DMD Fast Forward. Published on March 23, 2009 as doi:10.1124/dmd.109.026526




# Evaluation of multiple in vitro systems for assessment of CYP3A4 Induction in Drug Discovery: Human hepatocytes, PXR reporter gene, Fa2N-4 and HepaRG cells.

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DMD Fast Forward. Published on March 23, 2009 as DOI: 10.1124/dmd.109.026526
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**Running Title**

**Evaluation of multiple in vitro systems for CYP3A4 Induction**

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28 text pages

5 Tables

6 Figures

33 Refs

239 words in Abstract

742 words in Introduction

1505 words in Discussion

**Abbreviations**

AhR, arylhydrocarbon receptor: CAR, constitutive androstane receptor: P450, cytochrome P450: CITCO, 6-(4-chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehydeO-(3,4-dichlorobenzyl)oxime: DDI, drug-drug interaction(s): $E_{max}$, maximum observed induction at an optimum concentration: $EC_{50}$, effective concentration that supports half maximal induction: HLM, human liver microsomes: PXR, pregnane X receptor: qRT-PCR, quantitative reverse transcription-polymerase chain reaction: RXR, retinoid X receptor: TDI, time-dependent inhibition:

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**Abstract**

Prototypic CYP3A4 inducers were tested in a PXR reporter gene assay, Fa2N-4 cells, HepaRG cells and primary human hepatocytes, along with negative controls, using CYP3A4 mRNA and activity endpoints, where appropriate. Over half of the compounds tested (14/24) were identified as time dependent inhibitors of CYP3A4 and high mRNA: activity ratios (>10) were consistent with CYP3A4 time dependent inhibition for compounds such as troleandomycin, ritonavir and verapamil. Induction response was compared between two human donors; there was an excellent correlation in the $EC_{50}$ estimates ($r^2$ = 0.89, p<0.001) and a weak but statistically significant correlation was noted for $E_{max}$ ($r^2$ = 0.38, p=0.001). $E_{max}$ and $EC_{50}$ estimates determined from the PXR reporter gene assay, Fa2N-4 and HepaRG cells were compared to those from hepatocytes. Overall, $EC_{50}$ values generated using hepatocytes agreed with those generated in PXR reporter gene assay ($r^2$ = 0.85, p<0.001), Fa2N-4 ($r^2$ = 0.65, p<0.001) and HepaRG ($r^2$ = 0.99, p<0.001) cells. However $E_{max}$ values generated in hepatocytes were only significantly correlated to those determined in Fa2N-4 ($r^2$ = 0.33, p=0.005) and HepaRG cells ($r^2$ = 0.79, p<0.001). 'Gold standard' P450 induction data can be generated using primary human hepatocytes but a restricted, erratic supply and inter-donor variability somewhat restricts routine application within a drug discovery setting. HepaRG cells are a valuable recent addition to the armoury of *in vitro* tools for assessing CYP3A4 induction and appear to be an excellent surrogate of primary cells.

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## Introduction

Induction of human cytochrome P450 (P450) enzymes *in vivo* can result in significant clinical consequences primarily via reduced exposure leading to suboptimal efficacy of co-administered drugs. CYP3A4 has been recognized as the target for the most clinically significant induction mediated drug-drug interactions (DDIs). For example the induction of CYP3A4 by some antibiotics leads to reduced ethinylestradiol levels from oral contraceptives resulting in breakthrough pregnancies and reduced cyclosporine levels can lead to organ rejection in transplant patients (Sinz et al., 2008). Therefore the need to evaluate and minimise the potential for P450 induction mediated DDIs is widely accepted within the pharmaceutical industry.

Induction of P450 by drugs and other xenobiotics occurs typically via activation of receptors that regulate transcription. The primary mechanism of CYP3A4 induction is via activation of the nuclear pregnane X receptor (PXR), a transcription factor that regulates the transcription of several CYP isoforms, UDP-glucuronosyl-transferases, and other drug metabolizing enzymes and transporters (Hewitt et al., 2007b).

Because of distinct species differences in both the extent and pattern of ligand response (Dickins, 2004), *in vitro* human assays are used to predict clinical effects and primary human hepatocytes in culture are the current system of choice (Hewitt et al., 2007a; 2007b). However, limited availability of high quality human hepatocytes and the inter-individual variability observed in the response to inducers has encouraged the search for alternative assay systems for use in drug discovery. The use of large batches of pre-characterized inducible cryopreserved human hepatocytes somewhat mitigate these issues. In addition, reporter gene assays for PXR (Wang and LeCluyse, 2003) lend themselves well to enhanced throughput screening for CYP3A4 induction liability yet have several potential drawbacks including that they offer a pseudo endpoint, only assay for one mechanism of CYP3A4 induction and the reporter gene construct is typically expressed in cell-lines with limited or no metabolic capability (Castell et al., 2006).

Several hepatic cell lines including HepG2, BC2 and HepaRG (Castell et al., 2006; Vermeir et al., 2005; Kanebratt and Andersson, 2008b) and the immortalised Fa2N-4 cell line (Mills et al., 2004; Ripp et al., 2006; Youdim et al., 2007; Hariparsad et al., 2008; Kenny et al., 2008) have recently undergone preliminary evaluation as alternatives to primary human hepatocytes for CYP3A4 induction studies. Studies have demonstrated Fa2N-4 cells to be responsive to prototypical inducers of CYP1A2 and CYP3A4 (Mills et al., 2004; Kenny et al., 2008) and the potency ($EC_{50}$) and magnitude of effect ($E_{max}$) of CYP3A4 inducers in Fa2N-4 cells could be used to predict the *in vivo* induction response (Ripp et al., 2006). More recently, the expression of PXR and arylhydrocarbon receptor (AhR) were determined to be similar between Fa2N-4 cells and human hepatocytes,

however both constitutive androstane receptor (CAR) and several hepatic uptake transporters including the organic anion transporting polypeptides were significantly lower in this cell line compared to primary cells (Hariparsad et al., 2008). This laboratory has recently confirmed that Fa2N-4 cells are a good surrogate for primary human hepatocytes when assessing AhR and PXR mediated CYP1A2 and CYP3A4 induction respectively, but not CAR mediated CYP2B6 induction (Kenny et al., 2008). HepaRG are newly developed human hepatoma cells that express several P450s (including CYP1A2, 2B6, 2C9, 2E1 and 3A4), PXR, AhR and CAR at levels comparable to cultured primary human hepatocytes, unlike HepG2 cells (Aninat et al., 2006). In addition HepaRG cells express conjugating drug metabolizing enzymes as well as membrane transporters. They are thus a promising alternative to primary human hepatocytes not only for P450 induction studies but as an alternative model for liver metabolism and hepatic toxicity of xenobiotics (Aninat et al., 2006; Kanebratt and Andersson, 2008a, 2008b; Guillouzo et al., 2007).

As previously reported by this laboratory, it is beneficial to determine both mRNA and activity in the assessment of induction, as processes such as time-dependent P450 inhibition (TDI) can confound the interpretation of data based on activity alone (McGinnity *et al.,* 2006; Kenny et al., 2008). In order to relate message to activity endpoints in this work, determination of P450 TDI was carried out for all compounds.

The present study describes a head-to-head evaluation of several promising *in vitro* approaches for studying CYP3A4 induction. Prototypic CYP3A4 inducers were tested in the PXR reporter gene assay, Fa2N-4 and HepaRG cells and primary human hepatocytes, the current 'gold standard' *in vitro* system, together with negative controls, with the aim of comparing data and the assessing the applicability of the alternate *in vitro* test systems for use in drug discovery.

## Materials and Methods

### Chemicals and Reagents.

Carbamazepine, 6-(4-chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehydeO-(3,4-dichlorobenzyl)oxime (CITCO), clotrimazole, dexamethasone, mifepristone, β-naphthoflavone, nifedipine, omeprazole, phenobarbital, phenytoin, quinidine, rifampicin, rifapentine, reserpine, troglitazone, troleandomycin, verapamil, and β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), were purchased from Sigma-Aldrich (Gillingham, UK) and were of the highest grade available. Efavirenz, hyperforin, midazolam, 1′-hydroxymidazolam, paclitaxel, pioglitazone, rifabutin, ritonavir and sulfinpyrazone were purchased from Sequoia Research Products Ltd. (Oxford, UK). Lansoprazole was synthesised at AstraZeneca. Collagen type I coated 24-well plates, tissue-cultured treated flasks and trypsin-ethylene diamine tetracetic acid (EDTA), BD™ Hepatocyte culture medium, epidermal growth factor, 6ß-hydroxytestosterone and 6β-hydroxytestosterone-[D7] were obtained from BD Biosciences Discovery Labware (Bedford, MA). Glutamine, Fungizone® and Trizol™ were purchased from Gibco Invitrogen Corp (Carlsbad, CA). Acetonitrile, dimethyl sulfoxide (DMSO), ethanol and formic acid were purchased from Fisher Scientific (Loughborough, UK) and were of the highest grade available. Cryopreserved Fa2N-4 cells, Multi-Function Enhancing (MFE) plating media and MFE support media with supplement A were obtained from Xenotech Ltd (Lenexa, KA, USA). HepaRG cells were purchased as pre-seeded 24-well plates from Biopredic International (Rennes, France). DNase 1 RNase free kit and RNase free water were from Invitrogen (Paisley, UK). TaqMan One-Step RT-PCR Master Reaction Mix, MultiScribe Reverse Transcriptase, primers and probes were obtained from Applied Biosystems (Foster City, CA). All other chemicals were of high purity grade and purchased from either Sigma-Aldrich (St. Louis, MO) or JT Baker (Phillipsburg, NJ).

### Human Hepatocytes.

Primary cultured fresh human hepatocytes (Donor HH205 and HH215) (Table 1), were obtained from BD Biosciences Discovery Labware (Bedford, MA). Human hepatocytes plated in collagen I-coated 24-well plates were maintained in culture at least 48 h prior to the treatment in hepatocyte culture medium supplemented with 10 μg/L epidermal growth factor, 50 μg/mL gentamycin, 2 mM L-glutamine, and 0.75 μg/mL fungizone. Cells were treated in triplicate with 0.08 % DMSO vehicle or test compounds at concentrations as described in Table 2 for 72 h with medium change and replenishment every 24 h. Regular visual inspection of the hepatocyte cultures revealed a confluent monolayer with good cell morphology.

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The CYP3A4 induction assay was performed essentially as described by Zhang et al (2006). In brief, the induction was determined by measuring the 6ß-hydroxytestosterone metabolite formation from model substrate testosterone *in situ* and measuring mRNA expression using TaqMan™ real time RT-PCR as described below.

**Determination of CYP3A4 TDI in Human Liver Microsomes.**

CYP3A4 TDI was assessed for all compounds in human liver microsomes (HLM) using an adapted version of the single time-point, single concentration, automated method described by Atkinson *et al.,* (2005). Each compound was tested at the top concentration incubated in the induction studies, with the exception of phenobarbital, which was tested at a final concentration of 500 µM to ensure solubility. Briefly, a 30 min pre-incubation was performed containing: HLM (1 mg/ml) in phosphate buffered saline (0.1 M; pH 7.4), NADPH (1 mM) and test compound (in DMSO 1% v/v). A 20-fold dilution was performed into NADPH (1 mM) and midazolam (10 µM) and the second reaction allowed to proceed for 15 min. Aliquots (50 µl) were quenched in ice-cold methanol (100 µl) and prepared for LC-MS-MS as described below. Projected $IC_{50}$ values were calculated as described previously (Atkinson *et al.,* 2005).

Quenched samples were centrifuged (2000 *g*; 15 min, 4 ˚C) and supernatants (10 µl) injected from a 96-well plate onto a Symmetry C18 (3.5 µm) column (2.1 x 100 mm, Waters, Hertfordshire, UK) maintained at 40 °C. HPLC separation was performed using Jasco PU-2085plus semi-micro pumps coupled to Jasco DG-2080-54 degasser, MX-2080-32 dynamic mixer and a CO-2067plus column oven (Jasco, Essex, UK). The mobile phase consisted of solvent A (0.1% formic acid in acetonitrile) and solvent B (0.1% formic acid in water), using a linear gradient of 10% A (0–0.1 min), 54% A (0.1–3.0 min), 100% A (3.0 – 3.5 min), 10% A (3.51 min) with a run time of 5.5 min and flow rate 0.5 ml/min. This was coupled to a triple quadruple Platinum Ultima (Waters, Hertfordshire, UK) operating in ESI+ mode, with Masslynx v.4.1 running in MRM mode monitoring for 1′-hydroxymidazolam (342.35 > 203.23, 31 V, 24 eV) which was quantified using an authentic standard.

**PXR reporter gene assay.**

Induction of CYP3A4 was assessed for all compounds in a PXR reporter gene assay as described by Persson et al., (2006). In brief, transiently transfected HepG2 cells were thawed and seeded at 2.33 million cell/ml in a 96-w plate. Test compounds were serial diluted (DMSO vehicle @ 0.025% final v/v) and added to the cells to generate a dose response for an $EC_{50}$ determination. Cells were then left to incubate at 37 °C with compound for 72 h. After 72 h luclite lyophilized substrate was added to all 96 wells of the

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plate. Cells were lysed and a signal generated during a 10 min RT incubation. The 96-well plates were placed in a luminescence plate reader and counts measured. Fold induction was calculated by comparing magnitude of response in treated wells relative to that observed in control (DMSO only) wells.

**Fa2N-4 cells.**

Induction of CYP3A4 was assessed for all compounds in Fa2N-4 cells as described by Kenny *et al.,* (2008). In brief, Fa2N-4 cells were grown on tissue culture treated flasks (75 $cm^2$), maintained in MFE support media with supplement A (1 µl/ml) at 37 ˚C under an atmosphere of $CO_2$ :$O_2$ (5:95%) at 95% relative humidity. Support media was changed every 2 days and cells were passaged weekly as they reached confluence. For passaging, cells were trypsinised (5 ml / flask) for 15 min, harvested in MFE plating media (40 ml), centrifuged (120 *g* for 5 min), and re-suspended in fresh MFE plating media. Cells were then diluted to 0.2 x $10^6$ cells/ml and either re-seeded in culture treated flasks (~2 x $10^6$ cells/flask) or seeded on collagen coated 24-well plates (0.1 x $10^6$ cells/well; 0.5 ml/well). After 24 h, MFE plating media was replaced with MFE support media containing supplement A. All Fa2N-4 cells used in the experiments were between passages 2 and 8. Fa2N-4 cells were pre-cultured for 2 days on 24-well plates in MFE support media containing supplement A. A range of 6 concentrations was prepared for each test compound, selected to allow full induction dose response curves to be generated. Test compounds in media (0.5 ml) were added to appropriate wells of a 24-well plate containing pre-cultured Fa2N-4 cells, this was replaced with freshly prepared solutions of media and test compound every 24 h, for up to 72 h.

**HepaRG cells.**

HepaRG cells were obtained as pre-seeded 24-well plates. Induction of CYP3A4 was assessed for all compounds in HepaRG cells as described by Kanebratt and Andersson (2008b). In brief, after a 48-72 h shipment, cells were allowed to recover at 37° C for 48 h in after-shipment medium (low % DMSO + serum) followed by an overnight recovery in differentiation media. After the cells had recovered in differentiation medium, they were maintained for 48 h in basal medium (no DMSO or serum) with daily changes of media. After 48 h of maintenance in basal medium, cells were treated daily with test compounds at varying concentrations for 48 h. The final concentration of DMSO in culture medium was 0.1% (v/v), and the vehicle control group received 0.1% DMSO. For the determination of CYP3A activities, HepaRG cells were incubated in situ with midazolam and analyzed by LC-MS-MS as described below. Quantification of CYP3A4 mRNA was carried out by one-step qRT-PCR, and all mRNA were normalized to total RNA in sample

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as described below. The fold induction of each CYP was calculated as described for the PXR–reporter gene assay.

**Determination of CYP3A Activity in Human hepatocytes, Fa2N-4 and HepaRG cells.**

CYP3A4 activity was assessed in human hepatocytes using testosterone 6β-hydroxylase activity using by LC-MS-MS analysis. After treatment, hepatocytes in culture were washed once with medium and then incubated with 200 μM testosterone at 37° C for 15 min. The reaction was stopped by removing 300 μL incubation medium, followed by mixing with 150 μL acetonitrile containing 0.1% formic acid and stable-isotope labeled 6ß-hydroxytestosterone internal standard. The 6β-hydroxy metabolite formation was measured using a PE Sciex API4000 or API4000 Qtrap LC/MS/MS system equipped with PerkinElmer pumps and a CTC autosampler (Shelton, CT).

Midazolam 1′ -hydroxylation was used to assess CYP3A in Fa2N-4 and HepaRG cells as described by Kenny et al., (2008). Briefly, at the end of the induction period, test compound and media were aspirated from the cells and replaced with pre-warmed media (0.5 ml) containing midazolam at a final substrate concentration of 25 μM. Cells were then incubated on an orbital shaker for gentle agitation (~ 30 rpm; 37 °C). Aliquots (100 μl) of media were removed at 20 min, quenched in ice-cold methanol (100 μl) and stored at -30 °C until analysis for 1′-hydroxymidazolam by LC-MS-MS as described above. Finally, residual media containing substrate was aspirated, cells lysed in RLT buffer (600 μl; Qiagen, West Sussex, UK) containing β-mercaptoethanol (10 μl/ml) and stored at -80˚C until required for mRNA analysis.

**CYP3A4 mRNA analysis.**

The remaining human hepatocytes in each well after enzyme reaction were washed once with phosphate buffered saline. A 1 mL aliquot of Trizol$^{TM}$ was added to each well and total RNA was extracted in accordance with the manufacturer's instruction. The content of total RNA was determined at 260 nm and purity of RNA was estimated using a ratio of 260/280 nm. The RT-PCR assay was performed with Applied Biosystem's Gene Expression kit and in a 25 μL aliquot of TaqMan One-Step RT-PCR Master Reaction Mix containing CYP3A4 and house-keeping gene β-actin forward and reverse primers, TaqMan probe, MultiScribe Reverse Transcriptase and 50 ng of total RNA. The PCR amplification was performed and the transcription was determined using an ABI 7300 Real Time PCR System.

The methodology used for CYP3A4 mRNA analysis of Fa2N-4 and HepaRG cells was as described by Kenny et al., (2008). Briefly, Fa2N-4 and HepaRG cell lysates (stored in RLT

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buffer on culture plate) were defrosted on ice and mRNA isolated using the RNeasy 96 kit (Qiagen, West Sussex, UK) according to the manufacturers instructions for isolation of total RNA from animal cells using vacuum technology, including an on-column DNase 1 digestion to minimise genomic DNA contamination (RNase-Free DNase Set, Invitrogen, Paisley, UK). Total RNA was quantified fluorometrically using a Quant-iT RiboGreen RNA reagent and assay kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Purified mRNA samples (5 µl) were analysed by one-step quantitative RT-PCR performed on an Mx3005P real-time cycler with MxPro software (Stratagene, Amsterdam, The Netherlands) using QuantiTect Multiplex RT-PCR No ROX Master Mix Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions using optimised primer and probe concentrations. Relative quantification of gene expression level was determined by interpolation of threshold cycle (Ct) values to a standard curve. This standard curve was generated from a dilution series of human hepatocyte total mRNA. Standard mRNA was pooled from human hepatocytes induced with rifampicin (10 µM), omeprazole (50 µM) and phenobarbital (2 mM) for 72 h. Data was normalised to total RNA content, as determined in the RiboGreen assay.

**Data transformation, Statistical Analysis and Curve Fitting.**

Unless otherwise specified, all results were expressed as the mean of triplicate determinations. The parameters $E_{max}$ and $EC_{50}$ were determined from dose response data, where $E_{max}$ was the maximum observed induction at an optimum concentration and $EC_{50}$ was the effective concentration that supports half maximal induction. To estimate $EC_{50}$ values, dose response data was fitted to a simple $E_{max}$ model with Hill function according to the following equation:

$$y = (E_{max} \cdot x^{\gamma})/(EC_{50}^{\gamma} + x^{\gamma})$$

using Origin 6.0 (MicroCal, Northampton, MA, USA). Similar to the approach taken by Ripp *et al.,* (2006), data points from wells with some evident toxicity, based on visual inspection of cell morphology, were excluded from the fits and the $E_{max}$ was set at the observed $E_{max}$ to prevent extrapolation of the curve fit beyond measured data. All CYP3A4 $E_{max}$ data for CYP3A4 was normalised to the rifampicin positive control as follows:

$$E_{max} \text{ (\% maximal rifampicin response)} = (E_{max} \text{ test} / E_{max} \text{ rifampicin}) \cdot 100$$

**Calculations.** The catalytic activity for CYP3A4 was calculated using standard curves and reported as pmol/min/$10^6$ cells. The fold induction was calculated as (enzyme activity of test drug treated cells)/ (mean of enzyme activity of negative control). Percentage of positive control (%) was calculated as (mean of enzyme activity of test drug treated cells -

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mean of enzyme activity of negative control)/(mean of enzyme activity of rifampicin treated cells - mean of enzyme activity of negative control) x 100 %. CYP3A4 mRNA fold induction was calculated by measuring the change in the treated sample divided by the control. Fold induction was calculated by comparing magnitude of response in treated wells relative to that observed in control (DMSO only) wells.

A measure of compounds' induction efficiency was defined as the efficacy ratio and calculated as follows:

$$\text{Efficacy ratio} = E_{max} / EC_{50}$$

## Results

*Identification of CYP3A4 Time-dependent inhibitors in human liver microsomes*

Table 3 shows 14 compounds identified as time-dependent inhibitors of CYP3A4 using HLM with the mean CYP3A4 $IC_{50}$ values determined as described in Materials and Methods. The CYP3A4 TDI assay correctly classified all the well-established mechanism-based inhibitors of CYP3A4 such as troleandomycin ($IC_{50}$ 0.5 μM), mifepristone (3 μM), ritonavir (4 μM) and verapamil (43 μM), but also highlights compounds that, to our knowledge, have not previously been reported as time-dependent inhibitors of CYP3A4 such nifedipine (projected $IC_{50}$ value of 300 μM).

*Comparison of activity and mRNA endpoints of induction*

As can be seen from Figure 1, the mRNA : activity ratio was between 1-10 in both donors for the majority of compounds. Very high mRNA : activity ratios (>10) observed in these experiments were consistent with the previously confirmed CYP3A4 TDI for compounds such as troleandomycin, ritonavir and verapamil. Interestingly, quinidine also demonstrated a high mRNA : activity ratio despite no evidence of CYP3A4 TDI.

*Comparison of $E_{max}$ values and $EC_{50}$ estimates between HH205 and HH215 using mRNA.*

The parameters $E_{max}$ and $EC_{50}$ were determined from the dose response data from both human hepatocyte donors (Table 4), where $E_{max}$ was the maximum observed induction at an optimum concentration and $EC_{50}$ was the effective concentration that supports half maximal induction. To estimate $EC_{50}$ values, dose response data from all the prototypic CYP3A4 inducers was fitted to a simple $E_{max}$ model with Hill function as described in Materials and Methods. Rifampicin was an inducer of CYP3A4 with a mean $EC_{50}$ of 0.3 μM and an $E_{max}$ of 55-fold using mRNA data from both donors (Table 4). A negative control for CYP3A induction β-naphthoflavone demonstrated, as expected, no inductive response in both donors. Figure 2A shows an excellent correlation between mRNA $EC_{50}$ estimates determined in both human hepatocyte donors. Figure 2B demonstrates a significant correlation between the maximum fold induction between both donors although a clear offset can be observed whereby the HH205 response was on average ~2 fold higher than HH215. This inter-donor variability in induction response can be normalised relative to the response for rifampicin (Figure 2C) and was used throughout the rest of this analysis. Figure 2D shows the correlation of a measure of induction efficiency defined as the efficacy ratio ($E_{max}$ : $EC_{50}$) between both donors.

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*Comparison of $E_{max}$ values and $EC_{50}$ estimates between PXR reporter gene assay and human hepatocytes.*

The parameters $E_{max}$ and $EC_{50}$ were determined from a dose response using eight concentrations in the PXR reporter gene assay. Table 5 summarises the $EC_{50}$ and $E_{max}$ data for the compound test set. Rifampicin was a potent activator of PXR with an $E_{max}$ of 19-fold and $EC_{50}$ of 0.9 µM. β-naphthoflavone, a negative control, showed no evidence of PXR activation. $EC_{50}$ values ranged from the most potent PXR activator, rifabutin ($EC_{50}$ 0.1 µM) to the least, phenobarbital ($EC_{50}$ 100 µM). Several compounds demonstrated similar maximal response to rifampicin including CITCO, omeprazole, rifapentine and ritonavir. The estimates of $E_{max}$ and $EC_{50}$ generated from the PXR reporter gene assay were compared to those generated in human hepatocytes. In contrast to $E_{max}$ where no significant correlation was observed the $EC_{50}$ values from the reporter gene assay appear to be in reasonable agreement to those generated in human hepatocytes (Figure 3).

*Comparison of $E_{max}$ values and $EC_{50}$ estimates between Fa2N-4 cells and human hepatocytes.*

The parameters $E_{max}$ and $EC_{50}$ were determined from a dose response of six concentrations using Fa2N-4 cells. Rifampicin was shown to be an inducer of CYP3A4 in Fa2N-4 cells with an mRNA $EC_{50}$ of 4.2 ± 1.5 µM and an $E_{max}$ of 33 ± 11-fold. β-Napthoflavone, a negative control for CYP3A4 induction, showed no evidence of CYP3A4 induction in Fa2N-4 cells. A bell shaped dose response curve was noted for rifapentine and whilst rifapentine was clearly a potent CYP3A4 inducer in Fa2N-4 cells ($EC_{50}$ of 3 µM and an $E_{max}$ of 27-fold), a decrease in induction was observed at the highest concentration of 20 µM. Bell shaped dose response curves were similarly observed for several other compounds (dexamethasone, paclitaxel, phenobarbital, pioglitazone, reserpine, ritonavir, sulfinpyrazone and verapamil). The estimates of $E_{max}$ and $EC_{50}$ generated from the Fa2N-4 cells appear in reasonable agreement to that generated in human hepatocytes (Figure 4A), however, the several compounds appeared to have ~ 3-fold lower $EC_{50}$ in Fa2N-4 cells than determined in human hepatocytes. A notable exception was rifampicin where the $EC_{50}$ was markedly higher in Fa2N-4 cells compared to human hepatocytes (4 µM vs. 0.3 µM). Figure 4B demonstrates that Fa2N-4 cells demonstrate a weak but significant correlation with the mean $E_{max}$ values from the human hepatocyte donors used in this study. Figure 4C shows a good correlation of the efficacy ratio ($E_{max}$ : $EC_{50}$) between Fa2N-4 cells and human hepatocytes, albeit rifampicin was a notable outlier.

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*Comparison of $E_{max}$ values and $EC_{50}$ estimates between HepaRG cells and human hepatocytes.*

The parameters $E_{max}$ and $EC_{50}$ were determined from a dose response of six concentrations using HepaRG cells. Rifampicin was shown to be an inducer of CYP3A4 in HepaRG cells with a mRNA mean $EC_{50}$ of 0.8 µM and an $E_{max}$ of 83-fold from duplicate experiments. β-Naphthoflavone, a negative control, showed no evidence of CYP3A4 induction. The estimates of $E_{max}$ and $EC_{50}$ generated from the HepaRG cells appeared in excellent agreement to those generated in human hepatocytes (Figure 5A and B), which translated to an excellent concordance between efficacy ratio ($E_{max}$ : $EC_{50}$) determined using HepaRG cells and hepatocytes (Figure 5C). Figure 6 compares CYP3A4 induction using PXR reporter gene assay, Fa2N-4 cell line, HepaRG cell line and primary human hepatocytes in culture following exposure to rifampicin.

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## Discussion

Human hepatocytes are commonly used to evaluate P450 induction via an enzyme activity endpoint, however TDI of P450 can confound data interpretation (McGinnity et al., 2006). In this work, over half of the compounds tested (14/24) were identified as time dependent inhibitors of CYP3A4 as determined by the HLM assay (Table 1), including well characterised drugs such as nifedipine. The clinical consequences of such weak TDI are likely to be insignificant, yet this property may compromise the activity readout in cultured cells. Weak TDI of CYP3A4 may be relatively common and compounds should be assessed for TDI before committing to resource intensive P450 induction assays. Moreover, these data show the utility of mRNA as an endpoint for P450 induction; it affords a larger induction window than activity (Figure 1) and should be a measure of the intrinsic induction potential of a compound in the absence of the confounding factor of TDI.

Due to the finite number of hepatocytes available, estimates of $EC_{50}$ and $E_{max}$ for both human hepatocyte donors were carried out from dose response curves with a limited number of concentrations (3). However it is clear from the excellent correlation of $EC_{50}$ estimates as determined from HH205 and HH215 that such estimates are reasonably consistent. Moreover, comparison between $EC_{50}$ estimates from the two donors in this work and other laboratories shows an overall good concordance. For example, rifampicin ($EC_{50,}$ of 0.1 and 0.4 μM for HH205 and HH215 respectively versus a literature range of 0.2 - 0.8 μM), troglitazone (0.8 μM) (HH215) vs. 0.3 - 5 μM), pioglitazone (9 and 7 μM vs. 2 - 13 μM), phenytoin (18 and 12 μM vs. 5 - 24 μM), carbamazepine (42 μM) (HH205) vs. 14 - 60 μM), dexamethasone (43 and 39 μM vs. 40 - 50 μM) and phenobarbital (142 μM (HH205) vs. 86 - 159 μM) (LeCluyse et al., 2000; Sahi et al., 2000; Faucette et al., 2004; Ramachandran et al., 1999). It should be noted that all $EC_{50}$ values quoted were apparent since no correction for non-specific binding was determined in the media and the proprietary nature of some media contents used in this analysis prevented any estimation of potential differences between all assay systems.

Figure 2B demonstrates a significant correlation between the maximum fold induction between both donors but it was demonstrably poorer versus the inter-donor $EC_{50}$ determinations and a clear offset can be observed, whereby the HH205 response was on average ~2 fold higher than HH215. Inter-donor variability can be reduced for some compounds, to some extent, by normalising against the response to prototypic inducer rifampicin (Figure 2C). Together these data suggest that variability between donors comes predominantly from $E_{max}$ and not $EC_{50}$ estimates which is consistent with previous literature reports (LeCluyse, 2001; Madan et al., 2003; Silva et al., 1998; Meunier et al., 2000). Moreover, significant inter-individual differences may be observed in the basal levels of CYP activity (and mRNA expression) which may lead to significant changes in

the fold induction observed between different donors, as there appears to be a ceiling on the maximum inducible activity (LeCluyse et al., 2000). This phenomenon was also exemplified in the data from HH205 and HH215 (data not shown).

Because human hepatocytes are such a precious and finite resource, limiting data points in a dose response curve can be a pragmatic compromise, but this in turn may obfuscate generating accurate estimates of $E_{max}$ and $EC_{50}$ and so represents a further limitation of using primary cells. Because of this and the variable inter-donor response to inducers, alternatives to primary human hepatocytes are required for use in drug discovery.

As expected, rifampicin was a potent activator of PXR in the reporter gene assay with an $E_{max}$ of 19-fold and $EC_{50}$ 0.9 µM, values similar to that previously reported ($E_{max}$ of 32-fold and $EC_{50}$ 1.2 µM) (Sinz et al., 2006; Luo et al., 2002). Encouragingly, for the compounds tested, $EC_{50}$ values determined via the PXR assay appeared to correlate reasonably with those determined from human hepatocytes (Figure 3A), but it was evident that the $E_{max}$ values do not correlate with $E_{max}$ from the human hepatocytes donors studied in this work (Figure 3B). Indeed, Luo and colleagues (2002) observed a relatively poor correlation between $E_{max}$ values in PXR and human hepatocytes. This may because of the involvement of more than one nuclear hormone receptor in CYP3A4 induction, the presence of other positive and negative feedback mechanisms and the metabolic capability of primary cells. That notwithstanding, the PXR assay is a simple, rapid, robust and enhanced throughput screen, that may offer value in assessing PXR mediated CYP3A4 induction within drug discovery programs, primarily to establish (Q)SAR relationships, assist in the design of compounds with reduced affinity to PXR and select appropriate compounds for further testing in human hepatocytes or an appropriate cell line.

It has previously been established by this laboratory and others that Fa2N-4 cells are a reasonable surrogate for primary human hepatocytes when assessing AhR and PXR mediated CYP1A2 and CYP3A4 induction respectively, but not CAR mediated CYP2B6 induction (Mills et al., 2004; Ripp et al., 2006; Youdim et al., 2007; Hariparsad et al., 2008; Kenny et al., 2008). The CYP3A4 $EC_{50}$ of rifampicin in Fa2N-4 cells was 4 µM, similar to the values reported previously (Ripp et al., 2006; Hariparsad et al., 2008) and approximately 10x greater than that observed in primary human hepatocytes, (0.3 µM). The disparity for rifampicin may therefore be due to specific differences in metabolism or drug transporter activity between the immortalised and primary cells (Hariparsad et al., 2008). However, this work demonstrates that for a range of compounds CYP3A4 $EC_{50}$ values appear to rank reasonably well between Fa2N-4 and human hepatocytes. Indeed the rifampicin data appears anomalous compared to the majority of compounds where $EC_{50}$ values determined from Fa2N-4 cells demonstrate somewhat lower $EC_{50}$ values (up

DMD Fast Forward. Published on March 23, 2009 as DOI: 10.1124/dmd.109.026526
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to 10 fold) compared to those observed from human hepatocytes (Figure 4A). Significant correlations between $E_{max}$ and efficacy ratio between Fa2N-4 cells and human hepatocytes were also observed (Figure 4B and C). Moreover, Ripp et al. (2006) have demonstrated that relative induction scores for compounds, determined from $E_{max}$ and $EC_{50}$ values from Fa2N-4 cells and unbound drug concentrations from clinical studies, were well correlated with *in vivo* decreases in AUC for co-administered CYP3A4 substrates. Overall the Fa2N-4 data suggest this model could be used for routine screening during discovery for PXR-mediated CYP3A4 induction. The absence of functional hCAR may also allow these cells to be used for mechanistic experiments to investigate the PXR-CAR cross-talk observed for many CYP3A4 and 2B6 inducers (Faucette et al., 2006).

The observation of bell shaped curves within the Fa2N-4 data was consistent with previous reports (Hariparsad et al., 2008). Approximately 40% of all compounds demonstrated some decrease in induction at the highest concentrations tested, perhaps suggesting Fa2N-4 cells were more sensitive to chemical insult relative to primary cells where no bell shaped curves were observed. It should be noted that bell shaped curves were also observed in HepaRG (30% frequency) and HepG2 (PXR) (36%) cells. Typically it was the same compounds that cause this phenomenon in more than one cell line.

HepaRG cells are derived from a hepatocellular carcinoma, which exhibits a differentiated hepatocyte-like morphology capable of expressing both phase I and II drug metabolizing enzymes as well as membrane transporters normally found in the liver (Aninat et al., 2006; Guillouzo et al., 2007). In addition to rifampicin induction of CYP3A4, HepaRG cells have also been demonstrated to be responsive to prototypical CYP1A and 2B6 inducers (Aninat et al., 2006; Josse et al., 2008; Kanebratt and Andersson, 2008b). In this work we have shown, for several prototypic CYP3A4 inducers, $E_{max}$ and $EC_{50}$ generated from the HepaRG cells appear to be in excellent agreement with those values generated in primary cells (Figure 5) and therefore HepaRG appear to be a very promising surrogate for determining the CYP3A4 induction potential of test compounds and candidate drugs. It has also been suggested that HepaRG cells express CAR (Guillouzo et al., 2007) but further investigation is required in order to determine if the CAR mediated induction is present in this cell line; if demonstrated, HepaRG cells offer a clear advantage over Fa2N-4 for use in drug discovery.

'Gold standard' P450 induction data can be generated using primary human hepatocytes but an erratic, restricted supply and inter-donor variability issues may restrict the routine application of this assay system within early drug discovery. The PXR reporter gene assay can be considered to be an enhanced throughput assay for screening away from CYP3A4 induction liability, for known PXR-specific activators. Fa2N-4 has the advantage versus the

PXR reporter gene assay of being able to assess CYP1A in addition to 3A4 induction. However, importantly, Fa2N-4 cells have been demonstrated to give no CYP2B6 response via CAR. The HepaRG cell line appears to be an excellent surrogate for CYP3A4 induction compared to primary cells and an valuable recent addition to the armoury of *in vitro* tools for CYP3A4 induction screening in drug discovery and this cell line warrants further investment.

DMD Fast Forward. Published on March 23, 2009 as DOI: 10.1124/dmd.109.026526
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**Acknowledgements**

The authors would like to thank Tommy Andersson, Etienne Lessard, and Denis Projean for several helpful discussions and Joe Goodwin for providing the compound solubility measurements.

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**Footnotes**

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## Legends for Figures

**Figure 1. Relationship between enzyme activity and mRNA $E_{max}$ values from HH205 and HH215.**

The $E_{max}$ values were determined in HH205 (A) and HH215 (B) using CYP3A4 activity and mRNA as the endpoints as outlined in Materials and Methods. Open circles represent CYP3A4 time-dependent inhibitors as determined in HLM and closed circles compounds which were negative in this assay. All compounds with a mRNA : activity ratio of >10 are labelled.

**Figure 2. Relationship between $EC_{50}$, $E_{max}$ and efficacy ratio ($E_{max}$ : $EC_{50}$) values estimated using CYP3A4 mRNA between human hepatocytes HH205 and HH215.**

The $EC_{50}$, $E_{max}$ values from CYP3A4 mRNA were determined in HH205 and HH215 as outlined in Materials and Methods. A. Correlation between mRNA $EC_{50}$ estimates determined in HH205 and HH215. B. Correlation between $E_{max}$ estimates determined in HH205 and HH215. C Correlation between $E_{max}$ estimates, normalised by setting the rifampicin CYP3A4 response as 100% determined in HH205 and HH215. D. Correlation of a measure of induction efficiency defined as the efficacy ratio ($E_{max}$ : $EC_{50}$) between HH205 and HH215. The dotted lines are unity. The solid lines indicate linear regression of the data. The data from this plot are tabulated in Table 4.

**Figure 3. Comparison between $EC_{50}$ and $E_{max}$ values estimated using human hepatocytes and PXR reporter gene assay.**

The $EC_{50}$ (A) and $E_{max}$ (B) values were determined in human hepatocytes from CYP3A4 mRNA and the PXR reporter gene assay as outlined in Materials and Methods. Human hepatocyte data were the mean values determined from HH205 and HH215. The dotted lines are unity. The solid line indicates linear regression of the $EC_{50}$ data ($r^2$ = 0.85, $p < 0.001$). The PXR data from this plot are tabulated in Table 5.

**Figure 4. Comparison between $EC_{50}$ and $E_{max}$ values estimated using human hepatocytes and Fa2N-4 cells.**

The $EC_{50}$ (A) and $E_{max}$ (B) values were determined in human hepatocytes from CYP3A4 mRNA and the Fa2N-4 cells as outlined in Materials and Methods. Human hepatocyte data were the mean values determined from HH205 and HH215. The relationship between efficacy ratio ($E_{max}$ : $EC_{50}$) calculated from human hepatocytes and Fa2N-4 cells is also

shown (C). Dotted lines are unity and solid lines indicate linear regression of the $EC_{50}$ ($r^2$ = 0.65, p< 0.001), $E_{max}$ ($r^2$ = 0.33, p= 0.005) and $E_{max}$ : $EC_{50}$ data ($r^2$ = 0.81, p< 0.001).

**Figure 5. Comparison between $EC_{50}$ and $E_{max}$ values estimated using human hepatocytes and HepaRG cells.**

The $EC_{50}$ (A) and $E_{max}$ (B) values were determined in human hepatocytes from CYP3A4 mRNA and the HepaRG cells as outlined in Materials and Methods. Human hepatocyte data were the mean values determined from HH205 and HH215. The relationship between efficacy ratio ($E_{max}$ : $EC_{50}$) calculated from human hepatocytes and HepaRG cells is also shown (C). Dotted lines are unity and solid lines indicate linear regression of the $EC_{50}$ ($r^2$ = 0.99, p< 0.001), $E_{max}$ ($r^2$ = 0.79, p= 0.005) and $E_{max}$ : $EC_{50}$ data ($r^2$ = 0.97, p< 0.001).

**Figure 6. Comparison of CYP3A4 induction using PXR reporter gene assay, HepaRG cell line, Fa2N-4 cell line and primary human hepatocytes in culture following exposure to rifampicin**

The dose response of CYP3A4 mRNA to rifampicin was determined using human hepatocytes, Fa2N-4 and HepaRG cells and the PXR reporter gene assay was as outlined in Materials and Methods. The maximal response in each assay system was normalised to 100%. Data from human hepatocytes is in closed circles, Fa2N-4 triangles, HepaRG open circles and PXR reporter gene assay in squares.

**Table 1. Human hepatocyte donor information**

| Donor | Age | Sex | Race | Cause of Death | Drug History | Other |
|---|---|---|---|---|---|---|
| HH205 | 47 | F | Cau | HG | 15 years of hypertension (med-compliant) | - |
| HH215 | 39 | M | Asian | CVA | Undiagnosed hypertension | Smoker |

M, Male; F, Female; Cau, Caucasian, HG: Intracranial Hemorrhage, CVA: cardiovascular accident.

**Table 2. Compounds and concentrations tested in human hepatocytes**

A range of three concentrations was selected for each compound based on literature CYP3A4 induction data for human hepatocytes. The number of concentrations was limited by hepatocyte availability. Compounds were soluble in media at the top concentrations as determined using the BD-Gentest solubility scanner, which detects compound precipitates via light scatter in the relevant culture media (Coan and Shoichet, 2008).

| Test Compounds | Concentrations (μM) | | |
|---|---|---|---|
| Carbamazepine | 250 | 25 | 2.5 |
| CITCO | 10 | 1 | 0.1 |
| Clotrimazole | 3 | 0.3 | 0.03 |
| Dexamethasone | 250 | 25 | 2.5 |
| Efavirenz | 10 | 1 | 0.1 |
| Hyperforin | 2 | 0.2 | 0.02 |
| Lansoprazole | 40 | 4 | 0.4 |
| Mifepristone | 0.1 | 0.01 | 0.001 |
| Nifedipine | 50 | 5 | 0.5 |
| Omeprazole | 25 | 2.5 | 0.25 |
| Paclitaxel | 50 | 5 | 0.5 |
| Phenobarbital | 2000 | 200 | 20 |
| Phenytoin | 250 | 25 | 2.5 |
| Pioglitazone | 50 | 5 | 0.5 |
| Quinidine | 250 | 25 | 2.5 |
| Reserpine | 10 | 1 | 0.1 |
| Rifabutin | 10 | 1 | 0.1 |
| Rifampicin | 10 | 1 | 0.1 |
| Rifapentine | 10 | 1 | 0.1 |
| Ritonavir | 1 | 0.1 | 0.01 |
| ß-naphthoflavone | 25 | 2.5 | 0.25 |
| Sulfinpyrazone | 150 | 15 | 1.5 |
| Troglitazone | 5 | 0.5 | 0.05 |
| Troleandomycin | 20 | 2.0 | 0.2 |
| Verapamil | 40 | 4 | 0.4 |

**Table 3. Compounds identified as time-dependent inhibitors of CYP3A4 in human liver microsomes**

Results are expressed as the mean $IC_{50}$ of duplicate determinations. The $IC_{50}$ measurements were carried out as described in Materials and Methods. All other compounds from Table 2, with the exception of lansoprazole, which was not tested, demonstrated no CYP3A4 TDI.

| Compound | % Inhibition | $IC_{50}$ (μM) |
|---|---|---|
| Troleandomycin | 68 | 0.5 |
| Mifepristone | 70 | 3 |
| Ritonavir | 53 | 4 |
| Hyperforin | 22 | 10 |
| Troglitazone | 50 | 20 |
| Resveratrol | 54 | 40 |
| Verapamil | 65 | 43 |
| Rifabutin | 25 | 84 |
| CITCO | 13 | 85 |
| Reserpine | 29 | 170 |
| Pioglitazone | 34 | 250 |
| Omeprazole | 20 | 300 |
| Nifedipine | 31 | 300 |
| Sulfinpyrazone | 7 | >2000 |

**Table 4. $EC_{50}$ and $E_{max}$ data for CYP3A4 induction in human hepatocytes HH205 and HH215.**

| | Activity | | | | | | mRNA | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HH205 | | | HH215 | | | HH205 | | | HH215 | | |
| Compound | $EC_{50}$ (μM) | $E_{max}$ (fold) | $E_{max}$ (% rif) | $EC_{50}$ (μM) | $E_{max}$ (fold) | $E_{max}$ (% rif) | $EC_{50}$ (μM) | $E_{max}$ (fold) | $E_{max}$ (% rif) | $EC_{50}$ (μM) | $E_{max}$ (fold) | $E_{max}$ (% rif) |
| Carbamazepine | 40 | 7.7 | 39 | | 6.3 | 63 | 42 | 23 | 30 | | 31 | 94 |
| CITCO | | 1.7 | 9 | | 3.1 | 31 | | 5.4 | 7 | | 7.8 | 24 |
| Clotrimazole | | 1.9 | 10 | | 1.7 | 17 | | 13 | 17 | | 4.1 | 12 |
| Dexamethasone | 43 | 8.6 | 43 | 12 | 3.4 | 34 | 43 | 71 | 93 | 39 | 13 | 39 |
| Efavirenz | | 2.6 | 13 | | 6.6 | 66 | | 5.1 | 7 | | 22 | 67 |
| Hyperforin | 0.3 | 3.2 | 16 | 0.1 | 11 | 110 | 0.3 | 12 | 16 | 0.3 | 13.5 | 41 |
| Lansoprazole | 6.8 | 7.3 | 37 | 3.1 | 6.8 | 68 | 6.9 | 42 | 55 | 3.3 | 8.6 | 26 |
| Mifepristone | | 1.1 | 6 | | 1 | 10 | | 2 | 3 | | 1.5 | 5 |
| Nifedipine | | 1.8 | 9 | | 5.1 | 51 | | 20 | 26 | | 9.6 | 29 |
| Omeprazole | | 1.3 | 7 | | 2.8 | 28 | | 21 | 28 | | 7.1 | 22 |
| Paclitaxel | | 4 | 20 | 0.3 | 4.5 | 45 | | 46 | 61 | 2.6 | 12 | 36 |
| Phenobarbital | 23 | 14 | 70 | | 13 | 130 | 142 | 17 | 22 | | 11 | 33 |
| Phenytoin | 19 | 16 | 80 | 13 | 11 | 110 | 18 | 15 | 20 | 12 | 12 | 36 |
| Pioglitazone | 8.5 | 10 | 50 | 5 | 12 | 120 | 9.2 | 18 | 24 | 7.2 | 18 | 55 |
| Quinidine | | 0.8 | 4 | | 0.5 | 5 | | 9.8 | 13 | | 7.2 | 22 |
| Reserpine | 0.6 | 1.4 | 7 | | 3.8 | 38 | 1.9 | 78 | 103 | | 24 | 73 |
| Rifabutin | 0.3 | 3.4 | 17 | | 6.1 | 61 | 1.6 | 57 | 75 | | 30 | 91 |
| Rifampicin | 0.6 | 20 | 100 | 0.6 | 10 | 100 | 0.1 | 76 | 100 | 0.4 | 33 | 100 |
| Rifapentine | 0.9 | 16 | 80 | 0.7 | 10 | 100 | 1.4 | 109 | 143 | 1.2 | 22 | 67 |
| Ritonavir | | 0.3 | 2 | | 0.5 | 5 | | 25 | 33 | | 15 | 45 |
| ß-Naphthoflavone | | 0.9 | 5 | | 0.6 | 6 | | 1.7 | 2 | | 0.5 | 2 |
| Sulfinpyrazone | 17 | 7.3 | 37 | 1.7 | 6.8 | 68 | 23 | 103 | 136 | 7.2 | 25 | 76 |
| Troglitazone | | 2.4 | 12 | 0.2 | 3.2 | 32 | | 4 | 5 | 0.8 | 8.7 | 26 |
| Troleandomycin | | 2.1 | 11 | | 1.2 | 12 | | 27 | 36 | | 15 | 45 |
| Verapamil | | 0.6 | 3 | | 0.4 | 4 | | 49 | 64 | | 29 | 88 |

Fold– fold induction; % rif - % CYP3A4 induction of positive control rifampicin

**Table 5 $EC_{50}$ and $E_{max}$ data for CYP3A4 induction in PXR reporter gene assay**

The parameters $E_{max}$ and $EC_{50}$ were determined from a dose response using eight concentrations. Data represents the mean values calculated from triplicate experiments.

| | $EC_{50}$ (μM) | $E_{max}$ (fold induction) | $E_{max}$ (% rifampicin) |
|---|---|---|---|
| Carbamazepine | 51 | 8 | 42 |
| ClTCO | 3 | 18 | 95 |
| Clotrimazole | 1.2 | 11 | 58 |
| Dexamethasone | 100 | 12 | 63 |
| Efavirenz | 3 | 9 | 47 |
| Hyperforin | 0.2 | 12 | 63 |
| Lansoprazole | 4 | 15 | 79 |
| Mifepristone | 1.2 | 9 | 47 |
| Nifedipine | 2 | 14 | 74 |
| Omeprazole | 12 | 23 | 121 |
| Paclitaxel | 16 | 5 | 26 |
| Phenobarbital | 100 | 9 | 47 |
| Phenytoin | 0.3 | 3 | 16 |
| Pioglitazone | na | 2 | 11 |
| Quinidine | nd | nd | nd |
| Reserpine | 1.2 | 11 | 58 |
| Rifabutin | 0.1 | 6 | 32 |
| Rifampicin | 0.9 | 19 | 100 |
| Rifapentine | 2 | 16 | 84 |
| Ritonavir | 1.4 | 18 | 95 |
| β-Naphthoflavone | na | 2 | 11 |
| Sulfinpyrazone | 12 | 12 | 63 |
| Troglitazone | na | 3 | 16 |
| Troleandomycin | 5 | 8 | 42 |
| Verapamil | nd | nd | nd |

na - not appropriate to define, nd - not determined

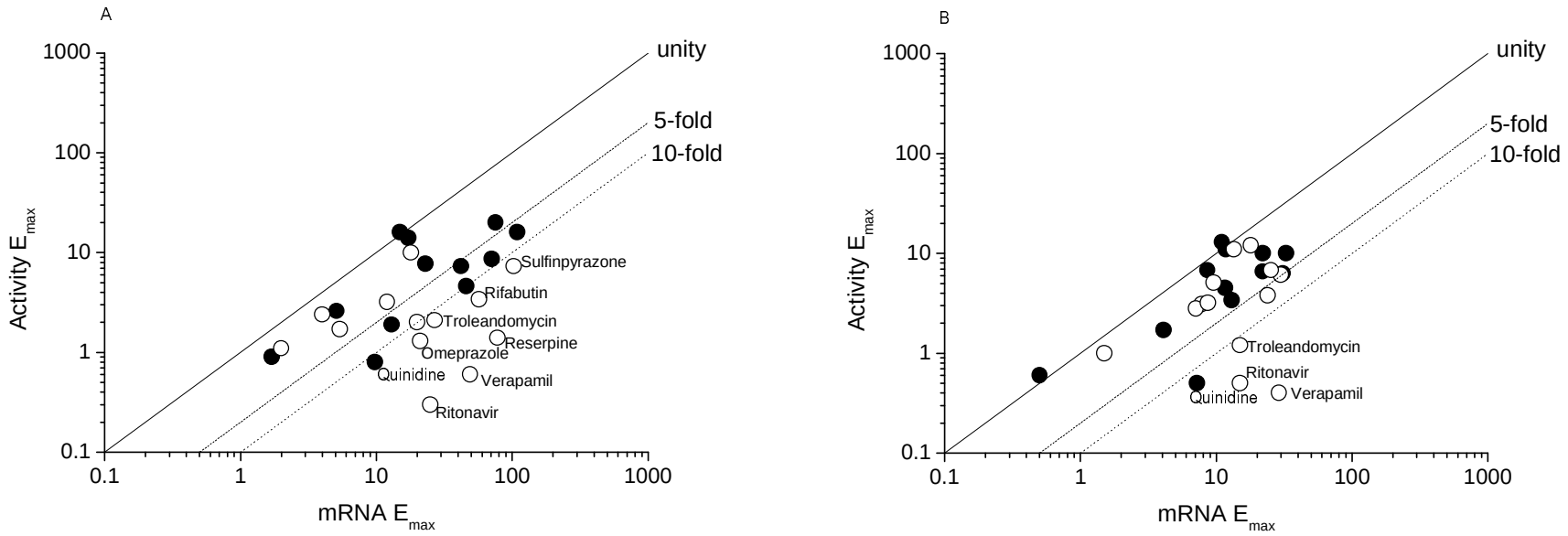


Figure 1

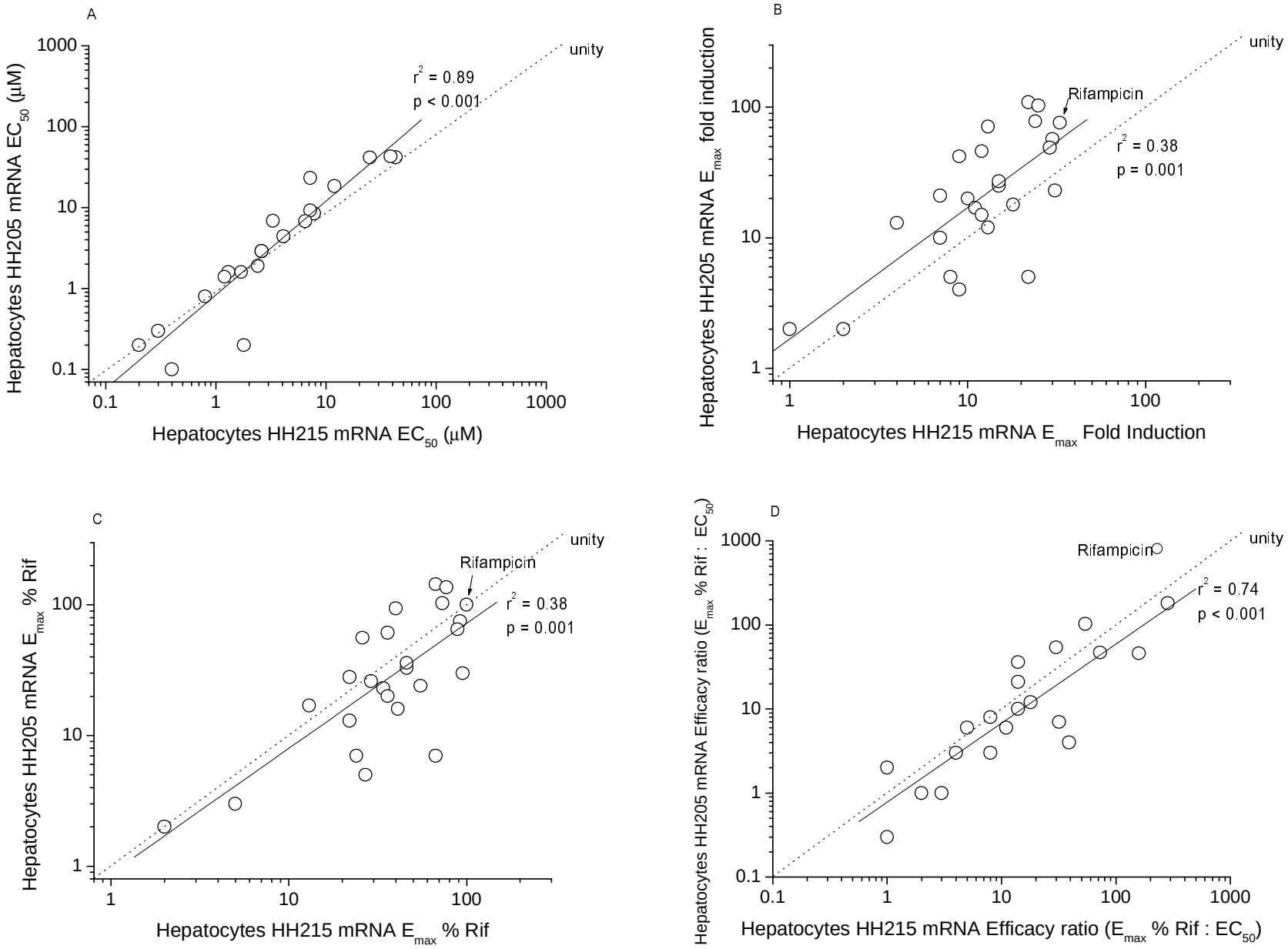


Figure 2

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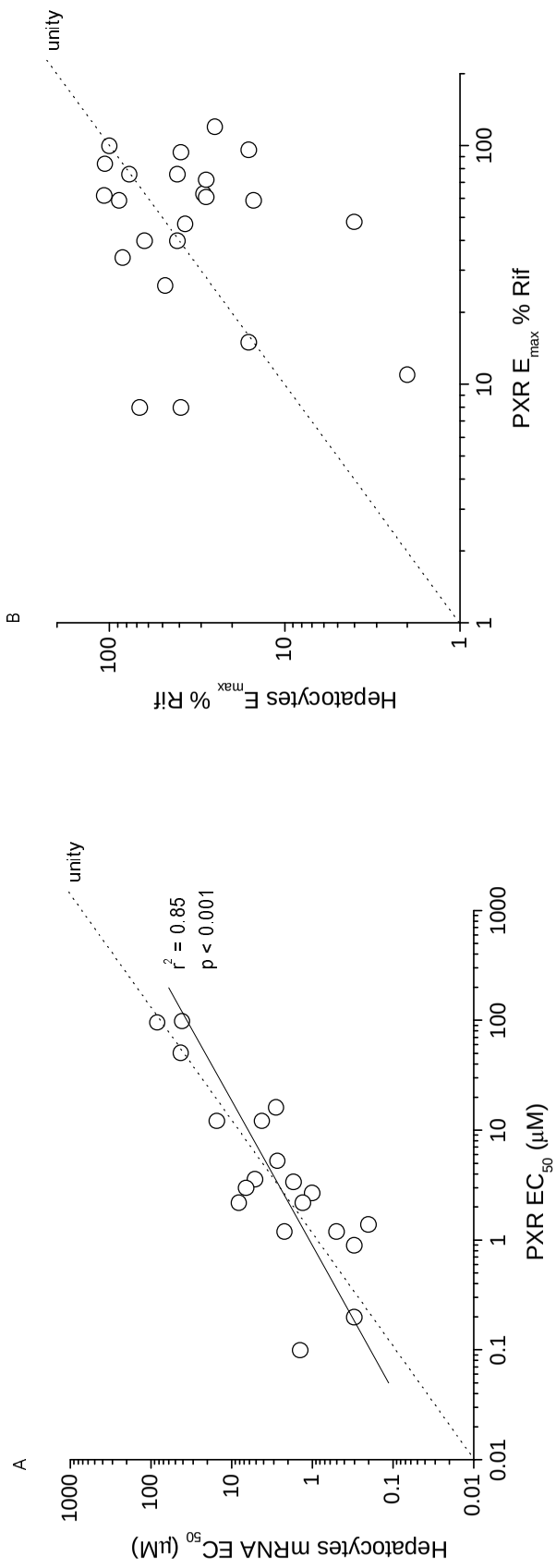


Figure 3

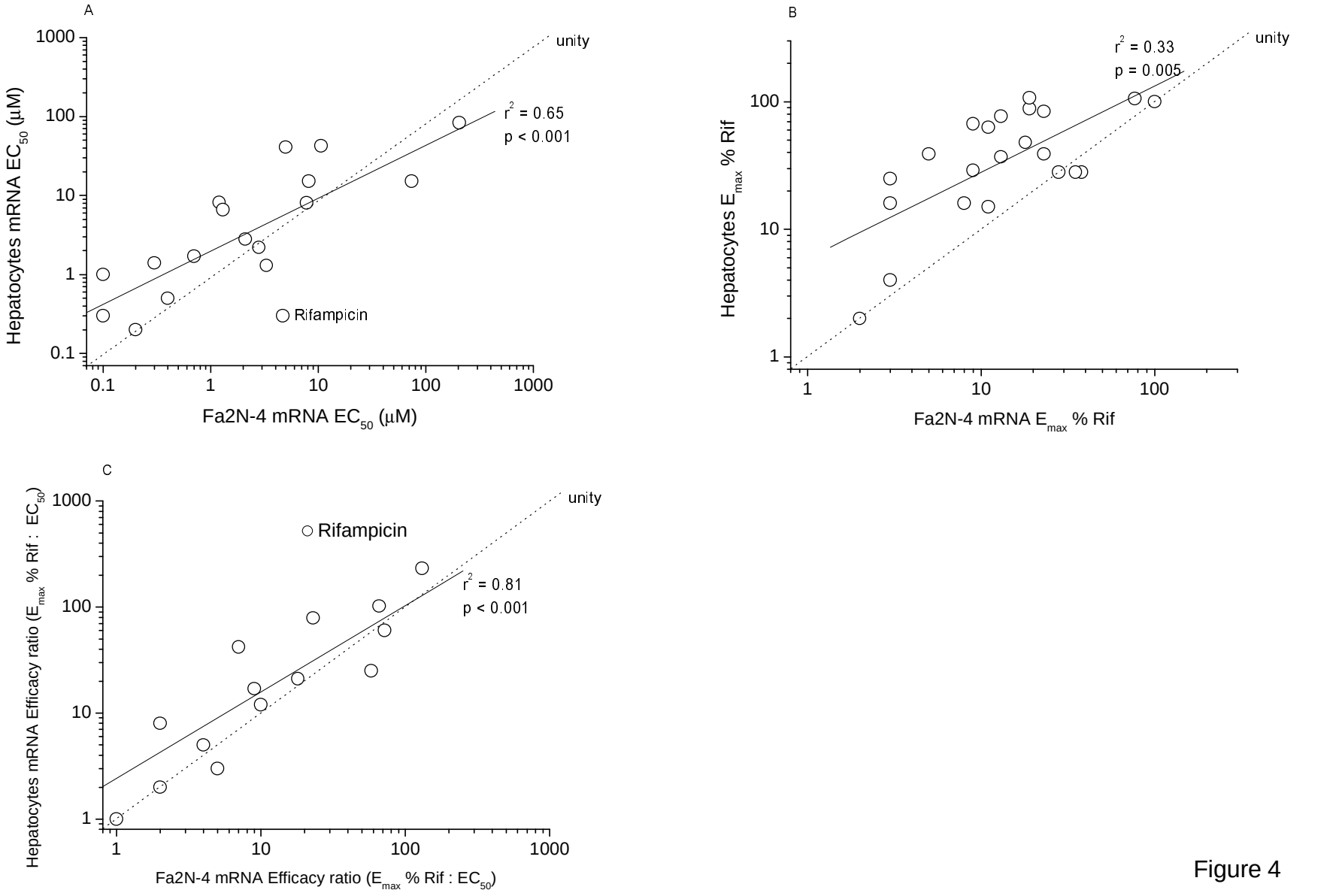


Figure 4

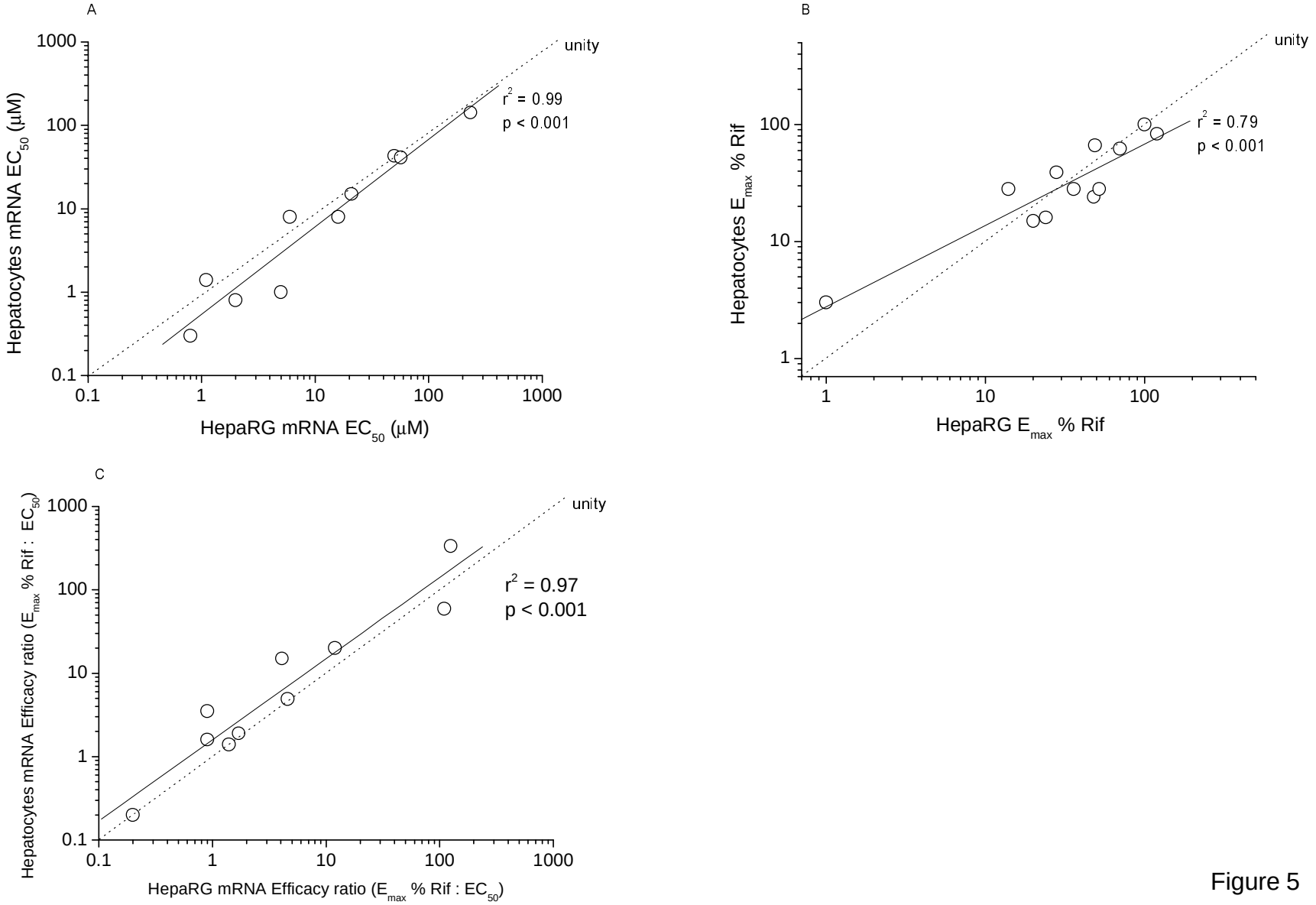


Figure 5

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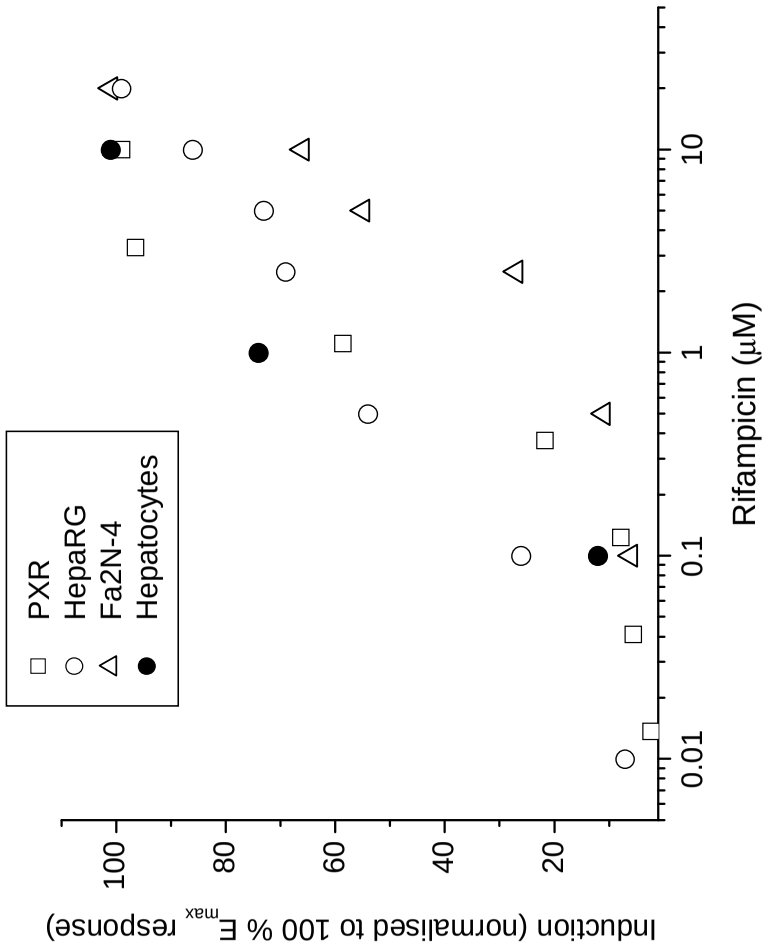


Figure 6