luciferase induction <sup>a</sup>	Concentration at max. induction (µM)
Active compounds	PCB 180	1.50 E-05	82 ± 6	30
	PCB 138	1.51 E -05	112 ± 4	30
	PCB 153	1.53 E-05	85 ± 11	30
	PCB 101	2.06 E-05	74 ± 5	30
	PCB 118	2.83 E-05	38 ± 2	30
	BPA <sup>b</sup>	2.71 E-05	37 ± 2	10
	TPP	1.36 E-06	62 ± 2	10
Inactive compounds	PCB 28, 52, 77, 126, 169, benzo[k]fluoranthene, 3OH-benzo[a]pyrene, fluoranthene, naphtalene and TCDD BHT, DHT, n-butyl paraben, n-benzyl paraben, n-pentyl paraben			

*a*, maximal luciferase induction is expressed as a percentage of maximal luciferase activity induced by SR12813 at 3 µM (mean value of triplicates ± SD). *b*, compound already tested by Mnif et al., 2007.



Table 4. Chemical analyses and SR12813-EQ determination in POCIS extracts

	Detected compounds	SREF <sup>a</sup>	Concentration (ng/POCIS)		
			F1	F2	F3
<i>Pharmaceuticals</i>	Carbamazepin	6.0E-04	1.6	0.1	n.d.
	Diclofenac	5.4E-04	0.5	3.7	n.d.
<i>Pesticides</i>	Terbutylazine	4.1E-03	2	n.d. <sup>b</sup>	n.d.
	Promethryn	1.0E-02	2	n.d.	n.d.
	Terbutryn	1.0E-02	1	n.d.	n.d.
	Lindane	2.7E-02	2	n.d.	n.d.
	Isoproturon	1.9E-02	6	n.d.	n.d.
	Linuron	3.1E-03	12	n.d.	n.d.
	Diuron	1.9E-03	23	n.d.	n.d.
	<i>o,p'</i> -DDT	1.7E-02	1	n.d.	n.d.
<i>Alkylphenols</i>	4tOP	5.8E-02	10	<1	<1
	4NP	3.0E-02	20	<3	<3
	BPA	5.8E-03	6	<1	<1
<i>Hormones</i>	E2	3.0E-03	n.d.	n.d.	2
$\Sigma$ Chem-SR-EQ <sup>c</sup>	( $\mu\text{g}/\text{POCIS}$ )		1.5E-03	1.4E-04	1.1E-04
Bio-SR-EQ <sup>d</sup>	( $\mu\text{g}/\text{POCIS}$ )		3.59	1.01	n.d.
Chem-SR-EQ / Bio-SR-EQ	(%)		0.037	0.013	-

<sup>a</sup>, SREF: SR12813 equivalence factor relative to SR12813, determined as described in the Materials and Methods section. <sup>b</sup> n.d.: not detected, <sup>c</sup> Chem-SR-EQs: chemical SR12813 equivalents based on chemical analyses, <sup>d</sup> Bio-SR-EQs: biological SR 12813 equivalents based on EC<sub>20</sub> effective sample concentration in the HG5LN-hPXR bioassay.

Table 5. Chemical analyses and SR12813-EQ determination in effluent extracts

Detected compounds		SREF <sup>a</sup>	Concentration (ng/L)					
			Site A		Site B		Site C	
			Crude	Filtered	Crude	Filtered	Crude	Filtered
<i>Pharmaceuticals</i>	Carbamazepin	6.8E-04	- <sup>b</sup>	6	-	559	-	248
	Diazepam	1.9E-03	-	n.d. <sup>c</sup>	-	11	-	n.d.
	Ketoprofen	4.0E-04	-	n.d.	-	950	-	303
	Diclofenac	5.4E-04	-	27	-	310	-	245
<i>Hormones</i>	E1	4.1E-03	-	83	-	8	-	58
	EE2	4.8E-03	-	n.d.	-	10	-	n.d.
<i>Pesticides</i>	Diuron	1.9E-03	-	70	-	430	-	349
	Linuron	3.1E-03	-	5	-	294	-	126
	Isoproturon	1.8E-03	-	12	-	0.4	-	9
	Lindane	2.2E-02	4.5	4	7.3	7	20.5	19
	<i>o,p'</i> -DDT	1.8E-02	0.3	n.d.	0.3	0.1	0.3	n.d.
<i>PCBs</i>	PCB 101	3.4E-03	10.8	5.3	3.3	2.3	16	9
	PCB 138	5.3E-03	3.2	n.d.	0.7	n.d.	7.5	4
	PCB 153	6.0E-03	14	10.2	3.1	2.4	19.4	15
	PCB 180	6.1E-03	0.8	n.d.	0.1	n.d.	0.7	n.d.
<i>Alkylphenols</i>	BPA	5.8E-03	6474	5100	232	230	13809	13600
	4tOP	6.1E-02	n.d.	n.d.	124	124	9	n.d.
$\Sigma$ Chem-SR-EQ <sup>d</sup>	( $\mu\text{g/L}$ )		0.038	0.030	0.009	0.012	0.081	0.081
Bio-SR-EQ <sup>e</sup>	( $\mu\text{g/L}$ )		23.0	22.8	0.76	0.85	9.45	10.7
Chem-SR-EQ / Bio-SR-EQ	(%)		0.16	0.13	1.20	1.39	0.86	0.76

*a*: SREF: SR12813 equivalence factor relative to SR 12813, determined as described in the Materials and Methods section. <sup>b</sup> - : only analysed in dissolved phase due to hydrophilic properties; *c*: n.d.: not detected or below quantification limits, *d*: Chem-SR-EQs: chemical SR12813 equivalents, *e*: Bio-SR-EQs: biological SR12813 equivalents based on EC<sub>20</sub> effective sample concentration in the HG5LN-hPXR bioassay.

Table 6. Estrogenic, (anti)androgenic, PAH-like and dioxin-like activities in POCIS and effluent extracts.

		Measured activities				
		Estrogenic	Androgenic	Anti-androgenic	PAH-like	Dioxin-like
		E2-EQ (ng/POCIS)	DHT-EQ (ng/POCIS)	FLU-EQ (µg/POCIS)	BaP-EQ (µg/POCIS)	TCDD-EQ (ng/POCIS)
<i>POCIS extracts</i>						
Procedural blank		n.d.	n.d.	n.d.	n.d.	n.d.
F1		0.44	n.d.	n.d.	47.5	n.d.
F2		0.06	n.d.	n.d.	15.8	n.d.
F3		n.d.	n.d.	n.d.	n.d.	n.d.
<i>LD</i>		<i>0.02</i>	<i>0.2</i>	<i>0.5</i>	<i>1.1</i>	<i>0.6</i>
<i>Effluent extracts</i>		E2-EQ (ng/L)	DHT-EQ (ng/L)	FLU-EQ (µg/L)	BaP-EQ (µg/L)	TCDD-EQ (ng/L)
Procedural blank		n.d.	n.d.	n.d.	n.d.	n.d.
A	crude	2.2	n.d.	n.d.	0.320	n.d.
	filtered	2.0	n.d.	n.d.	0.107	n.d.
B	crude	1.9	n.d.	n.d.	0.453	n.d.
	filtered	3.2	n.d.	n.d.	0.231	n.d.
C	crude	5.8	8.1	n.d.	0.608	n.d.
	filtered	8.2	14.1	n.d.	0.245	n.d.
<i>LD</i>		<i>0.09</i>	<i>0.98</i>	<i>0.45</i>	<i>0.004</i>	<i>2.7</i>

LD: limit of detection

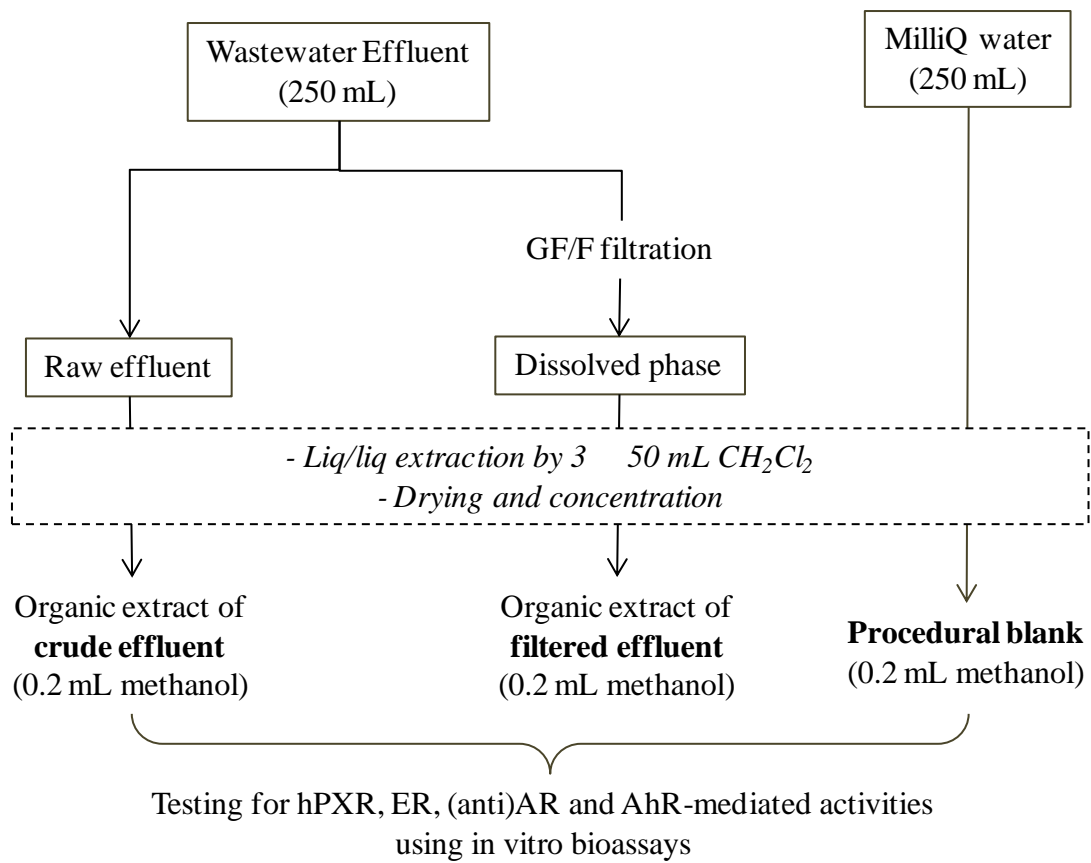


Fig. 1

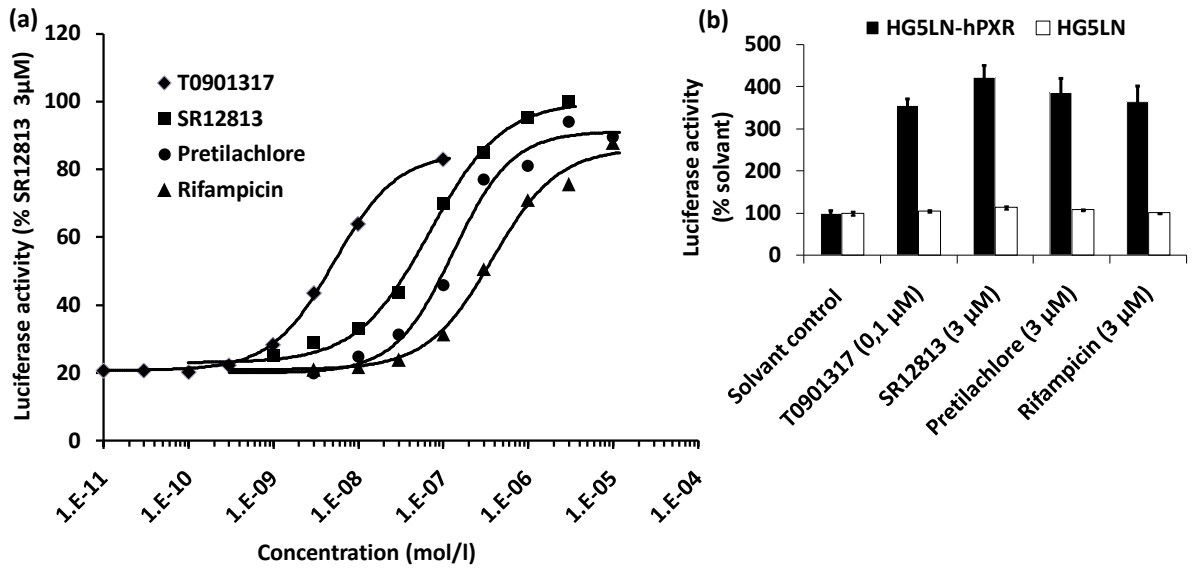


Fig. 2

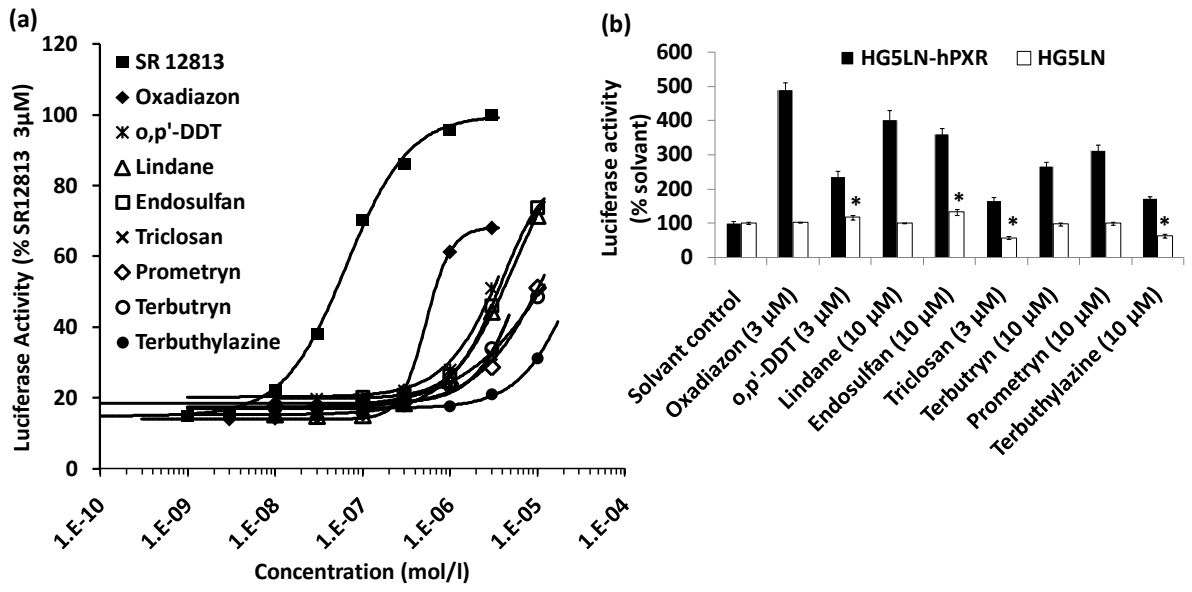


Fig. 3

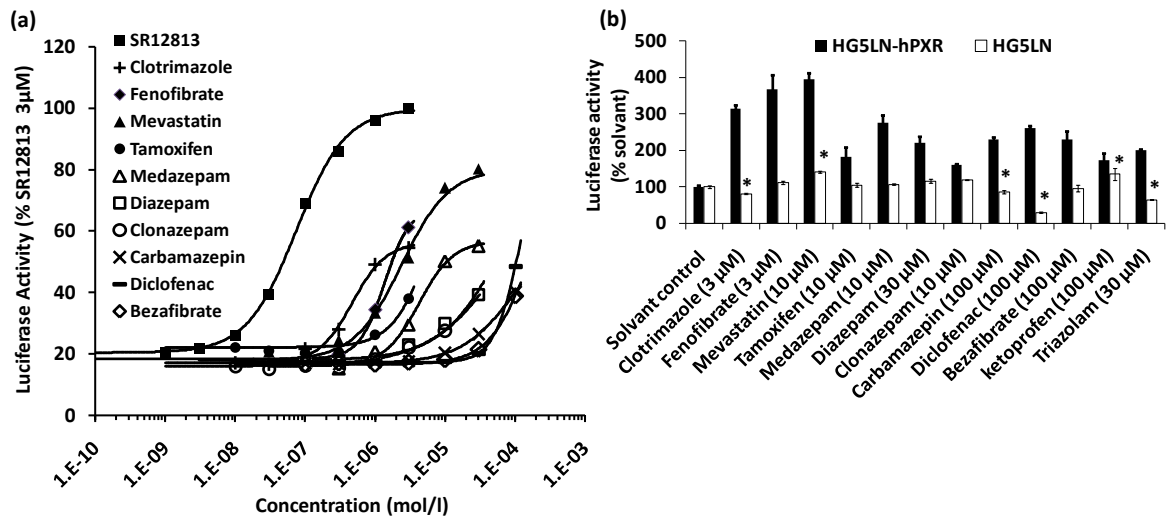


Fig. 4

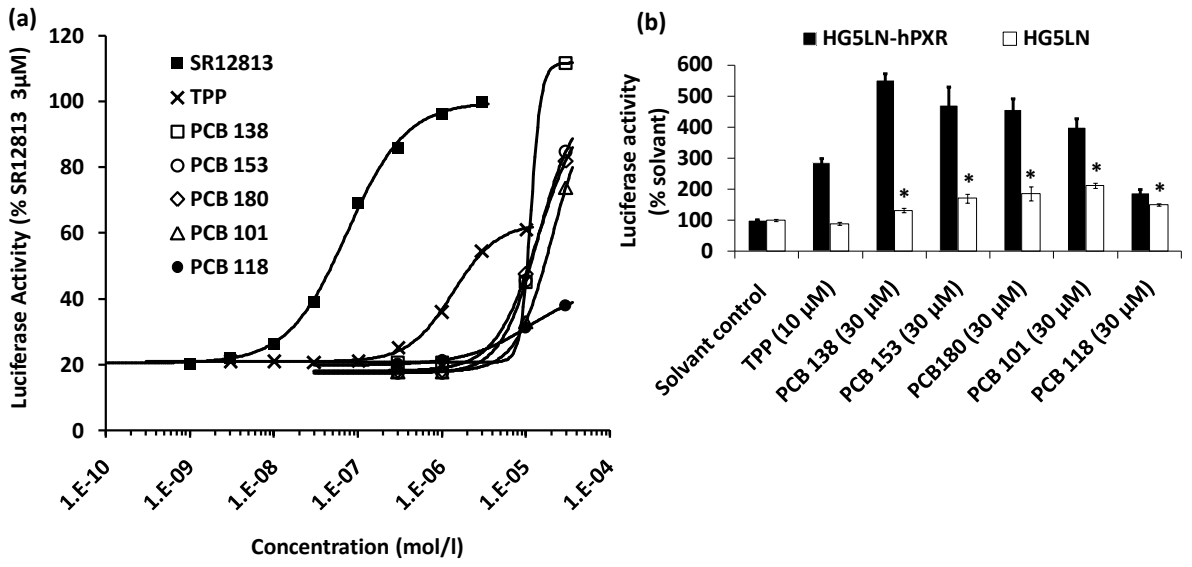


Fig. 5



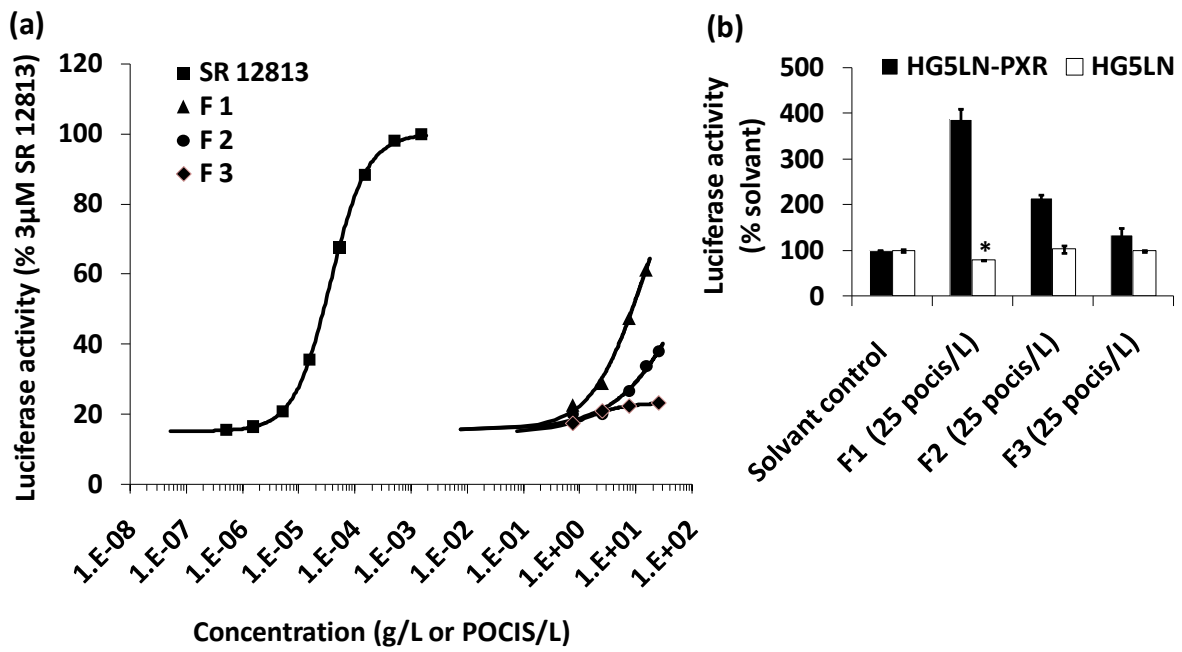


Fig. 6

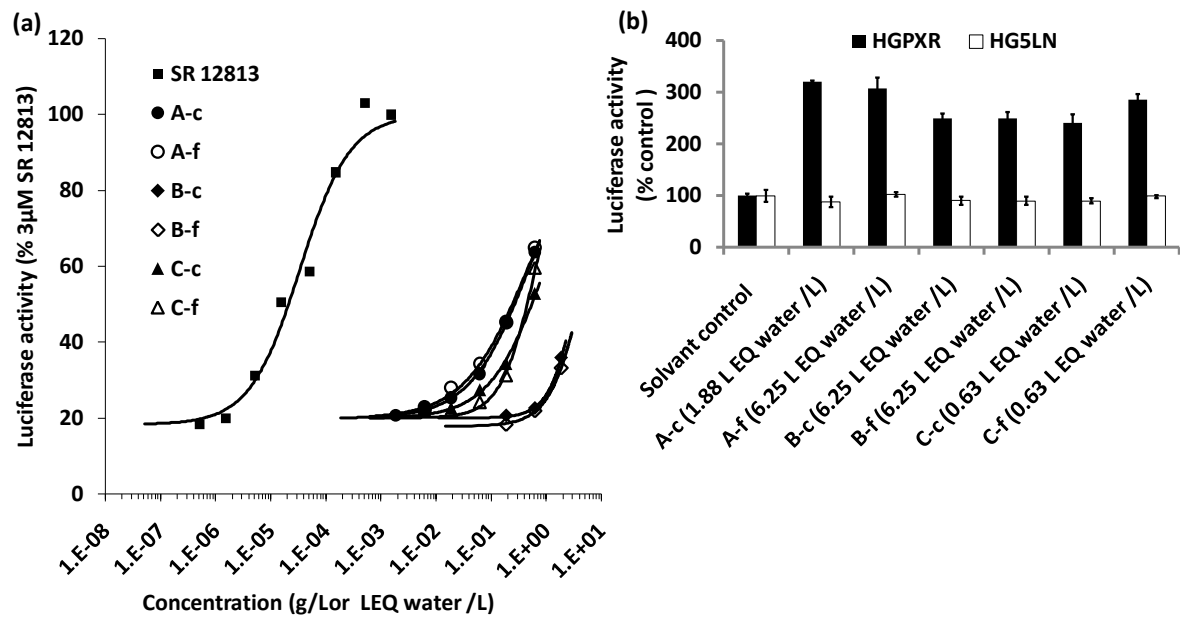


Fig. 7