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Application and interpretation of hPXR screening data: Validation of reporter signal requirements for prediction of clinically relevant CYP3A4 inducers

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ABSTRACT

A human pregnane X receptor (PXR) reporter-gene assay was established and validated using 19 therapeutic agents known to be clinical CYP3A4 inducers, 5 clinical non-inducers, and 6 known inducers in human hepatocytes. The extent of CYP3A4 induction (measured as RIF ratio in comparison to rifampicin) and EC50 was obtained from the dose–response curve. All of the clinical inducers (19/19) and human hepatocyte inducers (6/6) showed positive responses in the PXR assay. One out of five clinical non-inducers, pioglitazone, also showed a positive response. An additional series of 18 commonly used drugs with no reports of clinical induction was also evaluated as putative negative controls. Sixteen of these were negative (89%), whereas two of these, flutamide and haloperidol showed 16-fold (RIF ratio 0.79) and 10-fold (RIF ratio 0.48) maximal induction, respectively in the reporter-gene system. Flutamide and haloperidol were further demonstrated to cause CYP3A4 induction in human cryopreserved hepatocytes based on testosterone 6β-hydroxylation activity. The induction potential index calculated based on the maximum RIF ratio, EC50, and in vivo maximum plasma concentration was used to predict the likelihood of CYP3A4 induction in humans. When the induction potential index is greater than 0.08, the compound is likely to cause induction in humans. A high-throughput screening strategy was developed based on the validation results at 1 μM and 10 μM for the same set of drugs. A RIF ratio of 0.4 was set as more practical screening cut-off to minimize the possibility of generating false positives. Thus, a tiered approach was implemented to use the human PXR reporter-gene assay from early lead optimization to late lead characterization in drug discovery.

1. Introduction

Hepatic microsomal cytochrome P450 3A is the major cytochrome P450 family responsible for the metabolism of endogenous and xenobiotic substrates. In humans, CYP3A comprises 30–50% of the total microsomal cytochrome P450 in liver and small intestine [1,2]. To date, four human CYP3A isoforms have been identified, namely, CYP3A4, CYP3A5,

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Abbreviations: CAR, constitutive androstane receptor; CYP, cytochrome P450; DEX, dexamethasone; GR, glucocorticoid receptor; NCEs, new chemical entities; PB, phenobarbital; PCN, pregnenolone 16α-carbonitrile; PPAR, peroxisome proliferator activated receptor; PXR, pregnane X receptor; RIF, rifampicin; RXR, retinoic acid X receptor; VDR, vitamin D receptor.

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CYP3A7, and CYP3A43. Among them, CYP3A4 is the most abundant isoform, and is present in every adult liver sample screened [1]. Approximately 60% of marketed drugs are metabolized by CYP3A4 [3]. Due to the high abundance and the broad substrate specificity of CYP3A4, induction of this enzyme in humans could have severe consequences. For example, co-administration of cyclosporine A with CYP3A4 inducers such as rifampicin, sulfamethazine, and other anticonvulsant agents in organ transplant patients has resulted in a drastic decrease in cyclosporine A plasma concentrations, leading to organ rejection, or even death, among these patients [4].

Induction of CYP3A4 is regulated mainly at the transcriptional level. A molecular mechanism of CYP3A4 induction was revealed upon the discovery of the pregnane X receptor (PXR) [5–7] and the delineation of the upstream promoter and enhancers of CYP3A genes [8]. After binding to an agonist ligand in the cytosol, PXR translocates into the nucleus and forms a heterodimer with the retinoic acid X receptor alpha (RXRα), interacts with the response element(s) DR3 and/or ER6 located upstream of the CYP3A4 gene, and activates transcription [9].

The ligands for PXR are structurally diverse, and include therapeutic agents such as rifampicin, efavirenz, endogenous compounds such as steroids and bile acids, and the natural products as hyperforin [10]. The crystal structure of the human PXR ligand-binding domain has been solved, and shows that the ligand-binding site has a relatively large hydrophobic cavity, spaced throughout with a small number of polar residues [11]. The ligand-binding site also contains a 13-residue loop and 2 additional β-strands, which are not found in other nuclear receptors [11]. These features give PXR the flexibility to accommodate a very large number of structurally diverse chemicals. Recently, it was shown that several other nuclear receptors, such as the constitutive androstane receptor (CAR), the glucocorticoid receptor (GR) [12], and the vitamin D receptor (VDR) [13], may also interact with the CYP3A4 enhancer sequences, therefore, leading to various degrees of induction of the CYP3A genes. Several studies have demonstrated crosstalk between the CAR- and PXR-dependent signal transduction pathways. First, PXR and CAR share some ligands, including steroids and drugs [14]. Secondly, CAR was shown to transactivate through the CYP3A response element that serves as the PXR/RXR binding site [15,16]. However, based on the recent X-ray structure and structural modeling of CAR, it has been suggested that despite the crosstalk, CAR may play a limited role in the induction of CYP3A4 and is less promiscuous in terms of ligand-binding specificity [17–19].

Several in vitro assays have been developed to study CYP3A4 induction by various xenobiotics. Isolated primary human hepatocytes have been used to evaluate CYP induction; however, this approach is limited due to the low availability of human livers and the large individual variability in induction capacity. Precision-cut liver slices also have been used in CYP induction studies. While liver slices maintain the structural architecture of intact liver, this approach is also limited by the low availability of fresh human livers. The discovery of the signaling pathway of CYP3A induction has allowed the establishment of high-throughput screening assays for CYP3A4 induction. Luo et al. [20] have shown that data obtained from the human PXR reporter-gene assay correlate reasonably well with induction data from human hepatocytes. In this study, we have used clinically proven CYP3A4 inducers and non-inducers as well as putative non-inducers to evaluate the human PXR reporter-gene system, and correlate directly to CYP3A4 induction in humans. The human PXR reporter gene assay we describe predicts in vivo CYP3A4 induction with satisfactory accuracy and therefore can be used as a screening tool in lead optimization to improve the quality of drug candidates.

2. Materials and methods

2.1. Chemicals

Terbinafine was from ChemPacific Co. (Baltimore, MD). Troglitazone was purchased from Biomol International LP (Polymouth Meeting, PA). Ritonavir was obtained from Sequoia Research Product, Ltd. (Berkshire, UK). Tamoxifen was purchased from MP Biomedicals, LLC (Irvine, CA). Rifabutin was from Apin Chemicals (Oxon, UK). Fluoxetin was obtained from BD Biosciences (Rockville, MD). Rifampicin, carbamazepine, dexamethasone, captopril and haloperidol were purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Efavirenze and aprepitant were synthesized in house. The rest chemicals used in this study were from Sigma–Aldrich Corporation (St. Louis, MO).

2.2. Plasmid construction

The DNA fragment I of CYP3A4 5′ flanking region (−7836 to −7208) was amplified by PCR from GenomeWalker DNA library (Clontech, Moutain View, CA) using an Advantage 2 PCR kit. The forward primer 3A7836Mlu has the sequence 5′-GCA TAC GCG TTC TAG TGA GAT GGT TCA TTC CTT TC-3′ with an engineered restriction enzyme site Mlu I. The reverse primer 3A-7208OP has the sequence 5′-ACC TCA GCC AGC AAT GTC GTC AAC AGG TTA AAG GAG AAT GG-3′, and is a chimeric oligo which covers the region from −7208 to −7231 and the region from −1068 to −1084. The fragment II of CYP3A4 5′ flanking region (+53 to −1802) was amplified by PCR using reverse primer 3A + 53Xho (5′-GAT TCT CGA GTG TTG CTC TTT GCT GGG CTA TGT GC-3′), which has an engineered XHO I site, and forward primer 3A-1084OP (5′-TTT AAC CTG TTG ACG ACA TTG CTG GCT GAG GTG GTT GG-3′), which is complementary to primer 3A-7208OP, covering region from −7208 to −7222 and region from 1062 to −1084. The two PCR reactions were mixed at 1:1 ratio and diluted 1:100. The final PCR was performed on this mixture using primers 3A7836Mlu and 3A + 53Xho. The PCR product contains both fragments I and II with a size of 1.7 kb on 1% agarose gel. This band was excised, extracted, and digested with Xho I and Mlu I, then ligated into a pGL3 basic vector (Promega, Madison, WI) at the same restriction enzyme sites. The resulting vector was called pGL3B-3A4 after confirmation by DNA sequence analysis.

The human pregnane X receptor gene was amplified from marathon-ready cDNA (Clonetech) with primer pair Hup-1 5′-ATG GAG GTG AGA CCC AAA GAA AGC TGG-3′ and Hlow-1 5′-

GCT CAG CTA CCT GTG ATG CCG AAC AAC T-3′. The 1.3-kb PCR fragment was subcloned into pcDNA4/HisMax-TOPO vector (Invitrogen, Carlsbad, CA) and the newly constructed plasmid was called pcDNA4/HisMax-TOPO-hPXR. The sequence of the final plasmid was confirmed by DNA sequence analysis.

2.3. Transient transfection

HepG2 cells were seeded at a density of 0.8×10^5 cells/well into 48-well plates in GIBCO® Dulbecco's modified Eagle's medium (Invitrogen Life Science, Carlsbad, CA) supplemented with 10% fetal bovine serum and antibiotics penicillin G (50 units/mL) and streptomycin sulfate (50 μg/mL) 24 h prior to transfection. The cells were co-transfected with 250 ng of pGLB-3A4, 40 ng pcDNA4/HisMax-TOPO-hPXR, and 20 ng pRL-TK internal control vector (Promega Corporation, Madison, WI) using FuGENE 6 transfection reagent (Roche, Pleasanton, CA). Six hours after transfection, cells were treated once for 48 h with various concentrations of the test compounds in DMEM medium without antibiotics. After 48 h, the cells were harvested and analyzed for luciferase expression with a dual luciferase assay kit (Promega).

2.4. PXR data analysis

The firefly luciferase activity was normalized to the *Renilla* luciferase activity to determine the transfection efficiency for each well. The fold-induction was calculated as follows: fold-induction = normalized luciferase activity for treatment group/normalized luciferase activity of vehicle control group. To control for batch-to-batch variation, the ratio of the fold-induction for each treatment to the maximum fold-induction for rifampicin (RIF ratio) was calculated.

To get a complete dose–response curve, compounds were dosed at the highest concentrations allowed by solubility. *Renilla* luciferase activity was used to monitor any cytotoxicity. When the *Renilla* luciferase activity dropped below 40% of the vehicle control group value, the data was considered to be unreliable due to severe cytotoxic effect and was not reported.

EC50 values were calculated using GraphPad Prism (version 4.2) by sigmoidal curve-fitting using formula $Y = E_0 + (E_{max} - E_0)/(1 + 10\text{^}((\log EC50 - X)))$, where X is the logarithm of concentration, and Y is the response expressed as the RIF ratio. E_0 and E_{max} is the baseline and maximum response of a given compound, respectively. For compounds like efavirenz, sulfinpyrazone, tamoxifen, omeprazole, lansoprazole, and troglitazone, no plateau was observed for the dose–response curve. In theses cases, the top value was constrained to the highest reported RIF ratio.

2.5. Human hepatocyte induction

Two lots of cryopreserved human hepatocytes (Becton Dickson lot #47 and In Vitro Technologies lot #130) were plated at a density of 4×10^5 cells/well into Biocoat 24-well collagen I plates (BD Biosciences, Rockville, MD) 24 h prior to induction following the manufacturer's instruction. The hepatocytes were treated with various concentrations of test compounds for 48 h. Afterwards, the cells were washed with Waymouth's 725/1 medium (Mediatech, Inc., Manassas, VA) and treated with 0.5 mL Waymouth media containing 100 μM testosterone. Testosterone 6β-hydroxylation activity was determined at 30 min and 90 min incubations and by quantifying the amount of product formed using LC/MS/MS (API-3000).

2.6. Literature search

In vivo data on CYP3A4 induction by commercially available compounds was obtained by Medline literature search, or from the Physician's Desk Reference, the Food and Drug Administration website, and the Drug Interaction Database (Washington State University) [69].

3. Results

An in vitro pregnane X receptor activation assay using a luciferase reporter gene was developed to determine the potential of therapeutic drugs to induce human CYP3A4 gene expression. To determine whether the human PXR reporter-gene system can accurately predict CYP3A4 induction in human, a list of test compounds was selected from the literature for analysis in vitro. The compounds tested included 19 drugs known to cause induction in clinical studies (clinical inducers), 5 compounds shown not to cause induction in humans (clinical non-inducers), and 6 known inducers in human hepatocytes (Table 1). The following criteria were used to determine whether a drug is a clinical inducer, non-inducer, or inducer in human hepatocytes:

- Clinical inducers:
  - The perpetrator drug (drug to be tested) was co-administered with a victim drug (substrate for CYP3A4) and caused a decrease of the plasma concentration, a decrease of the exposure (AUC), or an increase of the clearance of the victim drug (drug interaction).
  - The drug to be tested is known to be metabolized by CYP3A4 and showed a decrease in plasma concentrations or exposure upon multiple dosing (autoinduction).
- Clinical non-inducers:
  - The perpetrator drug (drug to be tested) was co-administered with a victim drug (substrate for CYP3A4), but no change in the pharmacokinetics of victim drug was observed.
  - The drug to be tested is known to be metabolized by CYP3A4 but showed no change in pharmacokinetics following multiple dosing in patients.
- Inducers in human hepatocytes:
  - The drug showed induction of CYP3A4 mRNA or enzyme activity.

The selected drugs were then tested in the human PXR reporter-gene system at concentrations ranging from 30 nM to 1000 μM as described in Section 2 (see Fig. 1 for the specific range for each compound). The fold-induction and the RIF ratio were calculated and the maximum RIF ratio and EC50 for each drug were taken from the effect–concentration curve (Fig. 1). The data for each of the compounds tested is summarized in Table 1.

Table 1 – Summary of validation results in a human PXR reporter-gene assay using known clinical CYP3A4 inducers, clinical non-inducers, and inducers in human hepatocytes

	Induction at 1 μM (RIF ratio)	Induction at 10 μM (RIF ratio)	Maximum induction (RIF ratio)	EC50 (μM)	Representative Ref.
Clinical inducer					
Terbinafine	0.11	0.58	1.85	14	[30]
Troglitazone	0.11	0.53	1.48	11	[25,31,32]
Corticosterone	0.44	1.21	1.27	2.7	[33]
Efavirenz	0.07	0.46	1.12	9.8	[34–37]
Omeprazole	0.18	0.42	1.07	16	[29,38]
Phenobarbital	N.A.	N.A.	1.13	300	[39–41]
Rifampicin	0.72	1.00	1.00	0.4	[42–45]
Hyperforin	0.87	N.A.	0.87	0.2	[46]
Phenytoin	0.08	0.12	0.84	52	[39,47–49]
Ritonavir	0.56	0.83	0.83	0.7	[50]
Sulfinpyrazone	0.06	0.13	0.76	34	[51]
Rifabutin	0.35	0.59	0.59	0.7	[43,52,53]
Carbamazepine	0.06	0.08	0.38	47	[48,54,55]
Tamoxifen	0.05	0.11	0.31	13	[21,56]
Dexamethasone	0.15	0.15	0.28	47	[57–59]
Probenecid	0.09	0.11	0.24	37	[24]
Sulfamethazine	0.04	0.10	0.24	225	[60]
Aprepitant	0.05	0.19	0.19	4.3	[23]
Felbamate	0.09	0.12	0.16	225	[61,62]
Clinical non-inducer					
Pioglitazone	0.06	0.14	0.26	14	[25]
Imipramine	0.04	0.10	0.14	N.A.	[63]
Acyclovir	0.09	0.10	0.10	N.A.	[64]
Aspirin	0.06	0.06	0.07	N.A.	[65]
Fluoxetine	0.04	0.01	0.05	N.A.	[68]
Inducer in hepatocytes					
Lansoprazole	0.18	1.13	1.13	3.7	[27,28]
Nifedipine	0.26	0.61	0.78	3.5	[66]
Nicardipine	0.48	0.67	0.67	0.5	[66]
Troleandomycin	0.15	0.51	0.66	5.8	[20]
Phenylbutazone	0.06	0.09	0.59	36	[67]
Clotrimazole	0.17	0.58	0.58	2.4	[20]

EC50 values were calculated using GraphPad Prism software as described in Section 2.

The variability in the PXR reporter-gene assay was assessed across experiments. The maximum standard deviation (S.D.) for the vehicle control group was a RIF ratio of 0.021, and the maximum induction baseline for the vehicle control group was a RIF ratio of 0.071. Assuming that 3× the standard deviation is the noise of the experimental system, a RIF ratio of 0.14 (mean + 3 × S.D.) is the threshold for a positive signal above the noise. All 19 clinical inducers and 6 inducers in human hepatocytes showed a signal above RIF ratio of 0.14. Among clinical non-inducers, pioglitazone also showed a weak positive signal.

False-positive signals in the human PXR reporter-gene assay are also undesirable because such results would lead to the elimination of potential drug candidates early in the drug discovery process. To evaluate the potential of the human PXR reporter-gene assay to generate false-positive results, we tested 18 widely marketed drugs as putative negatives (clinical non-inducers). Most of the drugs chosen are CYP3A4 substrates. The putative negatives were selected based on the following criteria (Table 2):

- Drug–drug interaction studies identified during an extensive literature search on the given drug were carefully evaluated, but no interaction was reported that was due to CYP3A4 induction.
- If the given drug was a CYP3A4 substrate and pharmacokinetic data were available, no indication of autoinduction (decrease of plasma concentration, decrease in exposure, or increase of clearance) over time was observed.

The 18 putative negatives were tested in the human PXR reporter-gene assay at concentrations ranging from 30 nM to 100 μM as described for the other compounds. The RIF ratios at 1 μM and 10 μM, as well as the maximum induction for these 18 compounds, were determined and are summarized in Table 3. Haloperidol and flutamide appeared as positives in the PXR reporter-gene assay, with maximum RIF ratios of 0.48 and 0.79 at 10 μM, and EC50 values of 3 μM and 5 μM, respectively (Fig. 2A). Both haloperidol and flutamide were toxic to cells at the higher concentrations tested (30 μM and 100 μM).

Evaluation of testosterone-6β-hydroxylase activity in human primary hepatocytes is a widely accepted method to

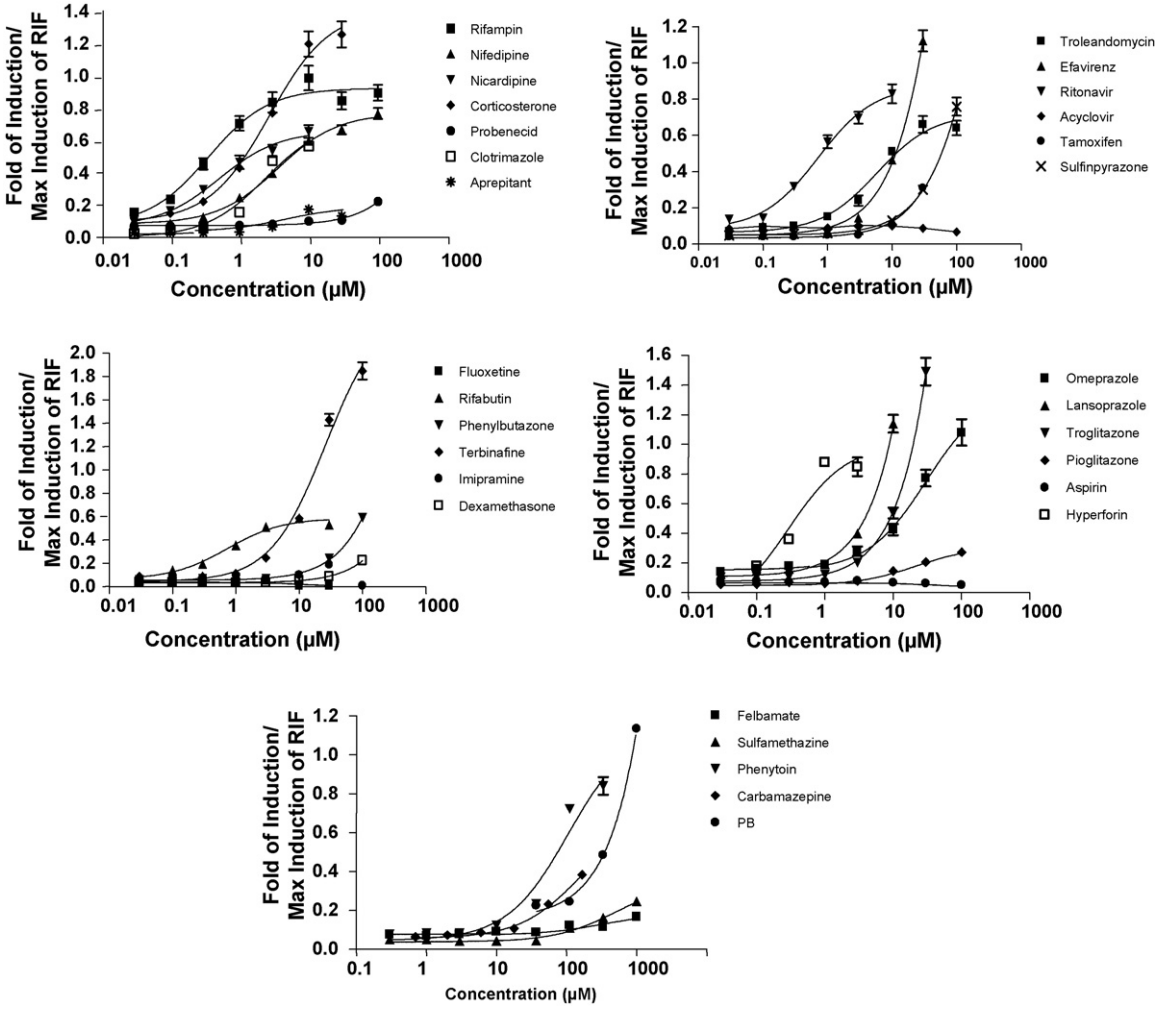

Fig. 1 – Analysis of CYP3A4 induction potential of commercial compounds by the human PXR reporter-gene system. HepG2 cells were transiently transfected with the pGL3B-3A4, pcDNA4/HisMax-TOPO-huPXR and pRL-TK plasmids. Cells were treated with various concentrations of compounds for 48 h. The induction of luciferase activity was calculated as fold to DMSO control. The data was mean ± S.E. of the triplicates in a single experiment, and expressed as fold increase relative to maximum induction of rifampicin.

evaluate CYP3A4 induction in vitro. Therefore, we tested whether haloperidol and flutamide are true CYP3A4 inducers in another test system or are false-positives generated by the human PXR reporter-gene assay. Plated human hepatocytes from two individual donors were treated with haloperidol and flutamide, as well as the benchmark compound, rifampicin, at concentrations ranging from 1 μM to 30 μM for 48 h, and induction was monitored by testosterone-6β-hydroxylase activity. Haloperidol caused fourfold and threefold induction in the two donors compared to vehicle-treated (0.1% DMSO) samples. Flutamide increased testosterone-6β-hydroxylase activity by 3.5-fold in both donors, as compared to vehicle-treated controls, as well. In comparison, rifampicin induced CYP3A4 activity by 6- and 7.5-fold, respectively (Fig. 2B). These results suggest that haloperidol and flutamide have bona fide, previously unidentified potential to induce CYP3A4.

To predict the likelihood of in vivo CYP3A4 induction by compounds showing induction in the human PXR reporter-gene assay, we used a numerical value, the induction potential (IP) index, to correlate the in vitro PXR potency with the in vivo exposure of the tested clinical inducers. The induction potential index is defined as

$$\mathrm{IP} = \frac{E_{\max} C_{\max}}{\mathrm{EC50} + C_{\max}}$$

where $C_{\max}$ is the maximum plasma concentration of the drug at the clinical dose and $E_{\max}$ is the maximum RIF ratio for the drug. The clinical doses, $C_{\max}$ values, induction potential index values, and the extent of in vivo induction for 12 clinical inducers and non-inducers are shown in Table 3. The in vivo induction was categorized into five groups: very strong inducers (++++), strong inducers (+++), moderate inducers (++), weak inducers (+), and non-inducers (–), according to the changes in plasma concentrations and exposure (AUC) observed in vivo. Based on the in vivo CYP3A4 induction data, we conclude that when the induction potential index is greater than 0.08, the

Table 2 – Validation of human PXR reporter-gene system using putative negative control compounds (approved drugs with no known CYP3A4 induction liability)

Drug name	RIF ratio at 1 μM	RIF ratio at 10 μM	Maximum induction (RIF ratio)
Acetominophen	0.10	0.10	0.11
Naproxen	0.04	0.04	0.06
Captopril	0.05	0.05	0.06
Haloperidol	0.12	0.48	0.48
Cyclosporine A	0.05	0.03	0.06
Flutamide	0.12	0.79	0.79
Caffeine	0.04	0.04	0.05
Theophylline	0.04	0.04	0.04
Tolbutamide	0.05	0.05	0.09
Dapsone	0.07	0.08	0.10
Antipyrine	0.06	0.09	0.09
Nadolol	0.12	0.13	0.13
Propanolol	0.08	0.10	0.10
Furosemide	0.13	0.16	0.16
Ibuprofen	0.07	0.07	0.08
Metoprolol	0.11	0.13	0.13
Desipramine	0.10	0.13	0.13
Timolol	0.12	0.18	0.18

The compounds were tested in the human PXR gene-reporter assay at concentrations ranging from 0.03 μM to 100 μM. Only data from the 1 μM and 10 μM concentrations and maximum induction ratios are shown.

compound is likely to cause induction in vivo. The potential for tamoxifen and dexamethasone to cause CYP3A4 induction was not accurately predicted in the PXR reporter-gene assay using the induction potential index and the reasons will be discussed in the next section. However, in general, evaluation of PXR reporter-gene assay results as described in this study nicely predicts potential clinical CYP3A4 induction liability, and can be used as a valuable tool to eliminate potent CYP3A4 inducers in the drug discovery stage.

4. Discussion

The potential value of using the human PXR reporter-gene assay as a means to predict CYP3A induction was realized soon after the discovery of the PXR receptor and its regulation of CYP3A transcription. An effort to correlate PXR activation and CYP3A induction in human primary hepatocytes was previously made [20]. In this study, we evaluated the correlation of PXR activation with clinical observations of

Table 3 – Induction potential index calculated using the human PXR reporter-gene assay and corresponding in vivo induction potency for clinical inducers and non-inducers

Drugs	Dose (mg)	Plasma Cmax (μM)	Induction potential index	Induction in humans[a]
Rifampicin	600	8	0.95	++++
Ritonavir	600	6	0.70	++
Efavirenz	400–600	12.6	0.62	++
Troglitazone	400	3.2	0.46	++
Phenytoin	300	60	0.43	+++
Terbinafine	250	3.1	0.34	++
Sulfinpyrazone	200	25	0.34	+
Rifabutin	300	0.44	0.23	+
Omeprazole (PMs)	40	3.5	0.20	++
Carbamazepine	200–600	39	0.18	+++
Probenecid	500	249[b]	0.17	+
Phenobarbital	100	56	0.17	+++
Aprepitant	80–125	3	0.08	+
Felbamate	2400	154	0.08	++
Flutamide	750	0.39	0.07	–
Omeprazole (EMs)	40	0.68	0.04	–
Pioglitazone	30	2.2	0.04	–
Tamoxifen	20	0.3	0.007	+
Haloperidol	20	0.025	0.004	–
Dexamethasone	8	0.17	0.001	+

[a] ++++, ΔAUC ≥ 80%; +++, 60% ≤ ΔAUC < 80%; ++, 40% ≤ ΔAUC < 60%; +, 20% ≤ ΔAUC < 40%; if data are available for multiple substrates, the maximum AUC change was recorded. If AUC is not available, plasma concentrations were used instead. See references in Table 1.
[b] Cavg.

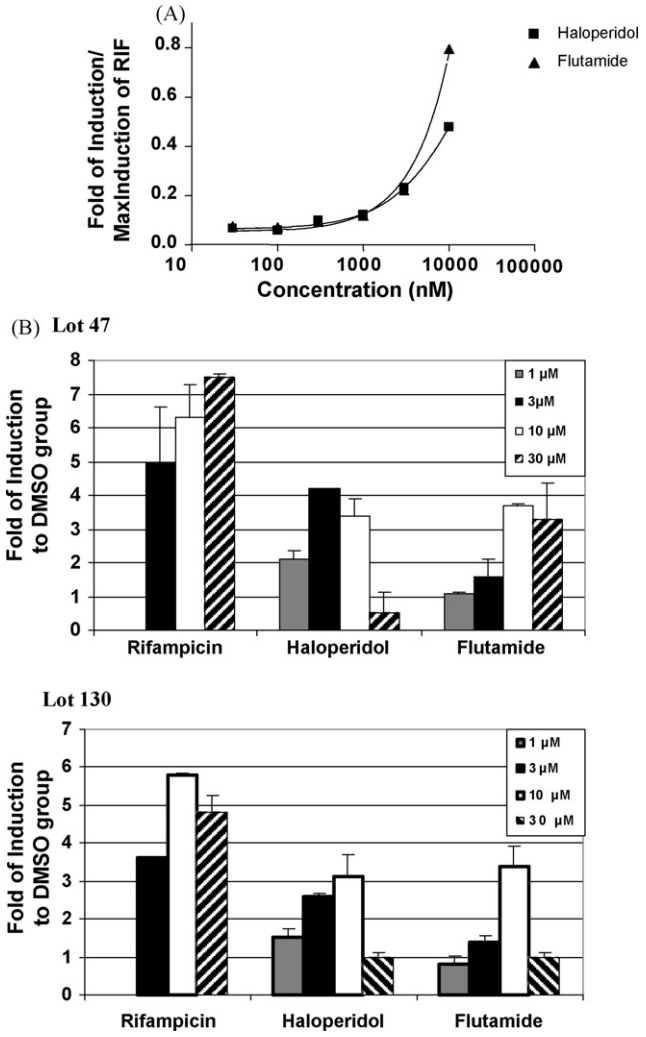

Fig. 2 – Haloperidol and flutamide are CYP3A4 inducers in cryopreserved human hepatocytes. (A) Haloperidol and flutamide activate PXR in transient transfected human PXR reporter-gene system. (B) Induction of 6β-testosterone hydroxylase activity by haloperidol and flutamide. The data were mean ± S.E. of the triplicates in a single experiment and expressed as fold of induction of 0.1% DMSO-treated vehicle control group.

CYP3A4 induction in humans. We also used the clinical CYP3A4 data to establish practical parameters for the interpretation of PXR reporter-gene assay results and predicting the likelihood of induction in vivo.

The drugs used as clinical inducers and non-inducers for testing in the PXR reporter-gene assay all have supporting literature data with in vivo drug–drug interaction studies. Six CYP3A4 inducers in human hepatocytes were tested along with the known clinical inducers. Among them, nifedipine, nicardipine, and troleandomycin are CYP3A4 inhibitors as well, but show strong CYP3A4 induction at the mRNA level. The selected drugs were evaluated in the human PXR reporter-gene assay over a wide range of concentrations, and both RIF ratio and EC50 values for each were calculated.

Bridging the gap between in vitro induction in a cell-based assay and CYP3A4 induction in humans presents challenges. Luo et al. [20] have shown that data obtained from the human PXR reporter-gene assay correlate reasonably well with induction data from human hepatocytes. Our study examined the correlation of data from the PXR reporter-gene assay with in vivo CYP3A4 induction data. Multiple factors determine whether or not a compound will cause CYP3A4 induction and alter the clearance of a co-administered drug. Pharmacokinetic and metabolism characteristics of the inducer and recipient drug should be taken into consideration when attempting to extrapolate in vitro data to in vivo induction results. Dose, duration of treatment, and plasma and liver concentrations of the inducer are critical factors that determine whether or not PXR activation and CYP3A4 induction will occur in vivo. To take some of these factors into account, a parameter, the induction potential index, was used to link in vitro potency to in vivo drug concentrations. The IP index values ranged from 0.95 to 0.001 for 20 inducers and non-inducers, and a cut-off 0.08 was set based on non-inducer data.

Using the IP index cut-off value of 0.08, all of the clinical inducers in this study were correctly predicted except for dexamethasone (DEX) and tamoxifen. The low activation of DEX in human PXR is consistent with a report [7] that DEX is a very weak or non-inducer in the human PXR reporter-gene assay, whereas it is a good inducer in the rodent system. It has been shown that in human hepatocytes, DEX is a weak or moderate inducer for CYP3A4 when treated with DEX alone, but it can synergistically enhance the induction by other PXR activators by up-regulating PXR expression through the glucocorticoid receptor [12]. Tamoxifen acts as a weak inducer in vivo and has been shown to decrease letrozole plasma concentration by 37% upon co-administration [21]. Desai et al. has shown that 4-hydroxytamoxifen, a tamoxifen metabolite, is more potent in the human PXR reporter-gene assay than the parent compound [22]. Therefore, the in vivo induction effect observed with tamoxifen was likely due to the additive effects of the parent drug and metabolite. As a result, the lack of CYP3A4 induction in the human PXR reporter-gene assay may due to the absence of metabolic capability in HepG2 cell line.

Felbamate, probenecid, and aprepitant also showed weak positive responses similar to that of pioglitazone in the human PXR reporter-gene assay. In humans, all of these compounds are weak inducers. For example, the AUC of midazolam dropped 19% when co-administered with aprepitant [23], and the AUC of carbamazepine decreased 19% when co-administered with probenecid [24], consistent with the weak positive human PXR reporter-gene assay results in vitro. However, pioglitazone was found to be a non-inducer in humans when co-administered with the CYP3A probe simvastatin [25]. It appears that the relatively low plasma concentration of pioglitazone at the clinical dose (C_{max} 2.2 μM) results in an IP value of 0.04. On the contrary, the high plasma concentrations of felbamate and probenecid at the recommended clinical dose (154 μM and 249 μM, respectively) have offset the high EC50 and low RIF ratio, leading to the induction observed in humans.

It is worth mentioning that omeprazole was not a clinical CYP3A4 inducer based on several drug–drug interaction

studies. For example, omeprazole did not change the plasma concentration and clearance of cyclosporine A after 2 weeks of treatment at a 20-mg daily dose [26]. However, it is well documented that omeprazole is a CYP3A inducer in human hepatocytes [27,28]. The positive omeprazole result in the reporter-gene system is consistent with the increased mRNA level in hepatocytes. Omeprazole is known to be rapidly metabolized by CYP2C19 and CYP3A4 in human. Therefore, lack of in vivo induction in patients could be due to low omeprazole concentration. Interestingly, Rosenshein et al. has published one case in which a 2C19 poor metabolizer (PM) showed induction of testosterone metabolism after omeprazole administration [29]. The Netherlands Pharmacovigilance Center has reported numerous cases that proton pump inhibitors, mostly omeprazole, cause gynecomastia, a disorder caused by low testosterone levels. One possible explanation for this observation is that omeprazole increases testosterone clearance by inducing CYP3A4. It would be interesting to determine whether these patients are CYP2C19 PMs.

For all of the compounds shown to be CYP3A4 inducers in human hepatocytes, the human PXR reporter-gene assay also predicted that they would induce CYP3A4. Among these compounds, nifedipine, nicardipine, and troleandomycin are CYP3A4 inhibitors as well as CYP3A4 inducers. In vivo, these drugs have not been shown to be clinical inducers since their induction effects (as judged by the increase in mRNA and protein levels) are apparently nullified by their inhibitory effects at the enzyme activity level.

To further evaluate whether the human PXR reporter-gene assay has any tendency to generate false-positive results, 18 putative negative controls were tested in addition to the clinical non-inducers. Among the 18 drugs tested, haloperidol and flutamide were shown to strongly activate PXR using the reporter-gene assay. Subsequently, these two drugs were shown to be CYP3A4 inducers in human hepatocytes. The low peak plasma concentrations (0.39 μM for flutamide and 25 nM for haloperidol) [70] could be the reason that these two drugs did not show in vivo induction at the clinical dose level.

In the drug discovery stage, no human pharmacokinetic data is available, and the projection of human pharmacokinetics only becomes feasible towards the end of the drug discovery stage. The prediction of CYP3A4 induction using the induction potential index has typically been performed right before a compound is recommended for development. In order to use the PXR reporter-gene assay in early lead optimization, a high-throughput screening strategy for the PXR reporter-gene assay was developed based on the same validation data (Table 1). Based on the noise level of the system (RIF ratio 0.14), at 10 μM, 58% of the clinical inducers and 88% of the hepatocyte inducers (including flutamide and haloperidol) showed positive results, and 100% of the clinical non-inducers were negative in human PXR reporter-gene assay. To further minimize the possibility of generating false-positive results, a cut-off RIF value of 0.4 was set for screening purpose. Using this cut-off, 60% of the clinical inducers and hepatocyte inducers will be picked up by reporter-gene system when screening at 10 μM, and no false positive was generated. Although EC50 cannot be determined from the screening data, the induction at 1 μM relative to that of 10 μM can provide some information in terms of the affinity of the compound for the PXR receptor. For example, the potent inducers rifampin and hyperforin all show strong induction at 1 μM. Thus, a tiered approach was adopted for reporter-gene assay. In lead optimization, compounds were screened at 1 μM and 10 μM. Compounds which had a RIF ratio greater than 0.4, especially at 1 μM, were flagged as potential inducers. The PXR reporter-gene assay was further used as a means to explore the structure–activity relationship for structural optimization. In lead characterization, a full concentration-effect profile will be generated. The maximum RIF ratio and the EC50 is determined, and combined with the predicted human Cmax, the induction potential index can be calculated. When the induction potential index value is greater than 0.08, the compound is predicted to be a potential inducer in vivo.

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