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Research Paper

Evaluation of Human Liver Slices and Reporter Gene Assays as Systems for Predicting the Cytochrome P450 Induction Potential of Drugs *in Vivo* in Humans

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Purpose. The aim of the study was to investigate the feasibility of predicting human *in vivo* cytochrome P450 (CYP) induction properties of drugs using *in vitro* methods.
Methods. The CYP induction potential of compounds was tested in human liver slices and in reporter gene assays for the aryl hydrocarbon receptor (AhR) and the pregnane X receptor (PXR).
Results. In human liver slices, CYP activities decreased dramatically over the experimental period, whereas mRNA levels could reliably be used to investigate CYP1A, 2C9, and 3A4 induction. However, the interindividual variations and demanding experimentation limit the use of liver slices in screening programs. Reporter gene assays are robust and reliable assays, amenable to high throughput screening. Several compounds activated AhR. The relevance of this activation, however, needs to be further investigated since there are no clear reports on drugs inducing CYP1A *in vivo*. The results from the PXR assay could be used to correctly classify compounds with known CYP3A induction properties when relating *in vivo* AUC_{tot} to PXR EC_{50} values.
Conclusions. Liver slices are a valuable model to study the regulation of a larger number of enzymes by single compounds. The PXR reporter gene assay could be used as a reliable screening method to predict CYP3A induction *in vivo*.

KEY WORDS: AhR; CYP induction; human liver slices; *in vitro–in vivo* correlation; PXR; reporter gene assay.

INTRODUCTION

The cytochrome P450 (CYP) enzymes have been shown to be responsible for metabolism of the majority of therapeutics and are therefore important for first-pass elimination (bioavailability), clearance, and drug–drug interactions. The pharmaceutical industry has, for this reason, invested resources in the early discovery process to identify screens for *in vitro* properties that could be used to predict *in vivo* pharmacokinetics before the compounds can be tested in man.

One undesirable property of a drug candidate is the induction of CYP enzymes, which can lead to clinically important drug–drug interactions and time-dependent pharmacokinetics. In clinical practice, enzyme induction can enhance clearance of the drug itself or a coadministered drug, which could result in loss of therapeutic effect. Drug-treatment-related induction of CYP enzymes is a well-known characteristic of a number of relatively old drugs, such as rifampicin and phenobarbital, and is mainly confined to CYP3A and, to a lesser extent, CYP2C9 (1–4). Troglitazone and bosentan are examples of drugs launched during recent years that have been associated with induction of CYP3A (5,6).

Induction of CYP genes generally occurs at the transcriptional level and is mediated by receptors such as the pregnane X receptor (PXR) and the aryl hydrocarbon receptor (AhR). PXR is a transcription factor that is widely accepted as the major determinant of CYP3A4 gene regulation by xenobiotics (7,8). Recently, PXR was also established to be a major determinant of CYP2C9 induction (9).

In contrast to CYP2C9 and 3A enzymes, CYP1A has not been reported to be induced by any drugs on the market at therapeutic doses. The mechanism of CYP1A induction has, however, been studied extensively (10,11). The CYP1A

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; huPO, human acidic ribosomal phosphoprotein; PXR, pregnane X receptor; TCDD, 2,3,7,8-tetrachloro-dibenzo-p-dioxin.

enzymes are regulated by AhR, and prototypical AhR ligands are planar, hydrophobic, and halogenated hydrocarbons such as 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) (12).

Significant species differences exist with respect to induction of both CYP1A and 3A4. For example, it is known that the prototypical CYP3A4 inducer rifampicin activates the human and rabbit PXR but not the rat or mouse PXR (13,14), and omeprazole and primaquine induce CYP1A1 protein in human and rat hepatoma cells but not in hepatoma cells from mouse (15). This discrepancy between species makes animal *in vivo* models inappropriate for induction studies, and reliable human *in vitro* models are required.

An extensive number of reports have studied induction using isolated human hepatocytes and liver slices (16–19). However, most studies have used prototypical inducers, and only a few reports have investigated whether the *in vitro* results correlate to clinical observations (20). The demand of the pharmaceutical industry for a standardized *in vitro* assay for evaluation of CYP induction properties of new chemical entities is immense. The aim of the present study was therefore to evaluate if an easy-to-use reporter gene assay could be utilized as a tool to predict *in vivo* CYP induction properties. Furthermore, the results in human liver slices were evaluated as a model to investigate induction in the intact tissue. The study was performed mainly with drugs or herbal remedies with known induction properties *in vivo* in humans.

MATERIALS AND METHODS

Chemicals

Acetaminophen, betamethasone, carbamazepine, cimetidine, chlorpromazine, dexamethasone, diazepam, diclofenac, indol-3-carbinol, indomethacin, naproxen, paracetamol, phenobarbital, phenytoin, primaquine, rifampicin, verapamil, and warfarin were from Sigma Chemical Co. (St Louis, MO, USA). Artemisinin and phenacetin were from Aldrich Chemical Co. (Milwaukee, WI, USA). Hyperforin was purchased from Apin Chemicals Ltd (Oxon, UK). Clotrimazole and lovastatin were from Calbiochem (San Diego, CA, USA). Lansoprazole, omeprazole, pantoprazole, primidone, rabeprazole, and troglitazone were provided by the Compound Management Department at AstraZeneca R&D Mölndal, and pravastatin was provided by AstraZeneca R&D Alderley Park. Midazolam and 4-hydroxydiclofenac were from Gentest (Woburn, MA, USA), and 1-hydroxymidazolam was from Ultrafine (Manchester, UK). TCDD was purchased from Larodan Fine Chemicals AB (Malmö, Sweden). The primers and probes used in this study were purchased from Applied Biosystems (Cheshire, UK), and TaqMan® Universal Master Mix was purchased from Applied Biosystems (Stockholm, Sweden). Fugene 6 Transfection Reagent was purchased from Roche Applied Science (Bromma, Sweden), and Luclite assay kit was from PerkinElmer Life Sciences (Stockholm, Sweden). Luciferase assay kit was from BioThema (Haninge, Sweden).

Preparation of Human Liver Slices

Human liver tissue was obtained as surgical waste from Sahlgrenska University Hospital (Göteborg, Sweden). Clinical characteristics of liver donors are presented in Table I. All tissues were obtained through qualified medical staff, with donor consent and with the approval of the local ethics committee. Samples of 12 human livers were collected at the hospital directly after resection and were immediately flushed with University of Wisconsin solution [ViaSpan®, consisting of 50.0 g/l poly(*O*-2-hydroxyethyl) starch, 35.83 g/l lactobionic acid, 14.5 g/l potassium hydroxide 56%, 3.679 g/l sodium hydroxide 40%, 1.34 g/l adenosine, 0.136 g/l allopurinol, 3.4 g/l potassium dihydrogen phosphate, 1.23 g/l magnesium sulfate $\times$ $7H_2O$, 17.83 g/l raffinose $\times$ $5H_2O$, and 0.922 g/l glutathione] and transported on ice to AstraZeneca R&D Mölndal. Tissue cylinders from liver samples were prepared using a 10-mm motor-driven tissue-coring tool. From the cylinders, liver slices (approximately 500 μm thick) were prepared in cold phosphate-buffered saline (PBS) containing 1% agarose using a water-cooled Krumdieck tissue slicer (Alabama Research and Development Corp., Munford, AL, USA). Slices were rinsed with cold oxygenated (95% O_2/5%

Table I. Clinical Characteristics of Liver Tissue Donors

Liver	Sex	Age	Smoker	Medications
1	M	75	No	Cilastatin, felodipine, imipenem, losartan, metoprolol
2	M	63	No information	Furosemide, meropenem, paracetamol, zolpidem
3	M	66	No	Atenolol, atorvastatin, bendroflumethiazide, cefotaxime, flunitrazepam, paracetamol, potassium chloride
4	F	62	No	Cefotaxime, fluorouracil, oxaliplatin
5	M	63	No	No information
6	F	71	No	No information
7	M	61	No	No information
8	F	59	No	No information
9	F	74	No information	Fluorouracil, lansoprazole, leukovorin, paracetamol
10	F	58	No	No information
11	M	75	>20 cigarettes/day	Sotalol
12	M	78	No	Acetylsalicylic acid, amiloride, folic acid, hydrochlorothiazide, iron sulfate, levothyroxine,losartan, nitroglycerine, simvastatin, vitamin B12

The diagnosis was metastases for all donors except liver 1, which was a liver adenoma.

CO_2) Krebs–Henseleit buffer and kept on ice prior to incubation.

Culture and Treatment of Human Liver Slices

Incubation of human liver slices was started within 4 h of surgery. Slices were placed in 6-well plates and preincubated for 1 h in 2 ml culturing medium (Williams' Medium E with fungizone 2.5 μg/ml, insulin 0.1 μM, penicillin 100 U/ml, streptomycin 100 μg/ml, dexamethasone 0.1 μM, and L-glutamine 4 mM) at 37°C in a humidified atmosphere with 5% CO_2. The plates were placed on an orbital shaker kept at a speed that allowed the slice to rotate but not touch the sides. Test compounds were dissolved in dimethyl sulfoxide (DMSO; final concentration 1%) and were added to the culture in concentrations indicated in Table III. The medium was changed, and the slices were redosed every 24 h. The incubations were terminated by freezing the slice on dry ice and ethanol.

RNA Extraction

Total RNA from human liver slices was isolated using RNA STAT-60 reagents (BioSite, Täby, Sweden). The slices were homogenized in RNA STAT-60 using a Polytron PT 1200 homogenizer (Kinematica AG, Lucerne, Switzerland) and, after that, were treated according to manufacturer's instructions.

To eliminate any contamination of chromosomal DNA, the isolated total RNA was treated with DNase using RQ1 RNase-Free DNase (Promega, Madison, WI, USA) or DNA-free™ (Ambicon, Cambridgeshire, UK). cDNA was prepared from 2 μg of DNase-treated RNA using the SuperScript™ First-Strand Synthesis System for reverse transcriptase–polymerase chain reaction (PCR) (Invitrogen, Stockholm, Sweden).

Real-Time PCR

Real-time PCR for human CYP mRNA levels was performed employing a TaqMan® 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) and gene-specific double fluorescent-labeled probes (sequences displayed in Table II). The probes were labeled with VIC as the 5′-fluorescent reporter and TAMRA at the 3′-end as the quencher. The sets of primers and probes were designed to span exon junctions to prevent detection of any possible contamination of genomic DNA. The reaction mixture (25 μl per well) contained 1 μl of cDNA, 1× TaqMan Universal Master Mix, optimized concentrations of primers and probes (see Table II), and diethyl pyrocarbonate-treated distilled water. The thermal cycle conditions had initial steps of 50°C for 2 min and a 10 min step at 95°C followed by 40 PCR cycles of 95°C for 15 s and 60°C for 1 min. Each sample was analyzed twice in triplicate, and data were analyzed using the Sequence detector V1.6 program (Applied Biosystems).

Standard curves were constructed by serial 10-fold dilutions, ranging from 0.001 to 10 pg, of a plasmid containing the cDNA of interest as template. For normalization of the mRNA data, human acidic ribosomal phosphoprotein (huPO) was used as the endogenous control. Because equal amplification efficiency in the product formation of CYP1A1, 1A2, 2C9, and 3A4 was achieved, both when using plasmids and diluted liver samples as standard curves, plasmids were selected as standards for determining the arbitrary units for respective targets. The amount of mRNA was determined relative to that from samples treated with DMSO.

Statistics

The difference in mRNA levels in control slices and treated slices at the same time points was calculated using the paired Student's *t* test.

Activity Measurements in Human Liver Slices

Microsomes were prepared from two to six slices from each individual and time point. The protein concentration was measured with Bio-Rad Protein Assay based on the method of Bradford, and the absorbance was measured at 595 nm on a Wallac Victor^2 1420 Multilabel counter (PerkinElmer Life Sciences, Boston, MA, USA). The microsomes, in a final concentration of 0.5 mg/ml, were incubated with a cocktail of phenacetin, diclofenac, and midazolam (final concentration 20, 5, and 4 μM, respectively) in a potassium phosphate buffer

Table II. Sequences and Concentrations of Primers and Probes Used in TaqMan® Analysis

Primer/probe	Final concentration (nM)	Sequence of primers and probes
huPO Forward	200	5′ CCATTCTATCATCAACGGGTACAA 3′
huPO Reverse	200	5′ AGCAAGTGGGAAGGTGTAATCC 3′
huPO Probe	100	5′ TCTCCACAGACAAGGCCAGGACTCGT 3′
1A1 Forward	900	5′ GTTCTACAGCTTCATGCAGAAGATG 3′
1A1 Reverse	900	5′ TTGGCGTTCTCATCCAGCT 3′
1A1 Probe	250	5′ AAAACCTTTGAGAAGGGCCACATCCG 3′
1A2 Forward	300	5′ CTGTGGTTCCTGCAGAAAACAG 3′
1A2 Reverse	300	5′ CCCTTCTTGCTGTGCTTGAAC 3′
1A2 Probe	250	5′ CCGGACACTGTTCTTGTCAAAGTCCTGA 3′
2C9 Forward	900	5′ CAGCAATTTCTTAACTTAATGGAAAAG 3′
2C9 Reverse	900	5′ CGGGAAGTAATCAATGATAGGAGA 3′
2C9 Probe	250	5′ ATCAAGATTTTGAGCAGCCCCTGGATC 3′
3A4 Forward	900	5′ CATTCCTCATCCCAATTCTTGAAGT 3′
3A4 Reverse	900	5′ CCACTCGGTGCTTTTGTGTATCT 3′
3A4 Probe	250	5′ CGAGGCGACTTTCTTTCATCCTTTTTACAGATTTTC 3′

(100 mM, pH 7.4), final volume 200 μl, in a 96-well tissue culture test plate (TPP, Trasadingen, Switzerland). The three substances were dissolved in methanol so that the final concentration of methanol was 2.5% (v/v), which inhibited the specific CYP activities less than 20%. The mixture was preincubated for 5 min at 37°C with shaking, and the reaction was started by addition of NADPH (1 mM). Incubation was carried out for 30 min and stopped by addition of 100 μl ice-cold methanol. The samples were put in −20°C for 15 min and then centrifuged for 10 min at 3200 × *g*, 4°C, in a Rotanta TR centrifuge (Hettich, Tuttlingen, Deutschland). The supernatant was transferred to a new plate for analysis of the metabolites, namely, paracetamol (CYP1A2), 4-OH-diclofenac (CYP2C9), and 1-OH-midazolam (CYP3A). The samples were analyzed at two separate occasions by liquid chromatography–mass spectrometry (LC/MS). Chromatography was performed on a Zorbax Extend C18 (2.1 × 50 mm, 3.5 μm, Agilent, Palo Alto, CA, USA) employing an Agilent 1100 pump with gradient elution at 200 μl/min. Analysis on the second occasion comprised, in addition, a Zorbax Extend C18 precolumn (2.1 × 10 mm, 3.5 μm, Agilent) entailing improved selectivity for the early-eluting analyte paracetamol. The mobile phase consisted of (A) 0.2% (v/v) formic acid and 2% (v/v) acetonitrile in water and (B) 0.2% formic acid in acetonitrile. The organic modifier content was increased linearly from 3 to 75% B over 3 min, followed by a step gradient to 3% B for 2.2 min. The front was diverted to waste by means of a six-port two-position switching valve (VICI AG, Schenkon, Switzerland). After 1 min, the column effluent was directed toward the mass spectrometer without splitting. Sample storage at 4°C and injection (5 μl) were performed with a CTC HTS autosampler (CTC Analytics, Zwingen, Switzerland). Detection was performed in positive ionization mode with an Ultima triple quadrupole mass spectrometer equipped with electrospray (Waters, Manchester, UK). The transitions chosen were 152.0 > 110.0 for paracetamol, 312.0 > 231.0 for 4-OH-diclofenac, and 342.1 > 167.9 for 1-OH-midazolam at cone voltages of 70, 50, and 60 V, respectively, and collision energies of 40, 20, and 20 eV respectively. The limits of quantitation (LOQ) were as follows (in nmol/l): 82 (13) for paracetamol, 14 (21) for 4-OH-diclofenac, and 27 (23) for 1-OH-midazolam at the first (second) occasion of analysis. The difference in LOQ did not affect the results. Instrument control, data acquisition, and data evaluation were performed using MassLynx 4.0.

AhR Reporter Gene Assay

HepG2 cells stably transfected with pTX.DIR (21) and pSV_2-Neo were obtained from Lorenz Poellinger, Department of Cell and Molecular Biology, Karolinska Institutet (Stockholm, Sweden). The cells were seeded in 96-well plates (Wallac Isoplate TC) in culturing medium (RPMI 1640 with Glutamax I and 25 mM HEPES supplemented with 10% fetal bovine serum (FBS) and geneticin, 800 μg/ml) at a density of 40,000 cells per well and were incubated overnight. Serial dilutions of the test compounds were performed in culturing medium without geneticin with a final DMSO concentration of 0.1%. TCDD was run as a control on all plates. Media was removed from the cells, and test compounds were added to the plates and incubated for 24 h in 37°C. Following incubation, the luciferase activity was determined using a Luciferase assay kit. The luminescence was measured in a Wallac Victor^2 1420 Multilabel counter. Dose–response curves for the activation of AhR in the reporter gene assay were calculated with XLfit 2.0.9, model 205 (sigmoidal dose-response with one binding site).

PXR Reporter Gene Assay

The CYP3A4 luciferase reporter was prepared by cloning the xenobiotic responsive enhancer module (−7836/−7208), identified by Goodwin *et al.* (22), and the −362/+53 proximal promoter from CYP3A4 into the pGL3 basic vector (Promega).

The hPXR1 coding sequence (accession no. AF061056) was generated by PCR from human universal Quickclone cDNA (Clontech, BD Biosciences, Stockholm, Sweden) and cloned into pcDNA3.1/hygro (Invitrogen).

HepG2 cells were seeded in culturing flasks (2.25×10^6 cells per 75 cm^2 flask) in culture medium (Dulbecco's Modified Eagle Medium with Glutamax II supplemented with 1% nonessential amino acids, 2 mM L-glutamine, 10% FBS, and 1 mM sodium pyruvate) and incubated overnight in 5% CO_2-humidified, 37°C atmosphere. The cells in each 75 cm^2 flask were transiently transfected with 200 ng of expression vector pcDNA3.1hyg(−)huPXR1 and 16 μg of CYP3A4 enhancer (−7836/−7164) proximal promoter (−362/+53) pGL3b reporter construct using Fugene 6 Transfection Reagent according to the supplier's recommendations. Following transfection, the cells were harvested and seeded in 96-well plates (Wallac Isoplate TC; 22,500 cells per well) in fresh culturing medium. Test compounds were diluted in DMSO and added to the plates serially diluted in culturing medium with a final DMSO concentration of 0.1%. Rifampicin was used as a control in all experiments. The plates were incubated for 68 h at 37°C, and Luclite assay kit was used to determine luciferase activity following the manufacturer's instruction utilizing a Wallac Victor^2 1420 Multilabel counter. Dose–response curves for the activation of PXR in the reporter gene assay were calculated with XLfit 2.0.9, model 205 (sigmoidal dose-response with one binding site).

Viability of Cells in AhR and PXR Reporter Gene Assay

After 24 or 68 h of incubation, cells were washed with PBS containing 1 mM calcium and magnesium ions, and a luciferase assay was performed according to manufacturer's instructions (ATPLite, Packard Bioscience, Groningen, Netherlands). Luminescence was read in a Wallac Victor^2 1420 Multilabel counter. Results were used to exclude incubations where viability was less than 70%.

RESULTS

Human Liver Slices

The levels of CYP1A1, 1A2, and 2C9 mRNA in control human liver slices decreased dramatically during the first 24 h of incubation but were then relatively stable during the 24 to 72 h incubation period. Compared with CYP1A1, 1A2, and 2C9, the decrease in CYP3A4 mRNA levels was not as

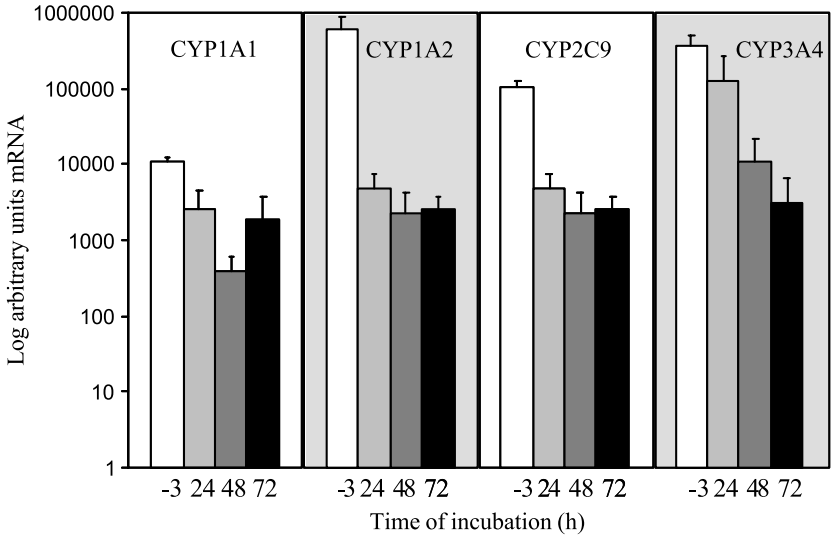

Fig. 1. Normalized amount (arbitrary units) of mRNA in human liver directly after resection (time = −3 h) and in human liver slices after incubation with control media for 24, 48, and 72 h. Values are mean ± SD from three individual livers.

dramatic during the first 24 h, but the basal levels continued to decrease during the experimental period of 72 h (Fig. 1).

A substantial decrease was also seen for the CYP-dependent phenacetin *O*-dealkylase, diclofenac 4-hydroxylase, and midazolam 1-hydroxylase activities over the experimental period in control human liver slices (Fig. 2), and in some experiments, no activity could be detected after 48 and 72 h incubation.

The induction potential of compounds in human liver slices was estimated by comparing the mRNA levels in slices exposed to test compound (concentrations indicated in Table III) with control slices (incubated with 1% DMSO) at the same time point. TCDD and rifampicin were used as prototypical CYP1A and CYP3A4 inducers, respectively, in the human liver slice experiments. TCDD induced CYP1A1 mRNA 137-fold and CYP1A2 mRNA 86-fold after 48 h exposure. Rifampicin had a small but significant effect on CYP1A1 and increased the mRNA 1.5-fold after 72 h exposure, but did not affect CYP1A2 mRNA levels in human liver slices (Fig. 3). Rifampicin induced CYP2C9 mRNA 3.5-fold and CYP3A4 mRNA 24-fold in human liver slices after 72 h exposure. TCDD did not affect CYP2C9 or 3A4 mRNA levels in human liver slices (Fig. 4).

A large interindividual variability was seen in the basal levels of mRNA. In human liver slices exposed to TCDD for 48 h, CYP1A1 and 1A2 mRNA levels increased compared with control levels (Fig. 5A). However, in slices exposed to rifampicin for 72 h, the CYP2C9 and 3A4 mRNA levels overlapped with the levels in control slices (Fig. 5B).

Omeprazole and primaquine both exhibited a significant induction of CYP1A1 and 1A2 mRNA but at different time points, 72 and 24 h, respectively (Table IIIA). Clotrimazole elevated both CYP1A1 and 1A2 mRNA after 48 h, and troglitazone increased CYP1A1 mRNA after 72 h of incubation, but to a smaller extent than omeprazole and primaquine. The levels of CYP2C9 mRNA were 2-fold higher than controls in human liver slices incubated with omeprazole for 72 h, which was the only significant induction of CYP2C9

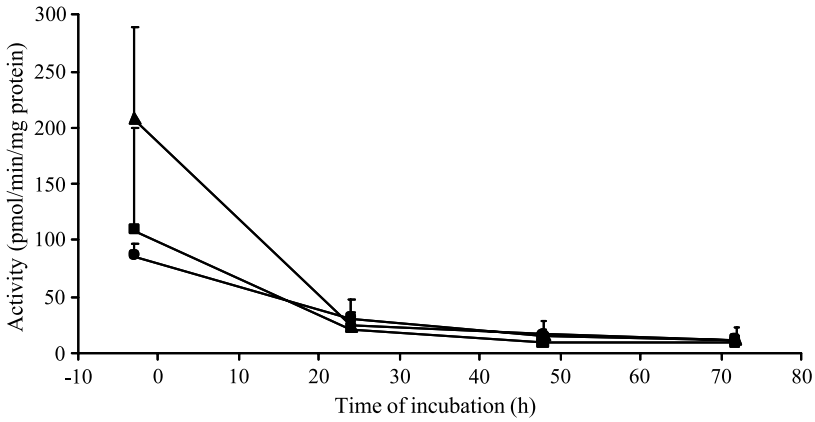

Fig. 2. Specific enzyme activities in human liver directly after resection (time = −3 h) and in human liver slices after incubation with control media for 24, 48, and 72 h. ● Phenacetin *O*-dealkylase activity, ■ diclofenac 4-hydroxylase activity, ▲ midazolam 1-hydroxylase activity (mean ± SD, $n \geq 3$).

Table III. Fold Induction of CYP1A1 and 1A2 (A) and CYP2C9 and 3A4 (B) mRNA in Human Liver Slices after Incubation with Test Compounds

(A)	Concentration (μM)	CYP1A1 fold induction	Exposure (h)	CYP1A2 fold induction	Exposure (h)
Carbamazepine	10	1.1 ± 0.3	24	1.3 ± 0.2*	48
Clotrimazole	10	1.9 ± 0.7*	48	2.0 ± 0.4**	48
Dexamethasone	10	1.2 ± 0.2	24	0.9 ± 0.2	24
Hyperforin	10	2.0 ± 1.9	24	1.4 ± 1.6	72
Lovastatin	10	0.9 ± 0.4	24	1.7 ± 0.9	72
Omeprazole	10	14 ± 10*	72	8.6 ± 3.3**	72
Phenobarbital	200	1.7 ± 1.4	48	1.7 ± 0.8	72
Phenytoin	10	1.4 ± 0.3*	72	1.4 ± 0.8	72
Primaquine	10	17 ± 13*	24	3.3 ± 1.9*	24
Rifampicin	25	1.5 ± 0.4**	72	1.1 ± 0.5	48
TCDD	0.00155	137 ± 125*	48	86 ± 68**	48
Troglitazone	10	2.7 ± 0.6**	72	2.2 ± 1.5	72
(B)	Concentration (μM)	CYP2C9 fold induction	Exposure (h)	CYP3A4 fold induction	Exposure (h)
Carbamazepine	10	1.4 ± 0.4	24	2.9 ± 1.9	72
Clotrimazole	10	1.6 ± 0.2**	48	4.3 ± 3.6	72
Dexamethasone	10	1.2 ± 0.1*	24	1.2 ± 0.2*	24
Hyperforin	10	2.0 ± 2.0	48	6.5 ± 5.1	72
Lovastatin	10	1.1 ± 0.4	72	3.3 ± 1.6*	72
Omeprazole	10	1.9 ± 0.8*	72	5.5 ± 3.8*	72
Phenobarbital	200	2.7 ± 1.8	72	12 ± 13	72
Phenytoin	10	1.1 ± 0.2	24	1.9 ± 1.0	72
Primaquine	10	1.5 ± 0.6	24	1.6 ± 0.7	24
Rifampicin	25	3.5 ± 3.1*	72	24 ± 17**	72
TCDD	0.00155	1.3 ± 0.6	24	1.2 ± 0.6	24
Troglitazone	10	3.0 ± 2.4	48	21 ± 33	72

The highest significant fold induction for each enzyme and compound is given. Values are mean ± SD from three individual livers, except for carbamazepine and dexamethasone CYP3A4, 72 h, where $n = 2$.
* $p < 0.1$.
** $p < 0.05$.

besides rifampicin (Table IIIB). CYP3A4 mRNA was significantly induced by omeprazole and lovastatin after 72 h exposure (Table IIIB). The mean induction of CYP3A4 mRNA by troglitazone was 21-fold, but the interindividual variation was large.

AhR Reporter Gene Assay

The EC_{50} value for TCDD in the AhR reporter gene assay was 0.71 ± 0.24 nM. Lansoprazole, omeprazole, and indole-3-carbinol were the only compounds tested that

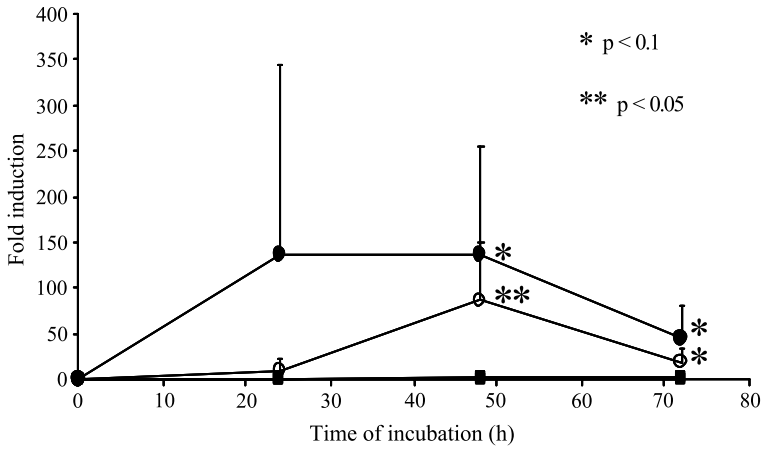

Fig. 3. The effect of 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) (1.55 nM) and rifampicin (25 μM) on CYP1A1 and 1A2 mRNA in human liver slices. ● TCDD, CYP1A1; ○ TCDD, CYP1A2; ■ rifampicin, CYP1A1; □ rifampicin, CYP1A2 (mean ± SD, $n \geq 3$). Rifampicin elevates CYP1A1 1.5-fold ($p < 0.05$, not marked in the figure).

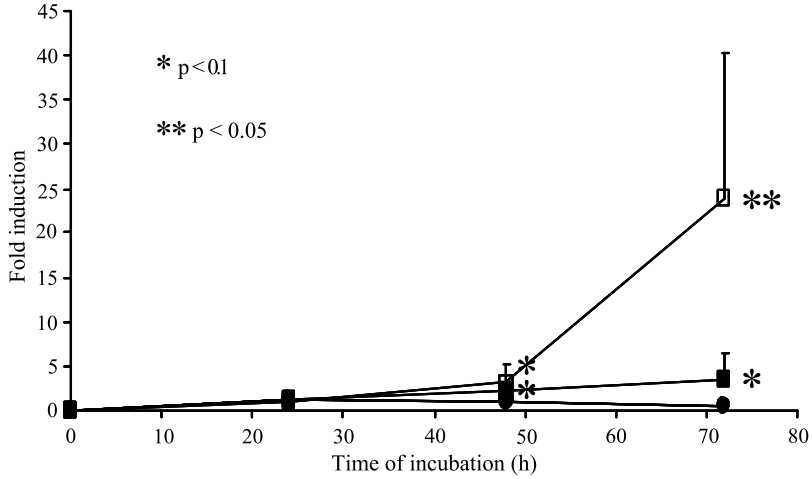

Fig. 4. The effect of TCDD (1.55 nM) and rifampicin (25 μM) on CYP2C9 and 3A4 mRNA in human liver slices. ● TCDD, CYP2C9; ○ TCDD, CYP3A4; ■ rifampicin, CYP2C9; □ rifampicin, CYP3A4 (mean ± SD, $n \geq 3$).

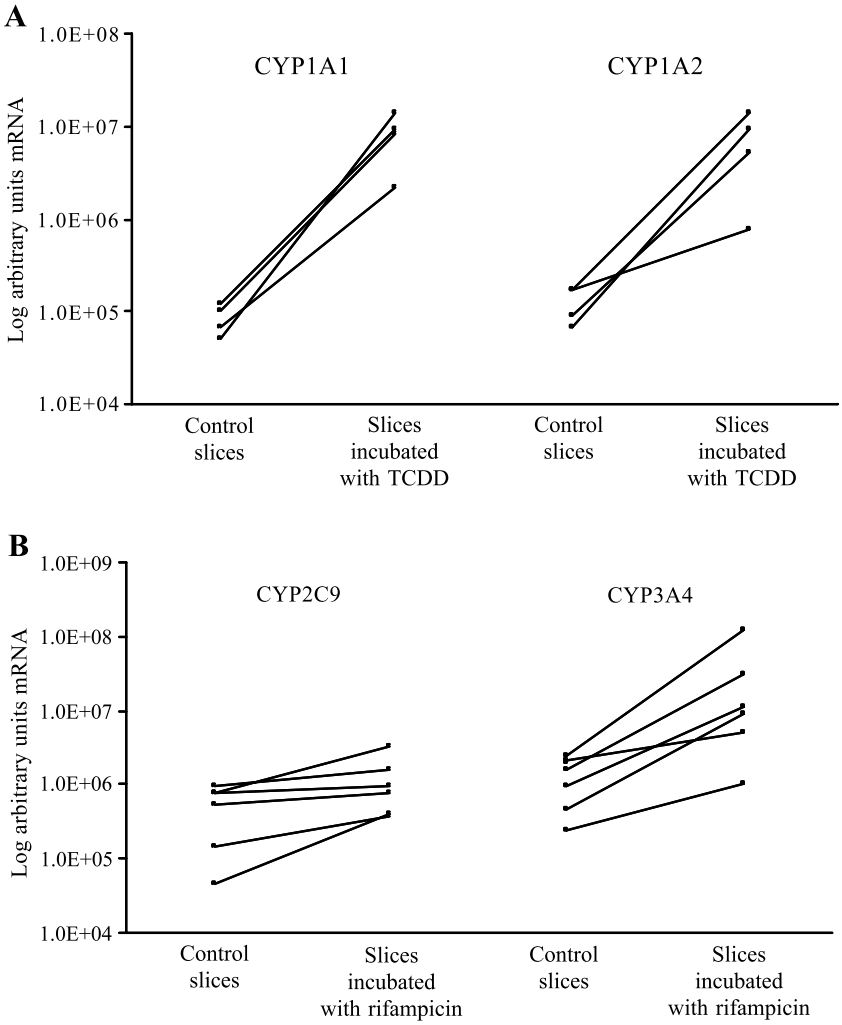

Fig. 5. mRNA levels in control and treated human liver slices from the same individuals. Arbitrary units of CYP1A1 and 1A2 mRNA after 48 h of incubation with control media or 1.55 nM TCDD (A) and CYP2C9 and 3A4 mRNA after 72 h of incubation with control media or 25 μM rifampicin (B).

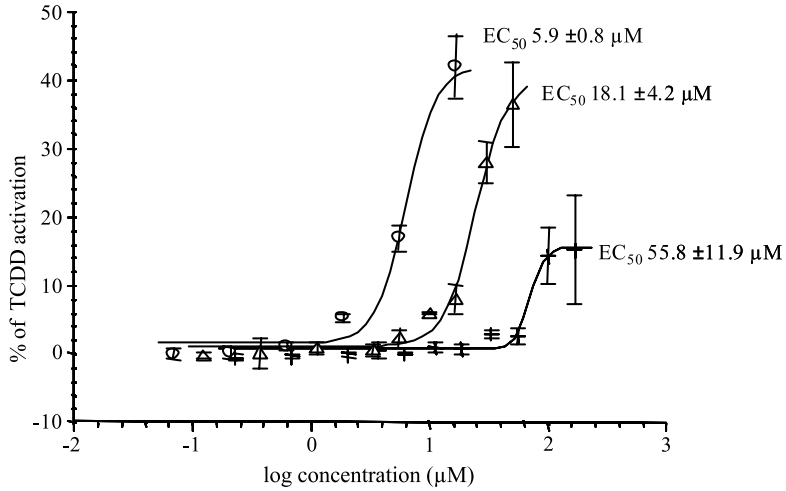

Fig. 6. Dose–response curves for lansoprazole (○), omeprazole (△), and indole-3-carbinol (+) in the AhR reporter gene assay. Average ± SD from four to seven experiments.

activated the receptor, exhibiting EC_{50} values of 5.9, 18.1, and 55.8 μM, respectively. The maximum concentrations used in the dose–response curves (Fig. 6) were limited by the observed cell toxicity of the compounds or their precipitation. The following compounds tested negative in the AhR reporter gene assay: artemisinin, betamethasone, carbamazepine, chlorpromazine, dexamethasone, diazepam, indomethacin, lovastatin, naproxen, pantoprazole, phenobarbital, phenytoin, primaquine, rabeprazole, rifampicin, troglitazone, and warfarin.

PXR Reporter Gene Assay

The activation of PXR in the reporter gene assay is shown in Table IV. The experiments were performed with concentrations giving full dose–response curves or to the limit of solubility or below concentrations resulting in cell toxicity. Of the 26 compounds tested, 18 activated the receptor. The most potent was hyperforin (EC_{50} = 0.003 μM) followed by rifampicin (EC_{50} = 0.20 μM). Diazepam activated the receptor to 19% of maximum rifampicin response, but a dose–response curve was unobtainable. The E_{max} values for the compounds tested varied considerably. Pantoprazole, lansoprazole, omeprazole, and hyperforin all exhibited values higher than the positive control (rifampicin).

Several approaches were used to correlate the activation of PXR in the reporter gene assay by the test compounds to known *in vivo* induction properties of CYP3A (Table V). Ranking of the tested compounds by EC_{50} values only, E_{max}/EC_{50}, or C_{max}/EC_{50} values, did not group the substances according to known CYP3A induction properties *in vivo* (data not shown). When compounds were ranked by AUC_{tot}/EC_{50} values (AUC and f_u values listed in Table IV), all *in vivo* inducers were grouped correctly with the exception of primidone (Fig 7A). Ranking by AUC_u/EC_{50} grouped all *in vivo* inducers but primidone and troglitazone (Fig. 7B). The induction factor, calculated with AUC_{tot}, did not rank the compounds properly (Fig. 7C), whereas the induction factor calculated with AUC_u ranked all compounds except primidone and troglitazone correctly (Fig. 7D). The induction factor calculated with C_{max} or C_{maxu} also gave erroneous results (data not shown).

DISCUSSION

In this study, the AhR and PXR reporter gene assays were evaluated as methods to predict the *in vivo* CYP1A and 3A4 induction properties of a range of compounds with known induction profiles *in vivo*. Furthermore, human liver slices were evaluated as an *in vitro* model to study CYP induction in the whole tissue.

Access to fresh human tissue meant that enzyme activities and mRNA could be measured already 1–2 h after liver resection and at different time points over a total period of 72 h. Unnecessary deterioration of the tissue prior to experiments because of handling time was therefore minimized. The results showed a substantial decrease both for enzyme activities and mRNA levels in the first 24 h of incubation. Over the experimental period, the activities in several experiments decreased to undetectable levels, whereas mRNA could always be detected in control human liver slices. These results are in line with previous studies on CYP apoprotein stability in human liver slices reported by Martin *et al.* (18). Meunier *et al.* (23) have reported similar decrease in CYP activities in human hepatocytes. After 96 h of culture, only 10–20% of the initial activities were retained. The dramatic loss of functional CYP1A2, 2C9, and 3A4 enzymes in human liver slices makes induction data on enzyme activity less reliable. mRNA levels were more consistent and therefore used as endpoints in the induction experiments in this study.

In human liver slices, the time course for induction of CYP mRNA exhibited different profiles. The time point for maximum fold induction of CYP1A1 and 1A2 mRNA was drug-dependent, whereas the compounds inducing CYP3A4 mRNA all exhibited the largest increase after 72 h exposure. Meunier *et al.* (23) showed that the induction of CYP activities exhibit specific profiles over time also in human hepatocytes. These results emphasize the need for induction properties to be followed over a period of time when investi-

Table IV. Receptor Activation by Test Compounds in the PXR Reporter Gene Assay

Substance	EC_{50} (μM)	E_{max} (% of maximum rifampicin induction)	*In vivo* AUC (h × μmol/l)	Reference *in vivo* AUC	f_u
Artemisinin	5.38 ± 3.66	17.5 ± 11.7	5.53	(41)	0.36
Betamethasone	19.9 ± 1.59	27.6 ± 8.93	0.19	(42)	0.36
Carbamazepine	15.6 ± 7.64	22.7 ± 6.45	1193	(43)	0.26
Chlorpromazine	NR	NR	0.78	(44)	0.03
Cimetidine	NR	NR	0.07	(45)	0.8
Dexamethasone	5.53 ± 3.99	12.4 ± 8.42	210	(46)	0.93
Diazepam	NR	18.8	16.5	(47)	0.02
Hyperforin	0.0030 ± 0.0027	120 ± 28.0	4.35	(48)	<0.0005
Indole-3-carbinol	NR	NR			
Indomethacin	17.6 ± 7.90	36.3 ± 10.5	28.8	(49)	0.01
Lansoprazole	3.02 ± 1.95	150 ± 13.0	5.01	(28)	0.03
Lovastatin	NR	NR	0.08	(50)	0.04
Midazolam	NR	NR	0.19	(51)	0.02
Naproxen	NR	NR	6201	(52)	0.001
Omeprazole	8.63 ± 3.14	147 ± 40.0	1.11	(28)	0.05
Pantoprazole	6.84 ± 2.16	152 ± 15.8	9.93	(28)	0.02
Phenobarbital	169 ± 26.9	71.7 ± 15.9	1497	(53)	0.49
Phenytoin	8.03 ± 5.18	20.6 ± 9.59	468	(54)	0.07
Pravastatin	NR	NR	0.16	(55)	0.5
Primaquine	13.6 ± 6.10	25.8 ± 10.3	3.27	(25)	0.066
Primidone	NR	NR	12.2	(56)	0.96
Rabeprazole	1.49 ± 0.58	81.1 ± 23.9	2.12	(57)	0.03
Rifampicin	0.20 ± 0.09	100	34.1	(58)	0.2
Troglitazone	3.45 ± 0.84	45.0 ± 15.3	16.5	(59)	0.01
Warfarin	49.5 ± 16.0	54.6 ± 17.5	86.4	(60)	0.01
Verapamil	3.19 ± 1.42	41.7 ± 13.8	1.27	(61)	0.1

Values are mean ± SD from three to six experiments. Published values of *in vivo* exposure (AUC) and fraction unbound (f_u) for the same drugs are listed.
NR, no response in the assay.

gating CYP induction of new chemical entities in human liver slices and hepatocytes.

Drugs such as clotrimazole, phenytoin, rifampicin, and troglitazone increased CYP1A1 and 1A2 mRNA less than 3-fold in human liver slices. These changes are low when compared with the 137- and 86-fold induction of CYP1A1 and 1A2 mRNA levels by TCDD. Furthermore, clotrimazole, phenytoin, rifampicin, and troglitazone did not activate AhR, which indicates that the increase in mRNA may be via other mechanisms than receptor activation. Primaquine did not activate AhR but was found to give the highest induction of CYP1A1 mRNA (17-fold) in human liver slices, except for TCDD, and also induced CYP1A2 mRNA but to a lesser extent. Primaquine has previously been characterized as a CYP1A inducer in human hepatocytes and HepG2 cells (15,24). In line with the present report, Fontaine *et al.* (24)

Table V. Different Models to Evaluate Test Compounds as Possible CYP3A Inducers *in Vivo* Based on Results from PXR Reporter Gene Assay and *in Vivo* Data

Model	Basis	Advantage/disadvantage
EC_{50}	The concentration that gives 50% of maximum response in the PXR reporter gene assay	Only *in vitro* potency in PXR reporter gene assay regarded
E_{max}/EC_{50}	The maximum activation (E_{max}) related to the EC_{50} values from the PXR reporter gene assay	Incorporates both potency and extent of induction in the PXR reporter gene assay
C_{max}/EC_{50}	Relating total or protein unbound *in vivo* C_{max} of the drug to EC_{50} values from the PXR reporter gene assay	Human *in vivo* data are needed or need to be predicted
AUC/EC_{50}	Relating total or protein unbound *in vivo* AUC of the drug to EC_{50} in the PXR reporter gene assay	Human *in vivo* data are needed or need to be predicted
$I = E_{min} + \frac{(E_{max} - E_{min})}{1 + \left(\frac{EC_{50}}{X}\right)^{k}}$	The induction factor, I, relates *in vivo* concentration or exposure, X, to EC_{50} and E_{max} values from the PXR reporter gene assay in an equation for dose-response. E_{min} = the minimum activation in the PXR assay; k = the slope of the dose–response curve created from the results in the PXR assay	Incorporates both potency and extent of induction in the PXR reporter gene assay. Human *in vivo* data are needed or need to be predicted

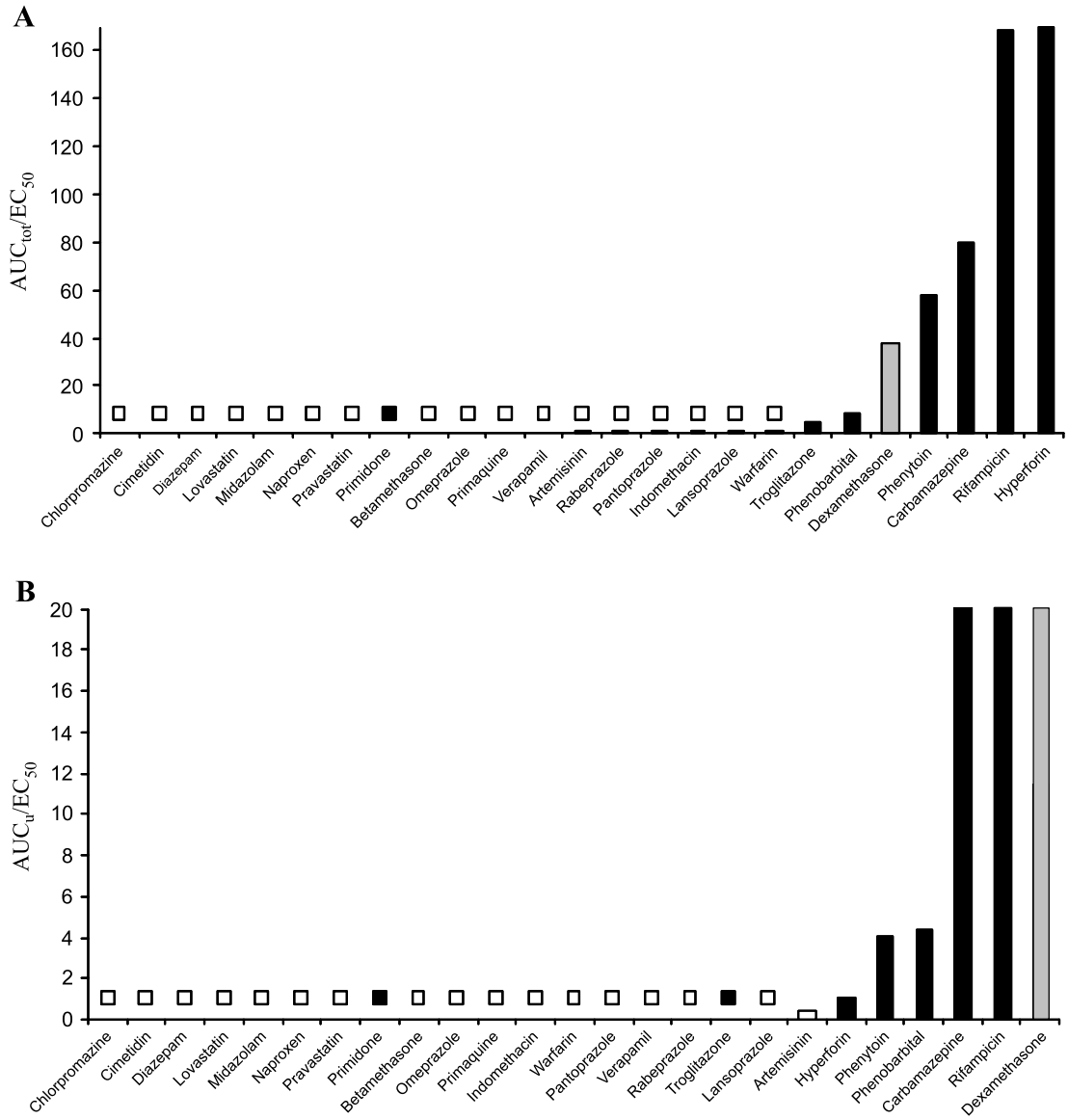

Fig. 7. Drugs ranked from lowest to highest value on the basis of *in vivo* AUC_{tot}/EC_{50} in PXR assay (A), *in vivo* AUC_u/EC_{50} in PXR assay (B), induction factor calculated using AUC_{tot} (C), and induction factor calculated using AUC_u (D). Closed bars = *in vivo* inducers; shaded bars = weak *in vivo* inducers; open bars = *in vivo* induction of CYP3A not found in literature. No values for the compounds farthest to the left because no EC_{50} values were obtained. AUC_{tot}/EC_{50} value for hyperforin = 1432. AUC_u/EC_{50} value for dexamethasone = 35.3 and for rifampicin = 33.7.

showed that primaquine is not a ligand for human AhR. Furthermore, there are, to our knowledge, no studies that indicate that primaquine induces CYP1A *in vivo*. This could be a result of the low plasma levels of primaquine (≈0.4 μM) (25), which are well below the concentrations used in this and in earlier *in vitro* studies of CYP1A induction by primaquine.

Omeprazole and lansoprazole have previously been shown to induce CYP1A enzymes in human *in vitro* systems (17,26). In this study, omeprazole and lansoprazole activated AhR, and omeprazole (10 μM) also induced CYP1A1 and 1A2 mRNA in human liver slices. The EC_{50} for omeprazole in the AhR reporter gene assay was 18.1 μM, which is well above the therapeutic plasma concentrations (1.1–3.2 μM) (27). Previous *in vivo* studies of omeprazole, given at therapeutic doses, report no induction of CYP1A when caffeine and theophylline were used as *in vivo* probes (28,29). The EC_{50} for lansoprazole in the AhR reporter gene assay was 5.9 μM, which is close to plasma C_{max} levels (2.0–4.8 μM) (27) especially in poor metabolizers. Lansoprazole has, however, been reported not to induce CYP1A *in vivo* at therapeutic doses using the same *in vivo* probes as for omeprazole (28,29). Because there are no known drugs that induce CYP1A *in vivo* in humans at therapeutic doses, the relevance of *in vitro* CYP1A induction results is difficult to assess.

Although both CYP2C9 and 3A4 are regulated by PXR (7–9), the induction of CYP2C9 *in vivo* in humans has only been shown for some of the strongest *in vivo* CYP3A inducers, e.g., phenobarbital and rifampicin (2,4). Furthermore, the fold induction for CYP2C9 probes *in vivo* is lower than for CYP3A probes. This implies that only potent

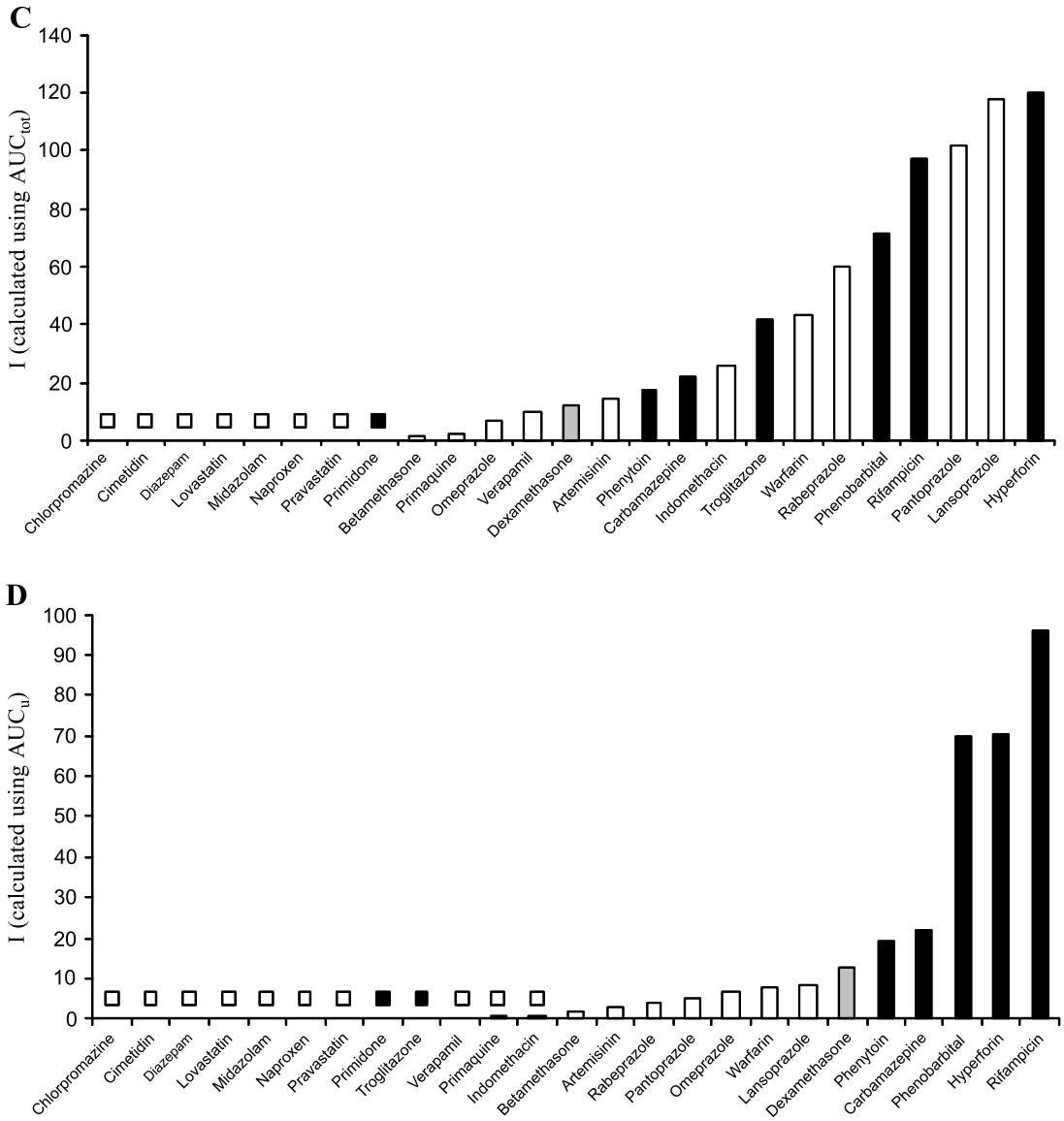

Fig. 7. Continued

CYP3A inducers will induce CYP2C9 and to a lesser degree than CYP3A. In the present study, the induction of CYP2C9 mRNA levels in human liver slices was also lower than for CYP3A4 induction, which mirrors the response *in vivo*. Similar results have also been presented by Rae *et al.* (30) and by Edwards *et al.* (16) who measured induction of mRNA levels in human hepatocytes and induction of apoprotein levels in human liver slices, respectively, by a range of compounds.

Rifampicin, which is the most potent CYP2C9 and 3A inducer *in vivo*, significantly induced the level of CYP2C9 and 3A4 mRNA in human liver slices. Even if rifampicin-treated slices always showed higher CYP2C9 and 3A4 mRNA levels than the control slices from the same individual, the large interindividual variation resulted in overlapping levels when the mRNA levels in rifampicin and control slices were compared. Carbamazepine, St. John's wort, phenobarbital, phenytoin, and troglitazone exhibit lower than 3-fold CYP3A induction *in vivo*, and the induction of CYP3A4 mRNA in human liver slices is in the same range. The weak response in CYP3A4 mRNA by phenytoin in spite of known induction properties *in vivo* may be explained by the concentration used *in vitro* (10 μM), which is below the concentration (40–80 μM) reported to cause induction *in vivo* (31). Omeprazole significantly induced CYP3A4 mRNA in the liver slices but is not reported to be an *in vivo* inducer (32). However, as for CYP1A induction, this can be explained by the supratherapeutic concentrations used in the study.

All substances inducing CYP3A4 mRNA in human liver slices were also shown to activate PXR, except for lovastatin. Lovastatin induced CYP3A4 mRNA in the liver slice experiments and has previously been shown to activate PXR (7,8). The results for this substance are therefore inconclusive. Primidone was not tested in human liver slices but did not activate PXR despite its known CYP3A induction properties

in vivo. The reason for the lack of effect by primidone is most likely because of the fact that this substance is metabolized to phenobarbital *in vivo* (33), which may be the principal inducer.

In the human liver slice experiments, a large interindividual variation was seen. Liver slices represent individuals exhibiting large variation in basal levels of mRNA as well as responsiveness to potential inducers. These large variations have previously been demonstrated both for human liver slices and hepatocytes. Edwards *et al.* (16) showed that after treatment with rifampicin (50 μM), the induction of CYP3A4 apoprotein in human liver slices varied between 0 and 1840% of control values. Martin *et al.* (18) studied mRNA, protein, and enzyme activity in both human liver slices and hepatocytes and reported large variability in both basal levels as well as induction response. The amount of CYP3A4 apoprotein varied between 15.5 and 87.5 pmol/mg protein in cultured human hepatocytes and liver slices. The variation in the inducibility of CYP3A4 activity was also large: 0.3- to 6.3-fold in liver slices and 0.6- to 133-fold in hepatocytes. Komoroski *et al.* (19) reported similar variation in the induction of CYP3A4 mRNA and activity in human hepatocytes, which varied between approximately 15- to 30- and 3.2- to 7.9-fold, respectively. Interindividual variation is also evident for other enzymes, such as CYP1A2 and 2C9 (19,34).

PXR is known to be the major transcription factor determining the induction of CYP3A4. A good correlation between activation of PXR in a reporter gene assay and the CYP3A4 mRNA and activity in primary human hepatocytes was reported by Luo *et al.* (35), and Roymans *et al.* (36) reported a good correlation between CYP3A4 mRNA and enzyme activity in human cryopreserved hepatocytes. There is therefore a mechanistic basis for investigating whether PXR activation could predict CYP3A induction *in vivo*.

Several approaches based on results from the PXR reporter gene assay were used to classify the compounds (Table V). The simplest, EC_{50} from the PXR reporter gene assay, did not rank the test compounds according to known *in vivo* induction properties. Another way to rank the test compounds is according to E_{max}/EC_{50} values from the PXR reporter gene assay. This reflects the overall ability of the compound to induce the enzyme and incorporates both potency and extent of induction (7,34), but this did not improve the ranking. Instead of E_{max}, *in vivo* concentrations were related to PXR EC_{50}, but this did not rank the compounds properly. The AUC was related to PXR EC_{50}, indicating that the induction could be connected to *in vivo* exposure rather than to maximum concentration *in vivo*. When the compounds were ranked according to AUC_u/EC_{50}, the CYP3A *in vivo* inducers were grouped together with the exceptions of primidone and troglitazone. Ranking the compounds by AUC_{tot}/EC_{50} clustered all *in vivo* inducers except primidone. We also calculated the induction factor I, based on an equation for dose-response, because this takes both potency and extent of induction in the PXR reporter gene assay into consideration and also relates the *in vitro* results to the *in vivo* kinetics. Although the induction factor was calculated using C_{max}, C_{maxu}, AUC_{tot}, and AUC_u, neither of these improved the classification of the compounds.

When substances were ranked according to AUC/EC_{50}, troglitazone was grouped together with non-inducers when plasma protein binding was taken into account but was grouped together with the other *in vivo* inducers when the total AUC was used. Troglitazone is highly protein bound in plasma, which may affect the *in vivo* prediction. Protein binding is a cornerstone in scaling exercises assuming that only protein-unbound substance is available to the cell. In the models for scaling clearance from *in vitro* data, large underpredictions are often obtained when protein binding is included for highly protein bound compounds (37). Further studies are needed to elucidate the relevance of protein binding in the prediction of *in vivo* induction from *in vitro* results.

Based on the EC_{50} results from the PXR reporter gene assay and reported *in vivo* exposure, it was possible to group the compounds as inducers or non-inducers. An AUC_{tot}/EC_{50} value above 3 or an AUC_u/EC_{50} value above 0.5 would indicate that the substance is a potential CYP3A4 inducer *in vivo*. However, this is a qualitative prediction, and extending the prediction to a quantitative assessment of change in AUC is a more difficult task. This is partly because the *in vitro* studies merely predict changes in clearance, and for drugs given orally, the AUC is dependent on both clearance and bioavailability. There are several examples showing greater decrease in AUC after an oral dose than after an iv dose of drugs metabolized by CYP3A in subjects treated with rifampicin. Rifampicin caused a 9.7-fold decrease in AUC after an oral dose and a 2.2-fold decrease after an iv dose of midazolam (38). The decrease in AUC for *S*-verapamil was 30-fold after an oral dose and 1.3-fold after an iv dose (39). For nifedipine, the decrease in AUC was 12-fold after an oral dose and 1.4-fold after an iv dose (40). These studies indicate that a major part of the decrease in AUC of CYP3A substrates after rifampicin treatment is caused by a decrease in bioavailability, most likely because of increased first-pass metabolism in the intestine and in the liver.

In conclusion, the interindividual variation in response of CYP enzymes to potential inducers in human liver slices reported here and elsewhere as well as in experiments on isolated hepatocytes is a major concern when a large number of compounds are to be evaluated for induction properties. Furthermore, the experimental setting is not applicable to high throughput screening, which thus limits the use of liver slices and cultured human hepatocytes in early drug discovery. However, liver slices and cultured human hepatocytes are valuable models to study the regulation of a larger number of enzymes by a single compound identified as a possible CYP inducer. In contrast to liver slices and cultured human hepatocytes, reporter gene assays are amenable to high throughput screening, and can be used to investigate a large number of substances over a concentration range at controlled conditions. The CYP1A induction recorded in the *in vitro* systems by well-known drugs seems to have no clinical significance because no clear induction *in vivo* by these compounds has been reported. Therefore, an evaluation of the relevance of the *in vitro* results on CYP1A induction *in vivo* is hampered. On the other hand, known CYP3A inducers *in vivo* were correctly classified by the results from the PXR reporter gene assay when related to *in vivo* exposure. The PXR reporter gene assay can thus be used as a reliable assay in the drug discovery process to screen for CYP3A inducers.

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