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HepaRG Cells as an *In Vitro* Model for Evaluation of Cytochrome P450 Induction in Humans

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Abbreviations: CYP, cytochrome P450; PXR, pregnane X receptor; CAR, constitutive androstane receptor; AhR, aryl hydrocarbon receptor; DMSO, dimethyl sulfoxide; FBS, foetal bovine serum; LDH, lactate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; huPO, human acidic ribosomal phosphoprotein; LC/MS, liquid chromatography/mass spectrometry; AUC, area under the plasma concentration versus time curve.

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Abstract

HepaRG is a highly differentiated cell line, which displays several hepatocyte-like functions including drug metabolising enzymes. In this study the HepaRG cells were characterised and evaluated as an *in vitro* model to predict cytochrome P450 (CYP) enzyme induction of drugs in humans. Exposure of HepaRG cells to prototypical inducers resulted in induction of CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4 mRNA as well as phenacetin O-dealkylase, bupropion hydroxylase, diclofenac 4'-hydroxylase, and midazolam 1'-hydroxylase activities. The observed induction is consistent with the previously reported expression of the nuclear receptors PXR, CAR and AhR, which are necessary for a CYP induction response. To avoid problems with toxicity and solubility, the induction potency of test compounds was evaluated by calculating the concentrations leading to a 2-fold increase of baseline mRNA or enzyme activity levels (F_2 values), instead of EC_{50} values from full dose-response curves. For CYP3A4 mRNA, the obtained F_2 values were related to the *in vivo* exposure (AUC) of the inducer (AUC/F_2). This score was then correlated with the decrease in AUC for a CYP3A probe drug, administered before and after treatment with the inducing agent. By using this method an excellent correlation (R^2=0.863) was obtained, which implies that the degree of CYP3A induction *in vivo* can be predicted from CYP3A4 mRNA induction in HepaRG cells. The present study shows that the HepaRG cells is a valuable model to be used for prediction of induction of drug metabolising CYP enzymes *in vivo* in humans.

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The cytochrome P450 (CYP) enzymes have been shown to be responsible for the metabolism of the majority of therapeutics and are therefore important for first pass elimination (bioavailability), clearance, and drug-drug interactions. Drug-drug interactions may be the result of both inhibition and induction of CYP enzymes. CYP inhibition studies using human liver microsomes or recombinant enzymes and model substrates for specific CYPs are well established *in vitro* methods for quantitative predictions of inhibition potency *in vivo* (Ito et al., 1998; Bjornsson et al., 2003). On the contrary, quantitative predictions of induction *in vivo* from *in vitro* results are less well established. Since induction is subject to important species differences, prediction of possible induction in man cannot rely on animal studies. Human hepatocytes have been suggested by several reports as a preferred model for induction studies (Li et al., 1997; Madan et al., 2003). Nevertheless, primary human hepatocytes are flawed with several drawbacks such as experimentally demanding methods, phenotypic changes during culture, scarce availability, interindividual variation and the need to evaluate multiple donors, which makes the model arduous. The demanding technique is probably the reason why no quantitative predictions of CYP induction *in vivo* using human hepatocytes have been published.

Induction of CYPs generally occurs at the transcriptional level and is mediated by receptors such as the pregnane X receptor (PXR), constitutive androstane receptor (CAR), 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 (Lehmann et al., 1998; Gibson et al., 2002), and has also been established to be important for CYP2C9 induction (Chen et al., 2004), while CAR have been proposed to regulate the expression of CYP2B6 (Sueyoshi et al., 1999; Wang et al., 2004). However, during recent years there has been increasing evidence of an extensive cross-talk between nuclear receptors, indicating that CAR and PXR are affecting the regulation of all three CYP enzymes (CYP2B6, CYP2C9, and CYP3A4) (Lin, 2006). The CYP1A 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 (Denison and Nagy, 2003).

Reporter gene assays for PXR and AhR have been developed and used as convenient high-throughput tools for the determination of a compound's ability to induce CYP3A and CYP1A. Results from PXR reporter gene assays were recently successfully used to classify clinically used drugs as CYP3A inducers or non-inducers (Persson et al., 2006; Sinz et al., 2006). However, since induction of CYP enzymes by drug molecules does not only depend on a single transcription factor but is mediated by a combination of several transcription factors in the cell, a human hepatocyte-like cell line would be a superior *in vitro* model for evaluation of CYP induction. A promising cell line is the HepaRG cells, which displays several hepatocyte-like functions including drug metabolising enzymes, nuclear receptors and hepatic drug transporters and can be differentiated into a hepatocyte-like morphology (Aninat et al., 2006; Le Vee et al., 2006). The HepaRG cells have also been shown to respond to prototypical CYP inducers such as 3-methylcholantrene, rifampicin, and isoniazid (Aninat et al., 2006).

The aim of the present study was to further characterise the CYP induction response in HepaRG cells and to develop a method that can be used to predict the extent of CYP induction *in vivo*.

Materials and methods

Chemicals. Carbamazepine, dexamethasone, diclofenac, dimethyl sulfoxide (DMSO), nifedipine, phenobarbital, rifampicin, sulfinpyrazone, and Williams' medium E without phenol red were purchased from Sigma Chemical Co. (St Louis, MO, USA). Paracetamol, phenacetin, and primaquine were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Hyperforin was purchased from Apin Chemicals Ltd (Oxon, UK). Williams' medium E with phenol red and foetal bovine serum (FBS) were obtained from Gibco BRL, Life Technologies (Täby, Sweden). Omeprazole and troglitazone were provided by AstraZeneca R&D Mölndal. 4'-hydroxydiclofenac was obtained from Gentest (Woburn, MA, USA) and 1'-hydroxymidazolam was purchased from Ultrafine (Manchester, UK). SuperScript III First-Strand Synthesis System for RT-PCR and Trizol reagent were acquired from Invitrogen (Stockholm, Sweden). Midazolam was purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Bupropion was purchased from Kemprotec Ltd. (Middlesbrough, UK) and hydroxybupropion was provided by Toronto Research Chemicals Inc. (Ontario, Canada). LDH opt, ref. LD401, was purchased from Randox Laboratories Ltd. (Crumlin, UK) and Triton X was purchased from Fisher Scientific GTF (Västra Frölunda, Sweden). The primers and probe used in this study were provided by Applied Biosystems (Cheshire, UK). Taqman Assay on Demand and Taqman® Universal Master Mix was purchased from Applied Biosystems (Stockholm, Sweden). All other chemicals were of analytical grade and highest quality available.

Cell Culturing and Induction. The differentiated HepaRG cells (passages 14 to 17) were purchased from Biopredic International, Rennes, France. 24-well plates, seeded with 0.05 million cells, were used for all experiments. The cells were initially grown in Williams' medium E with glutamax-I, supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml bovine insulin, and 50 µM hydrocortisone hemisuccinate. At confluence 2% DMSO was added to the medium in order to differentiate the cells into hepatocyte-like morphology. The cells were cultured in

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differentiation medium for 3 weeks before shipment to AstraZeneca. At arrival the medium was renewed and the cells were given 24 hours to recover before the medium was changed to basal HepaRG medium (Williams' medium E with glutamax-I, supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 µg/ml bovine insulin, and 50 µM hydrocortisone hemisuccinate). The cells were cultured in the basal medium for 5 days before the induction started, and the medium was renewed every 24 to 48 hours.

The cells were induced with rifampicin (0.004 to 4.0 µM), omeprazole (0.04 to 40 µM), troglitazone (0.024 to 25 µM), primaquine (0.04 to 40 µM), phenytoin (0.04 to 40 µM), phenobarbital (0.20 to 200 µM), carbamazepine (0.24 to 250 µM), dexamethasone (0.24 to 250 µM), nifedipine (0.24 to 250 µM), sulfinpyrazone (0.24 to 250 µM), and hyperforin (0.24 to 250 µM). Test compounds were dissolved in DMSO and added to the plates in basal HepaRG medium with a final DMSO concentration of 0.1% in all incubations. Control incubations contained basal HepaRG medium and 0.1% DMSO. For mRNA measurements, the cells were harvested in Trizol reagent (0.5 ml per well) after 24 hours exposure to the study compounds, experiments were repeated at three different occasions on separate batches of differentiated HepaRG cells. CYP activities were measured directly in the plates after 48 hours exposure to the study compounds, experiments were performed in duplicates and repeated at two different occasions on separate batches of differentiated HepaRG cells.

Viability. The viability of the cells in the dose-response experiments was tested by measuring the lactate dehydrogenase (LDH) activity before and after 24 hours exposure to the study compounds. LDH was measured by using a commercial reagent kit (LDH opt) and was performed with a Cobas Bio Centrifugalanalyser (Hoffman-La Roche, Basel, Switzerland).

RNA Extraction. Total RNA from HepaRG cells was prepared using Trizol reagent according to manufacturers' instructions. Quantity and purity of the RNA were determined spectrophotometrically using a GenQuant pro RNA/DNA calculator

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(Biochrom, Cambridge, UK). Electrophoretic separation of 0.5 µg total RNA on a 1% agarose gel run in TBE buffer (0.09 M Tris-borate, 2 mM EDTA, pH 7.8) at 80 mV for 1 hour, allowed integrity assessment of the isolated RNA. Two sharp ribosomal RNA bands and absence of RNA-debris was set as a quality criterion to proceed to cDNA synthesis.

cDNA was prepared from 1 µg of total RNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR with random hexamer primers according to the manufacturer's protocol.

Real-Time PCR. Real-time PCR for human CYP mRNA levels was performed employing a 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA) and manufacturer designed Assay on Demand for CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For human acidic ribosomal phosphoprotein (huPO) gene-specific double fluorescent labelled probes were used, reported previously (Persson et al., 2006). The reaction mixture (25 µl per well) contained 30 ng cDNA, 1xTaqman Universal Master Mix, optimised concentrations of primers and probes (huPO), or 1.25 µl Assay on Demand and RNase free water. The thermal cycle conditions were identical for all genes analysed and 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, 60 °C for 1 min. Each sample was analysed in triplicate and data was analysed using the 7500 Sequence detector software v1.3.1 (Applied Biosystems). GAPDH and huPO were used as endogenous controls and the amount of mRNA was determined relative to that from control samples.

Activity Measurements. For cells used for activity measurements induction medium was renewed after 24 hours. After 48 hours, induction medium was removed and the cells were washed twice with Williams' medium E without phenol red, thereafter 500 µl activity medium was added to each well. The activity medium consisted of Williams's medium E without phenol red (pH 7.4) and bupropion (100 µM) or a cocktail of phenacetin (26 µM), diclofenac (9 µM), and midazolam (3 µM). The compounds were

dissolved in methanol and added to a Falcon tube. The methanol was evaporated under nitrogen gas and the compounds dissolved in Williams' medium E without phenol red so that the activity medium did not include any organic solvent. After 15, 30, 45, and 60 min (for bupropion) or 0.5, 1, 2, and 3 hours (for the cocktail) 100 µl sample was taken out. 15 µl cold acetonitrile with 0.8% formic acid was added and the samples were analysed for the CYP2B6 metabolite hydroxybupropion or the CYP1A2 metabolite paracetamol, the CYP2C9 metabolite 4'-hydroxydiclofenac, and the CYP3A4 metabolite 1'-hydroxymidazolam.

After the last sample was taken, the final medium was removed and the cells were lysed with 200 µl 1% Triton X. The wells were scraped and the lysate was used for protein determination by a modified Lowry procedure (Markwell et al., 1978).

The samples were analysed at separate occasions by means of liquid chromatography/mass spectrometry (LC/MS). The LC system consisted of an HP 1100 series LC pump and column oven (Agilent Technologies Deutschland, Waldbronn, Germany) combined with an HTS PAL injector (CTC Analytics, Zwingen, Switzerland). For hydroxybupropion, 4'-hydroxydiclofenac and 1'-hydroxymidazolam LC separations were performed on a reversed-phase HyPurity C18 column (2.1x50 mm, 5 µm, ThermoQuest, Runcorn, UK) with a HyPurity C18 precolumn at 40 °C and with a flow rate at 750 µl/min. The mobile phase consisted of (A) 0.1% (v/v) formic acid and (B) 0.1% (v/v) formic acid in acetonitrile. The organic modifier content B was increased linearly from 5 to 80% B over 3 min, then back to 5% B in 0.2 min.

For paracetamol chromatography was performed on a Zorbax Eclipse XDB-C8 column (4.6x150 mm, 5 µm, Agilent Technologies, Täby, Sweden) with a HyPurity C18 precolumn, employing the same system and mobile phase. The organic modifier content B was increased linearly from 2 to 30% B over 5 min, then from 30 to 80% over 2 min, and then back to 2% B in 0.1 min. The retention times of hydroxybupropion, 4'-hydroxydiclofenac and 1'-hydroxymidazolam, and paracetamol were 2.0, 2.9, 2.4 and 6.4 min, respectively. Detection was performed with a triple quadrupole mass

spectrometer, API4000, equipped with electrospray interface (Applied Biosystems/MDS Sciex, Concord, Canada). The MS parameters were optimised using each analyte. The compound dependent parameters were as follows: the collision energy was set at 20, -15, 39, and 21 V for hydroxybupropion, 4'-hydroxydiclofenac, 1'-hydroxymidazolam, and paracetamol, respectively. Collision-activated dissociation gas at 5, 5, 7, and 5, respectively. The MRM transitions chosen were 256.1>237.8 for hydroxybupropion, 309.9>265.9 for 4'-hydroxydiclofenac, 342.0>202.7 for 1'-hydroxymidazolam, and 152.3>110.0 for paracetamol. A dwell time of 200 ms was used. Instrument control, data acquisition and data evaluation were performed using Applied Biosystems/MDS Sciex Analyst 1.4 software.

Metabolite formation was calculated in the linear range of the time curve and for hydroxybupropion the formation rate at 30 min was used. For paracetamol, 4'-hydroxydiclofenac, and 1'-hydroxymidazolam the formation rate at 2 hours, 1 hour, and 1 hour were used, respectively.

Curve-Fitting and Calculation of Induction Response. All curve fitting were carried out with XLfit 4.1.1. Dose-response data were fitted to a Hill equation for one site dose-response as follows:

$$I = E_{\min} + \frac{\left(E_{\max} - E_{\min}\right)}{1 + \left(\frac{EC_{50}}{C}\right)^{k}}$$

where E_{min} is the background, E_{max} the maximum effect, EC_{50} is the concentration giving 50% of E_{max}, k is the slope of the curve, and C is the drug concentration. A fit to the data points is created even if a plateau in the effect is not reached, but E_{max} values is only reported if a plateau is obtained. From this fit also the F_2 values were calculated. The F_2 value is the concentration leading to a 2-fold increase of the baseline levels. This approach was used by Weiss and Haefeli and found useful for evaluation of inhibition of P-glycoprotein (Weiss and Haefeli, 2006). The F_2 values were related to the *in vivo* exposure of the test drug, represented by the area under the plasma concentration

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versus time curve (AUC). This score, AUC/ F_2, was then ranked from lowest to highest value.

In vivo induction is measured by administration of a drug metabolised by the enzyme of interest, called a probe drug. Examples of probe drugs are caffeine for CYP1A2, tolbutamide for CYP2B6, and midazolam for CYP3A4. The probe drug is administered before and after administration of the potential inducer, and the induction is measured as the decrease in AUC of the probe drug.

The correlation between *in vitro* and *in vivo* induction in the present study was assessed by plotting AUC/F_2 versus the percentage decrease in AUC of a co-administered CYP3A probe drug. The data were fitted to an equation as follows:

$$y = \frac{\%\ decrease\ of\ \ in\ vivo\ AUC_{\max} * \left(AUC / F_2\right)}{\%\ decrease\ of\ \ in\ vivo\ AUC_{50} + \left(AUC / F_2\right)}$$

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Results

Viability. The viability of the cells was analysed by measuring LDH activity in the medium before and after 24 hours exposure. No effect on the cells, as evidenced by no increased LDH activity in the medium, was detected after incubations with carbamazepine, dexamethasone, omeprazole, phenobarbital, primaquine, rifampicin, and troglitazone. Hyperforin, nifedipine, phenytoin, and sulfinpyrazone increased LDH activity at all tested concentrations, however not in a dose related fashion and dose-response curves were still achieved. Hyperforin, nifedipine, and troglitazone displayed the highest induction at 62.5, 0.4, and 6.25 µM, respectively, while the induction response was weaker at the highest concentration (250, 2, and 25 µM, respectively). The weaker induction response by troglitazone at the highest concentration was not reflected by LDH leakage, suggesting that the induction response by troglitazone in this study was not affected by acute cell toxicity.

Induction of CYP mRNA. In initial experiments HepaRG cells were treated with omeprazole, TCDD (2,3,7,8-tetrachloro-dibenzo-p-dioxin), phenobarbital, and rifampicin. Results indicated that the induction of CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP3A4 mRNA peaked after 24 hours. No induction was seen for CYP2D6 mRNA, and the mRNA for CYP2D6 was not analysed in subsequent experiments. Therefore, the HepaRG cells were exposed to the study compounds rifampicin, omeprazole, troglitazone, primaquine, phenytoin, and phenobarbital for 24 hours for mRNA experiments. Representative dose-response curves for CYP1A1 and CYP1A2 mRNA induction by omeprazole and for CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4 mRNA induction by rifampicin are shown in Fig. 1. In Table 1 and 2 the F_2 values and the fold induction at the highest response measured for CYP1A1, CYP1A2, and CYP2B6 mRNA by the test compounds are presented. When a full dose-response curve was obtained and no sign of toxicity or solubility problems were observed, also the EC_{50} value was calculated. CYP1A1 and CYP1A2 mRNA was

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induced by omeprazole and primaquine whereas no effect were seen by the other tested compounds (Table 1). CYP2B6 mRNA was strongly induced by rifampicin, omeprazole, troglitazone, phenytoin, and phenobarbital, while primaquine did not affect CYP2B6 transcription (Table 2). Induction of CYP2C8, CYP2C9 and CYP2C19 mRNA was seen after exposure to rifampicin (Table 3) while omeprazole, troglitazone, phenytoin and primaquine did not significantly affect the CYP2C mRNA levels. Phenobarbital gave a weak induction of CYP2C mRNA, but the average fold induction at the highest concentration was low (2.6, 1.7, and 2.5-fold for CYP2C8, CYP2C9, and CYP2C19, respectively) and no average F_2 values could be calculated. A larger set of compounds was tested for CYP3A4 mRNA induction. Rifampicin, omeprazole, troglitazone, phenytoin, phenobarbital, carbamazepine, dexamethasone, hyperforin, nifedipine, and sulfinpyrazone all induced CYP3A4 mRNA, while primaquine did not affect CYP3A4 mRNA levels (Table 4).

Induction of CYP Dependent Activities. The induction of CYP specific enzyme activities was more pronounced after 48 than after 24 hours exposure to the test compounds, therefore 48 hours incubation was used for enzyme activity experiments. Induction of phenacetin O-dealkylase activity by omeprazole and bupropion hydroxylase, diclofenac 4'-hydroxylase, and midazolam 1'-hydoxylase activities by rifampicin gave clear dose-response curves over the concentration ranges tested (Fig. 2). In Table 5 the F_2 values, the fold induction at the highest response measured and, when obtained, EC_{50} values for phenacetin O-dealkylase, bupropion hydroxylase, and midazolam 1'-hydoxylase activities after treatment with rifampicin, omeprazole, troglitazone, phenytoin, primaquine and phenobarbital are presented.

Induction of the CYP1A2 dependent phenacetin O-dealkylase activity was only detected by omeprazole. Bupropion hydroxylase activity, which is catalysed by CYP2B6, was induced by rifampicin, phenytoin, and phenobarbital. Omeprazole and troglitazone did not result in measurable effects on bupropion hydroxylase activity although they induced CYP2B6 mRNA. CYP2C9 catalysed diclofenac 4'-hydroxylase activity was induced by

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rifampicin, the average fold induction at the highest concentration was 1.7-fold. CYP3A4 dependent midazolam 1'-hydroxylase activity was strongly induced by rifampicin and phenobarbital, and weakly by troglitazone.

***In Vitro-In Vivo* Correlation.** To investigate the relationship between CYP induction in HepaRG cells and induction *in vivo*, the same approach as for the previously reported PXR assay was applied (Persson et al., 2006), where the *in vivo* exposure of the test compound was related to the EC_{50} from the PXR reporter gene assay (AUC/EC_{50}). This method correctly identified compounds known to induce CYP3A *in vivo*. We recalculated the previously published PXR results to F_2 values, and found the same ranking and correct classification of the tested compounds when using F_2 values instead of the EC_{50} values, which indicates that the F_2 values could be a useful indicator for assessing induction potential.

In the present study, the *in vivo* AUC for the investigated drugs was related to F_2 values (AUC/F_2) obtained for the induction of CYP3A4 mRNA in the HepaRG cells (AUC, F_2, and AUC/F_2 values are listed in Table 4). By using the results from HepaRG cells, the same ranking of compounds was obtained as with the AUC/F_2 values from the PXR reporter gene assay (Persson et al., 2006). In Fig. 3, the AUC/EC_{50} and the AUC/F_2 from activation of PXR in the reporter gene assay, and the AUC/F_2 from CYP3A4 mRNA induction in HepaRG cells are shown normalised to rifampicin, which was set to 100%. The same ranking of compounds was obtained for all three ratios.

Induction of CYP3A4 mRNA in HepaRG cells by drugs was investigated as a quantitative measure of induction potency of the same drugs *in vivo*. Ten compounds (listed in Table 4) were selected on the basis that acceptable information on their induction potential *in vivo* is published. The *in vivo* induction is represented by the decrease in AUC for a CYP3A probe drug, administered before and after treatment with the inducing agent. The *in vivo* probes used for the correlation were midazolam for rifampicin (Backman et al., 1996a; Backman et al., 1998), phenytoin (Backman et al., 1996b), carbamazepine (Backman et al., 1996b), and hyperforin (St John's wort) (Wang

et al., 2001), verapamil for phenobarbital (Rutledge et al., 1988) and sulfinpyrazone (Wing et al., 1985), terfenadine for troglitazone (Loi et al., 1998), and triazolam for dexamethasone (Villikka et al., 1998). These probe drugs are moderate or high clearance drugs (Greenblatt et al., 1998; Wong et al., 1998; Lin, 2006), and the clearance and bioavailability is dependent on CYP3A4 metabolism. Omeprazole and nifedipine has not been found to induce CYP3A *in vivo* (Soons et al., 1992; Bowles et al., 1993). The correlation of AUC/F_2 values from CYP3A4 mRNA induction and the *in vivo* induction (Fig. 4) show an excellent correlation (R^2 = 0.863). When excluding hyperforin, a constituent of St John's wort, the correlation factor increased to 0.943 (Fig. 4).

Using unbound AUC values gave a slightly lower *in vitro-in vivo* correlation (R^2 = 0.859, data not shown). When the maximum plasma concentration *in vivo* (C_{max} and $C_{max,\,u}$) was used instead of AUC and AUC_u the R^2 values were 0.741 and 0.812, respectively.

The corresponding evaluation of the predictive value for the induction of the other investigated CYPs in the HepaRG cells is hampered by the lack of *in vivo* data.

Discussion

HepaRG is a new human hepatoma cell line developed from a liver carcinoma. The aim of the present study was to further evaluate the induction properties of the HepaRG cells and to develop a method to predict the magnitude of induction *in vivo*. In the present study we show that the drug metabolising enzymes CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4 are inducible in the HepaRG cells. The compounds tested are known to mediate the induction response via the AhR, CAR and/or PXR receptors. The results are thus consistent with the reported expression of relevant transcription factors in the HepaRG cells (Aninat et al., 2006).

The results are from three separate batches of differentiated HepaRG cells. The small variations in fold induction and F_2 values obtained after exposure to test compounds indicate that the HepaRG cells will give reproducible and consistent results.

CYP1A1 and CYP1A2 mRNA were highly induced in HepaRG cells by the tested compounds previously known to induce CYP1A. The EC_{50} value for induction of CYP1A1 mRNA by omeprazole (5.9 μM) was slightly lower than the EC_{50} value (18 μM) for the activation of AhR by omeprazole previously reported in a reporter gene assay (Persson et al., 2006). The F_2 values for the induction of phenacetin O-dealkylase activity was in the same order of magnitude as the F_2 values for induction of CYP1A1 and CYP1A2 mRNA. Although the fold induction of CYP1A1 and CYP1A2 mRNA was extensive after exposure to 40 μM omeprazole, the corresponding fold induction of phenacetin O-dealkylase activity at 48 hours exposure in the present study was only 3.5-fold. Omeprazole has in many *in vitro* studies been used as a positive control for activation of AhR and induction of CYP1A. However, omeprazole, or any other drug on the market that has been found to induce CYP1A or activate AhR *in vitro*, have not been found to induce CYP1A *in vivo* at relevant concentrations. Any translation of the *in vitro* results for CYP1A induction to the *in vivo* situation is thus uncertain.

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CYP2B6 mRNA and bupropion hydroxylase activity was highly inducible in HepaRG cells by rifampicin, phenobarbital and phenytoin. Rifampicin and phenytoin are considered to be selective activators of PXR and CAR, respectively, whereas phenobarbital activates both receptors (Moore et al., 2000; Wang et al., 2004). The present results, together with previously reported mRNA expression of PXR and CAR (Aninat et al., 2006), indicate that HepaRG cells express both a functional PXR and a functional CAR receptor.

In *in vivo* studies using CYP selective probes, the induction of CYP2C9 results in lower induction than induction of CYP3A when treating subjects with the same activators, such as rifampicin. This implies that CYP2C9 will be induced to a lesser degree than CYP3A by the same compounds. In accordance with the *in vivo* findings, the F_2 values were higher and the fold induction lower for CYP2C9 mRNA induction in HepaRG cells by rifampicin, compared to CYP3A4 mRNA induction. The same phenomenon has also been demonstrated in induction studies with primary human hepatocytes (Gerbal-Chaloin et al., 2001).

Induction of CYP2C8 and CYP2C19 mRNA in HepaRG cells by rifampicin is in the same range as induction of CYP2C9 mRNA, which corresponds with results from human hepatocytes (Gerbal-Chaloin et al., 2001). Both CYP2C8 and CYP2C19 are also induced *in vivo* by rifampicin (Zhou et al., 1990; Niemi et al., 2004; Park et al., 2004).

CYP3A4 was highly induced by all compounds known to activate PXR and/or CAR. The EC_{50} for rifampicin induction of CYP3A4 mRNA (0.42 µM) and midazolam 1'-hydroxylase activity (0.12 µM) in HepaRG cells, is consistent with the EC_{50} value obtained in the PXR reporter gene assay (0.20 µM) by the same drug (Persson et al., 2006), which suggest that PXR is the major receptor regulating the rifampicin induction of this gene product.

One important application of *in vitro* CYP induction studies in drug discovery is to predict the risk for induction of drug metabolism *in vivo*. In a previous study it was shown that the EC_{50} values from a PXR reporter gene assay could be used to distinguish between CYP3A inducers and non-inducers when related to the *in vivo* exposure of the test

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compound (Persson et al., 2006). In the present study, F_2 values from CYP3A4 mRNA induction related to *in vivo* AUC of the test compounds were found to give the same correct ranking of inducers and non-inducers. The results suggest that instead of EC_{50} values from full dose-response curves, F_2 values could be used to evaluate the induction response in the cell system. This is an advantage since an E_{max} many times cannot be achieved or may be confounded due to toxicity or solubility problems. For example, troglitazone did not affect viability but showed a decrease in CYP3A4 induction response at the highest concentration, which may affect the E_{max} value and as a consequence the EC_{50}.

The AUC/F_2 values for CYP3A4 mRNA induction were further evaluated as possible predictor of the magnitude of induction *in vivo*. In the present study we used results from published *in vivo* induction studies utilizing different probe drugs for measuring CYP3A4 dependent clearance. The probe drugs used in the current *in vivo* studies are all intermediate or high clearance drugs, metabolised and cleared mainly by CYP3A4. They should therefore be affected to a similar extent by CYP3A4 induction (Lin, 2006). Such an assumption is supported by the fact that the effect of rifampicin on AUC for the high/medium clearance drugs midazolam, verapamil, and triazolam after oral dosing is comparable (Backman et al., 1996a; Fromm et al., 1996; Villikka et al., 1997; Backman et al., 1998). An excellent correlation was found between the decrease of *in vivo* AUC for CYP3A probe drugs and AUC/F_2 for the tested drugs. The results thus indicate that the AUC/F_2 values for CYP3A4 mRNA response in HepaRG cells could be used as a reliable *in vitro* measure for evaluating CYP3A induction potency of compounds *in vivo*. In the *in vitro-in vivo* correlation analysis a component of St John's wort, hyperforin, was included. The manufacture of herbal remedies is not as rigorously controlled as production of drugs, which results in varying concentration of the components in different extracts. This means that *in vivo* data for hyperforin could be confounded by varying concentrations of hyperforin in the extracts. If hyperforin is excluded from the correlation a higher correlation factor is obtained, which could indicate that this might be the case.

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Besides hyperforin, the compound deviating the most from the correlation is dexamethasone, being a more potent inducer *in vivo* than what was predicted from AUC/F_2 values. The reason for this is obscure and proposes further investigations of this compound.

The Fa2N-4 cells were investigated by Ripp et al., (2006) as a model for predicting CYP3A induction *in vivo*. The *in vitro* E_{max} and EC_{50} values for a number of potential CYP3A drug inducers were related to *in vivo* C_{max} for the same drugs. The induction score obtained showed an excellent correlation with the degree of induction *in vivo*. In the present study on HepaRG cells, using C_{max} instead of AUC values for calculation of induction scores gave a poorer correlation with the degree of induction *in vivo* for the tested drugs.

According to the FDA guideline (http://www.fda.gov/cder/guidance/6695dft.pdf), a test compound should reach an induction level equal to or greater than 40% of a positive control to be considered as relevant for the *in vivo* situation. In the present study all compounds known to induce CYP3A4 *in vivo* reached at least 40% of rifampicin induction, except the well-known CYP3A inducer phenytoin. Phenytoin has previously been shown to be a weak inducer in PXR reporter gene assays and human hepatocytes, exhibiting an induction response close to or below 40% of rifampicin response (Luo et al., 2002; Persson et al., 2006). Substances like phenytoin could therefore be overlooked if using a conservative cut-off value for *in vivo* relevance. In the present study 40 µM phenytoin gave an induction response that were only 14% of maximum rifampicin induction. However, using the AUC/F_2 value for the CYP3A4 mRNA induction response in HepaRG cells correctly predicted the degree of induction *in vivo* by phenytoin. Furthermore, strong *in vitro* CYP3A4 inducers like omeprazole and nifedipine (50 and 92% of maximum rifampicin induction, respectively) were correctly classified as non-inducers when using AUC/F_2 values. These results emphasise the importance to relate the *in vitro* results to *in vivo* exposure for both seemingly “strong” and “weak” inducers in the *in vitro* system.

In conclusion, we have shown that HepaRG cells responds to PXR, CAR, and AhR activators, resulting in induction of CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4. Further it was shown that the F_2 values for CYP3A4 mRNA induction in HepaRG cells could be used not only to classify compounds as inducers or non-inducers of CYP3A, but also to predict the extent of induction *in vivo* in humans. The HepaRG cell line could thus be used as an important *in vitro* model for investigations of enzyme induction in drug discovery.

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Footnotes

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Legends for figures

Fig. 1. Representative results of mRNA induction in HepaRG cells. Induction of CYP1A1 and 1A2 by omeprazole (A), CYP2C8, 2C9, and 2C19 by rifampicin (B), and CYP2B6 and 3A4 by rifampicin (C).

Fig. 2. Representative results of induction of enzyme activities in HepaRG cells. Induction of phenacetin O-dealkylase (CYP1A2) activity by omeprazole (A), bupropion hydroxylase (CYP2B6) activity by rifampicin (B), diclofenac 4'-hydroxylase (CYP2C9) activity by rifampicin (C), and midazolam 1'-hydroxylase (CYP3A4) activity by rifampicin (D).

Fig 3. Ranking of *in vitro* results. The AUC/EC_{50} from PXR assay (white bars), AUC/F_2 from PXR assay (grey bars) (data from (Persson et al., 2006), and AUC/F_2 CYP3A4 mRNA in HepaRG cells (black bars). The study compounds were normalised to the rifampicin response, which were set to 100%.

Fig 4. Correlation of AUC/F_2 of CYP3A4 mRNA in HepaRG cells with % decrease of *in vivo* AUC for CYP3A probe drugs (whole line), $R^2 = 0.863$. The equation used is described under Materials & Methods. Correlation when hyperforin is excluded (dashed line), $R^2 = 0.943$. Compound abbreviations: CBZ, carbamazepine; DEX, dexamethasone; HYP, hyperforin; NIF, nifedipine; OME, omeprazole; PB, phenobarbital; PHY, phenytoin; RIF, rifampicin; SULF, sulfinpyrazone; TRO, troglitazone.

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Tables

Table 1. F_2 values and fold induction at highest concentration tested (given in parenthesis) for induction of CYP1A1 and CYP1A2 mRNA in HepaRG cells treated with rifampicin, omeprazole, troglitazone, phenytoin, primaquine, or phenobarbital. F_2 is the concentration resulting in a 2-fold increase of baseline mRNA levels. EC_{50} values are presented when achieved. Values are mean ± SD of three separate batches of differentiated HepaRG cells.

	CYP1A1 mRNA		CYP1A2 mRNA	
	F_2	Fold induction at highest concentration	F_2	Fold induction at highest concentration
Rifampicin	N.I.[a]	N.I.	N.I.	N.I.
Omeprazole	1.4±0.14[b] (EC_{50} = 5.9±1.5[b])	62±13 (40 µM)	1.0±0.2	38±19 (40 µM)
Troglitazone	N.I.	N.I.	N.I.	N.I.
Phenytoin	N.I.	N.I.	N.I.	N.I.
Primaquine	4.6±3.3	7.4±2.2 (40 µM)	1.7±1.0	20±16 (40 µM)
Phenobarbital	N.I.	N.I.	N.I.	N.I.

[a] N.I. = no induction recorded

[b] µM

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Table 2. F_2 values and fold induction at highest concentration tested (given in parenthesis) for induction of CYP2B6 mRNA in HepaRG cells treated with rifampicin, omeprazole, troglitazone, phenytoin, primaquine, or phenobarbital. F_2 is the concentration resulting in a 2-fold increase of baseline mRNA levels. EC_{50} values are presented when achieved. Values are mean ± SD of three separate batches of differentiated HepaRG cells.

	F_2	**Fold induction at highest concentration**
Rifampicin	0.16±0.11[a]	20±13 (4 µM)
Omeprazole	2.3±0.1	16±8.7 (40 µM)
Troglitazone	1.9±0.6	4.6±2.7 (6.25 µM)
Phenytoin	1.7±1.0	9.7±5.6 (40 µM)
Primaquine	N.I.[b]	N.I.
Phenobarbital	11±5.8	26±15 (200 µM)

[a] µM

[b] N.I. = no induction recorded

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Table 3. F_2 values and fold induction at highest concentration tested (given in parenthesis) for induction of CYP2C8, CYP2C9, and CYP2C19 mRNA in HepaRG cells treated with rifampicin. F_2 is the concentration resulting in a 2-fold increase of baseline mRNA levels. Values are mean ± SD of three separate batches of differentiated HepaRG cells.

	F_2	**Fold induction at highest concentration tested (4 µM)**
CYP2C8 mRNA	0.21±0.03[a]	2.9±0.58
CYP2C9 mRNA	0.25±0.03	2.3±1.4
CYP2C19 mRNA	0.41±0.25	2.9±2.1

[a] µM

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Table 4. F_2 values, fold induction at highest concentration tested (given in parenthesis), and AUC/F_2 for induction of CYP3A4 mRNA in HepaRG cells treated with rifampicin, omeprazole, troglitazone, phenytoin, primaquine, phenobarbital, carbamazepine, dexamethasone, hyperforin, nifedipine, or sulfinpyrazone. F_2 is the concentration resulting in a 2-fold increase of baseline mRNA levels. EC_{50} values are presented when achieved. Values are mean ± SD of three separate batches of differentiated HepaRG cells. Published values of *in vivo* exposure (AUC) for the same drugs are listed.

	F_2	**Fold induction at highest concentration**	**AUC/F_2**	***In vivo* AUC**	**Reference *in vivo* AUC**
Rifampicin	0.13±0.10[a] (EC_{50} = 0.25±0.02[a])	24±12 (4 µM)	262	34.1[b]	(Polk et al., 2001)
Omeprazole	2.0±0.33	12±2.2 (40 µM)	0.54	1.11	(Andersson et al., 1998)
Troglitazone	0.56±0.20	11±6.0 (6.25 µM)	29.5	16.5	(Ott et al., 1998)
Phenytoin	2.2±1.8	3.4±2.3 (40 µM)	218	468	(Spaans et al., 2002)
Primaquine	N.I.[c]	N.I.	N.I.	3.27	(Na-Bangchang et al., 2000)
Phenobarbital	15±3.9	14±4.0 (200 µM)	98.5	1497	(Reidenberg et al., 1995)
Carbamazepine	5.5±1.8	16±3.8 (250 µM)	228	1248	(Olling et al., 1999)
Dexamethasone	2.8±0.34	23±4.0 (250 µM)	0.10	0.29	(Loew et al., 1986)
Hyperforin	0.04±0.005	15±5.8 (0.04 µM)	168	6.24	(Biber et al., 1998)
Nifedipine	1.6±0.1	22±5.4 (62.5 µM)	0.30	0.49	(Hippius et al., 1995)
Sulfinpyrazone	2.1±1.0	24±13 (250 µM)	134	287	(Schlicht et al., 1985)

[a] µM

[b] (h*µmol/l)

[c] N.I. = no induction recorded

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Table 5. F_2 values and fold induction at highest concentration tested (given in parenthesis) for induction of phenacetin O-dealkylase, bupropion hydroxylase, and midazolam 1'-hydroxylase activities in HepaRG cells treated with rifampicin, omeprazole, troglitazone, phenytoin, primaquine, or phenobarbital. F_2 is the concentration resulting in a 2-fold increase of baseline activities levels. EC_{50} values are presented when achieved. Values are mean ± SD of duplicates in two separate batches of differentiated HepaRG cells.

	Phenacetin O-dealkylase activity		**Bupropion hydroxylase activity**		**Midazolam 1'-hydroxylase activity**	
	F_2	Fold induction at highest concentration	F_2	Fold induction at highest concentration	F_2	Fold induction at highest concentration
Rifampicin	N.I.[a]	N.I.	0.09±0.04[b]	5.9±2.4 (4 µM)	0.04±0.009 (EC_{50} = 0.12±0.02[b])	7.9±2.9 (4 µM)
Omeprazole	3.2±0.59	3.5±1.0 (40 µM)	N.I.	N.I.	N.I.	N.I.
Troglitazone	N.I.	N.I.	N.I.	N.I.	0.24±0.10	2.2±1.5
Phenytoin	N.I.	N.I.	0.41±0.28	3.4±2.1 (40 µM)	N.I.	N.I.
Primaquine	N.I.	N.I.	N.I.	N.I.	N.I.	N.I.
Phenobarbital	N.I.	N.I.	7.0±3.8	6.2±4.3 (200 µM)	25±10	6.2±2.4 (200 µM)

[a] N.I. = no induction recorded

[b] µM

Fig. 1A

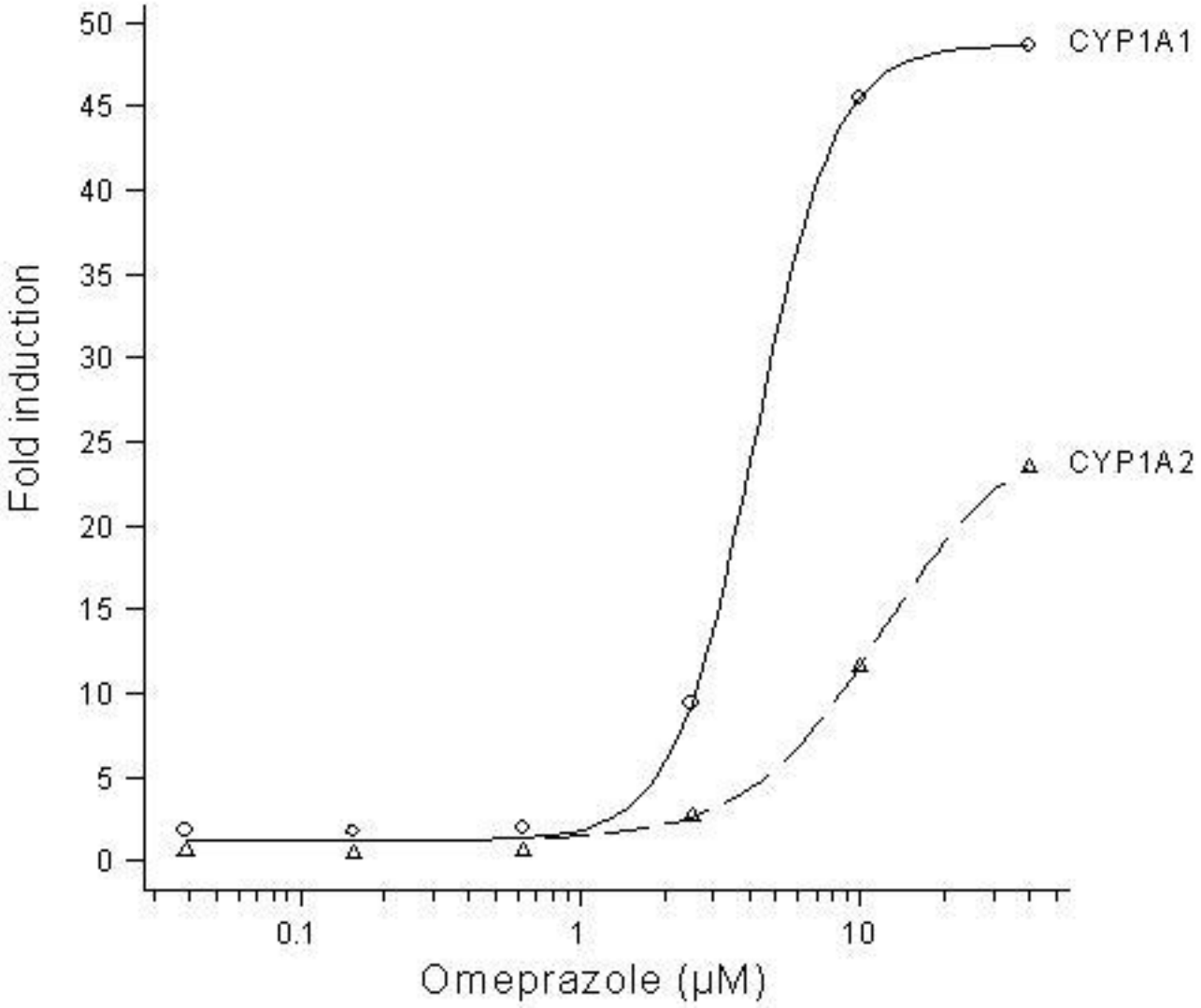

Fig. 1B

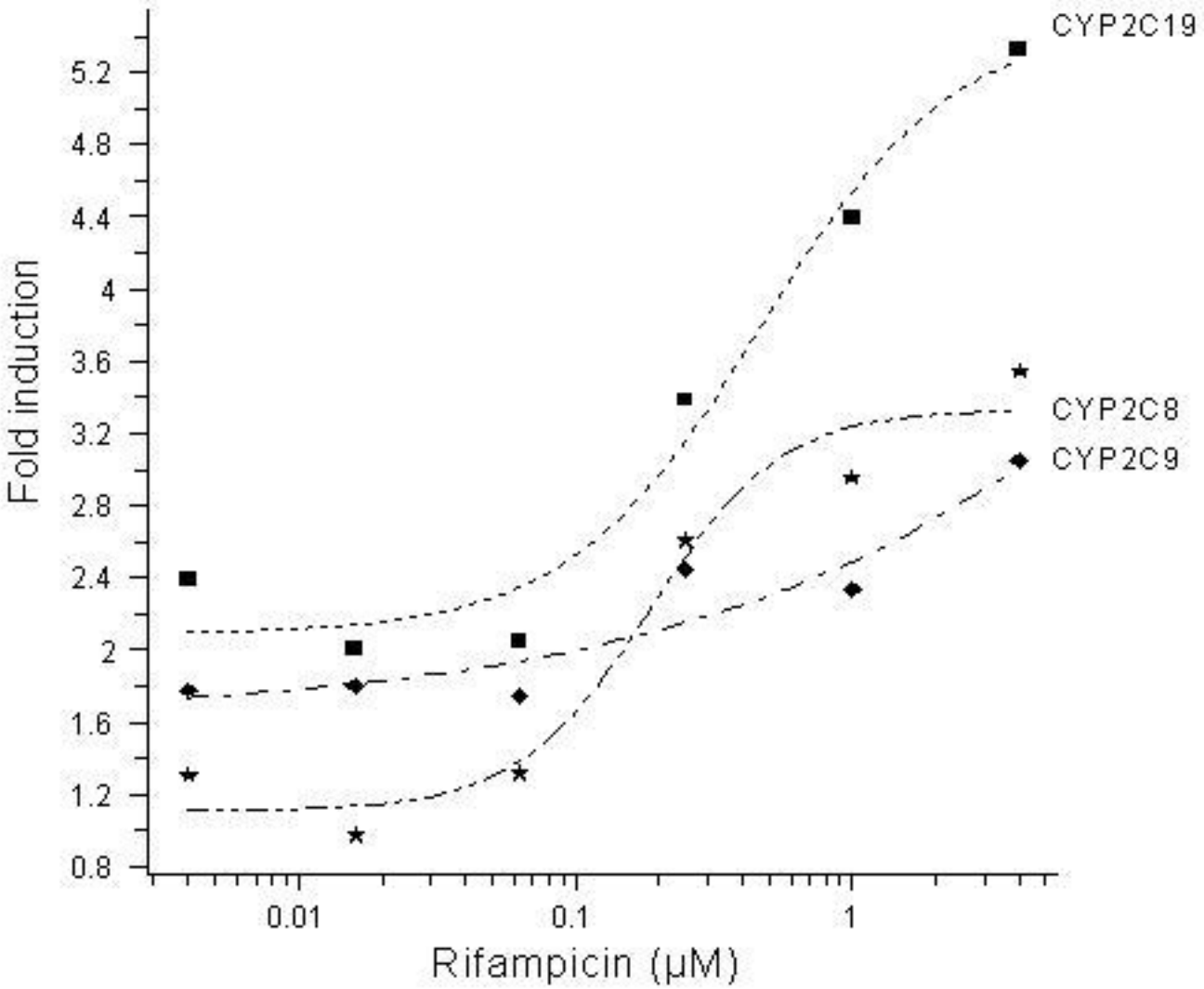

Fig. 1C

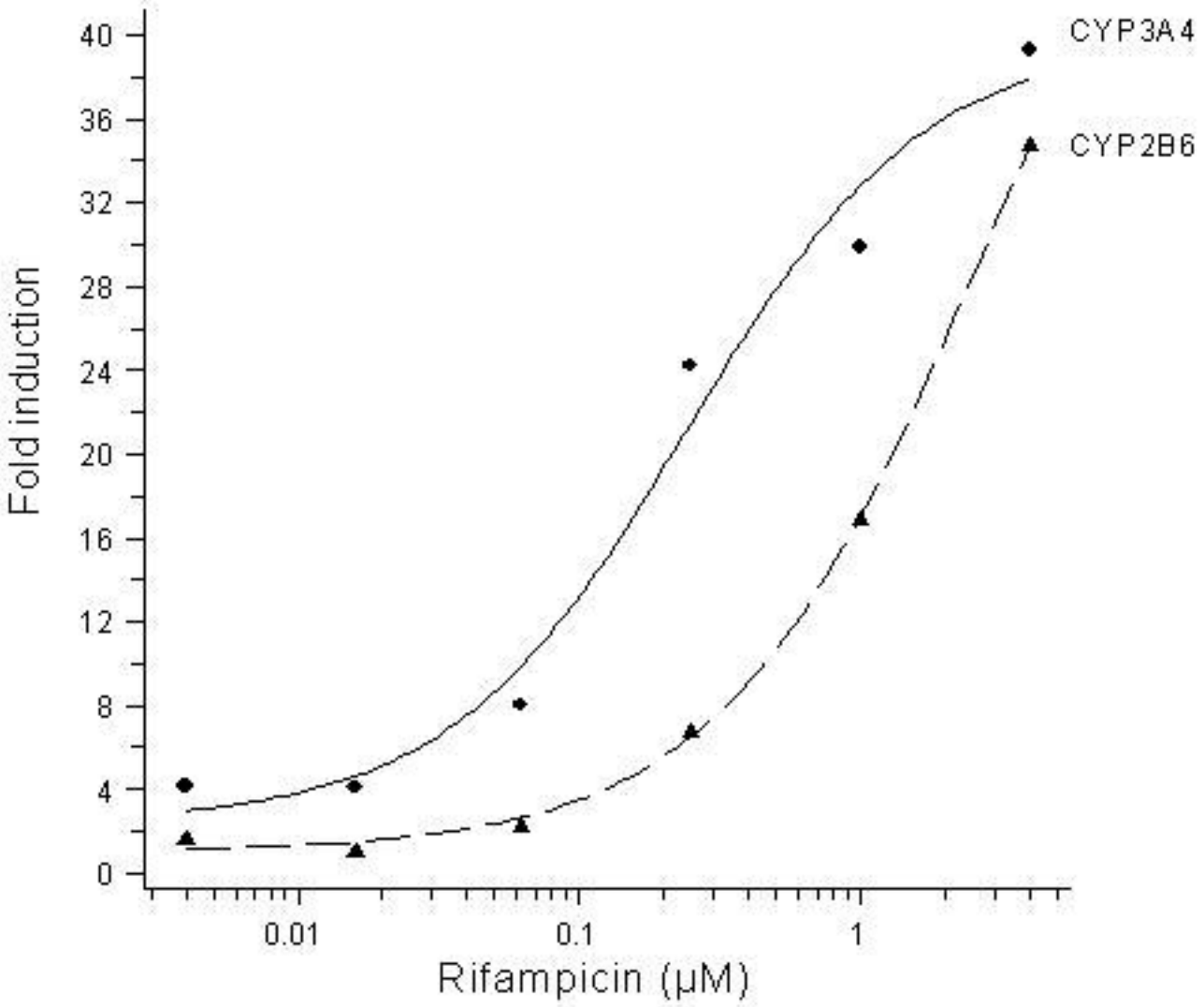

Fig. 2A

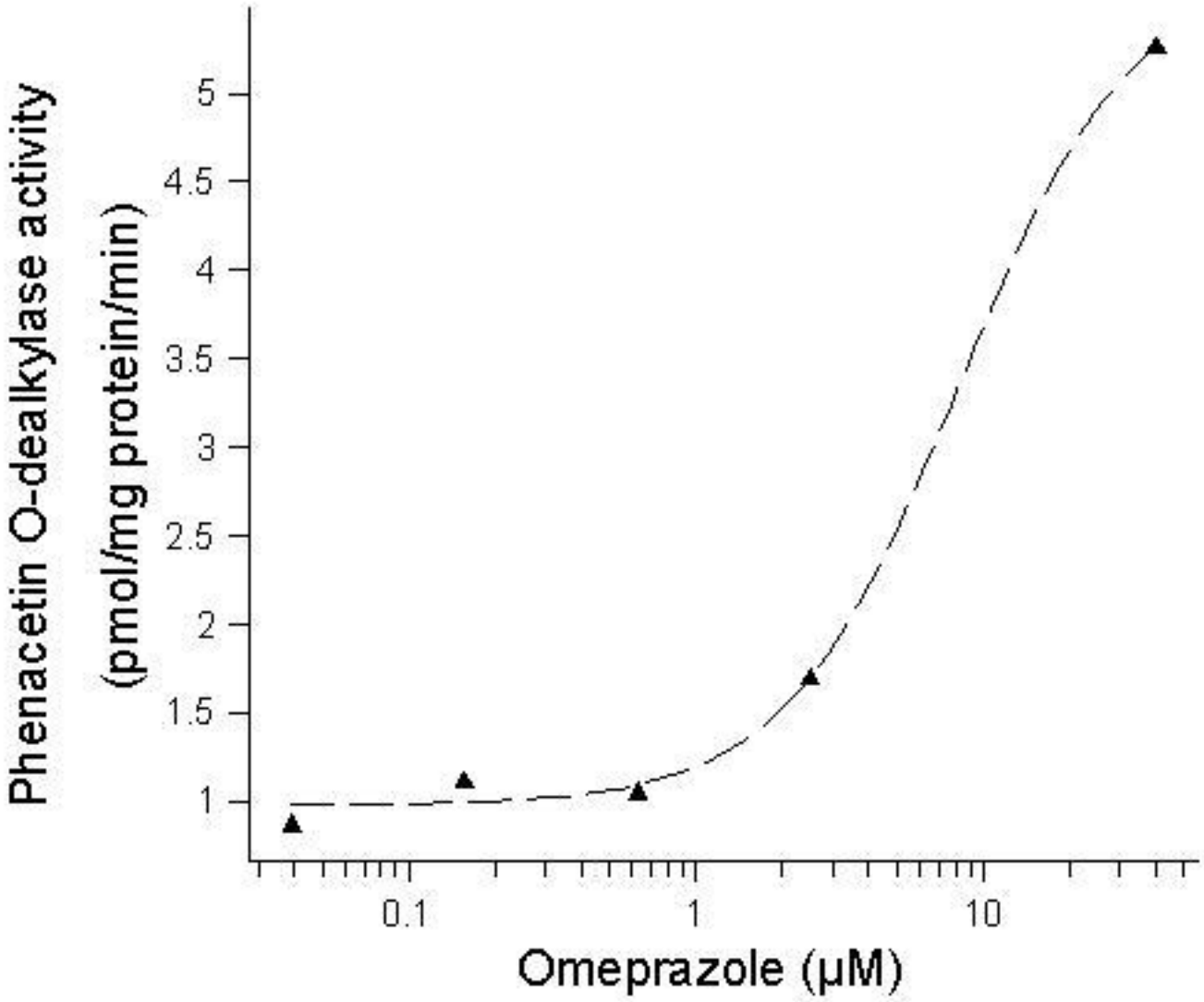

Fig. 2B

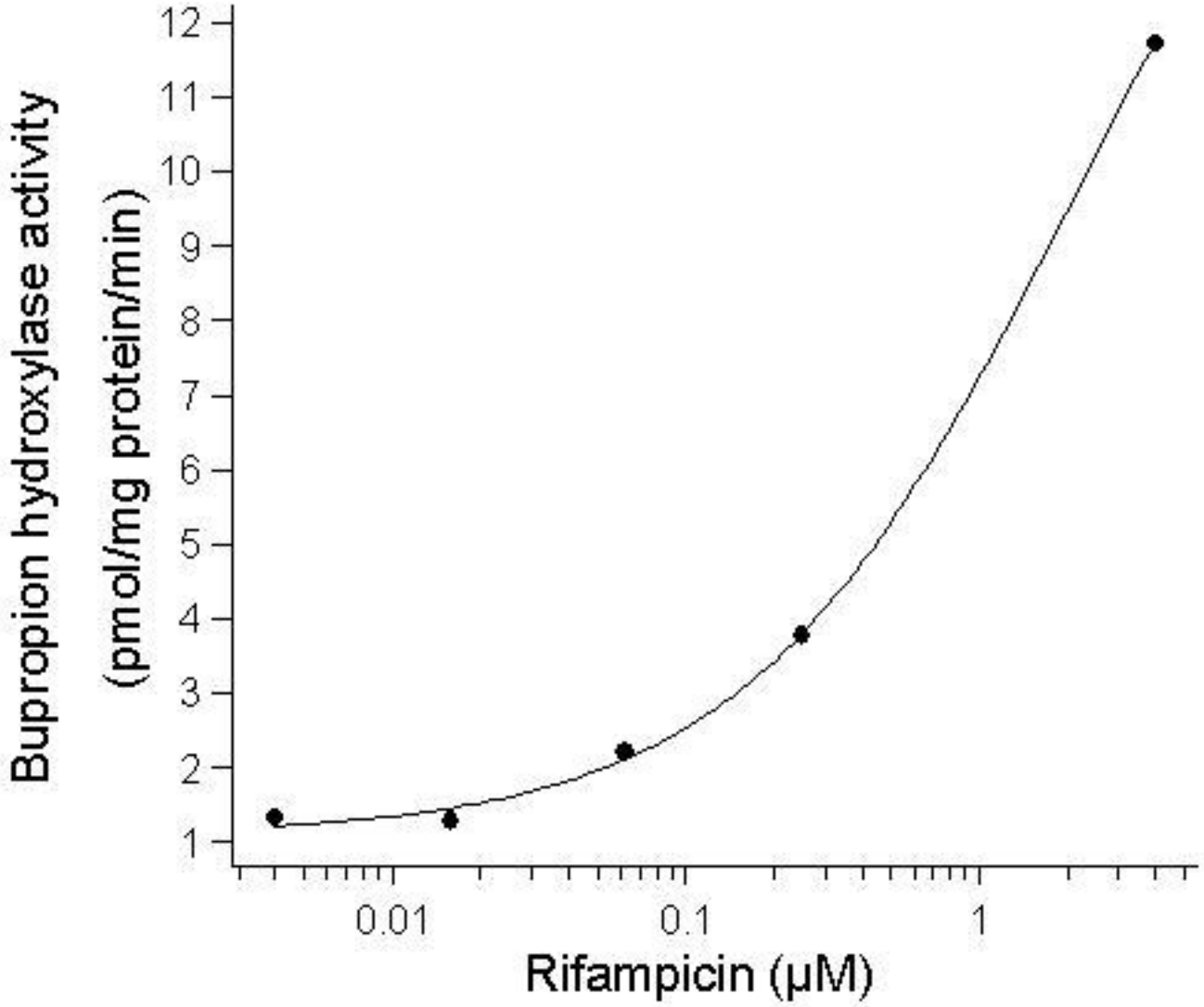

Fig. 2C

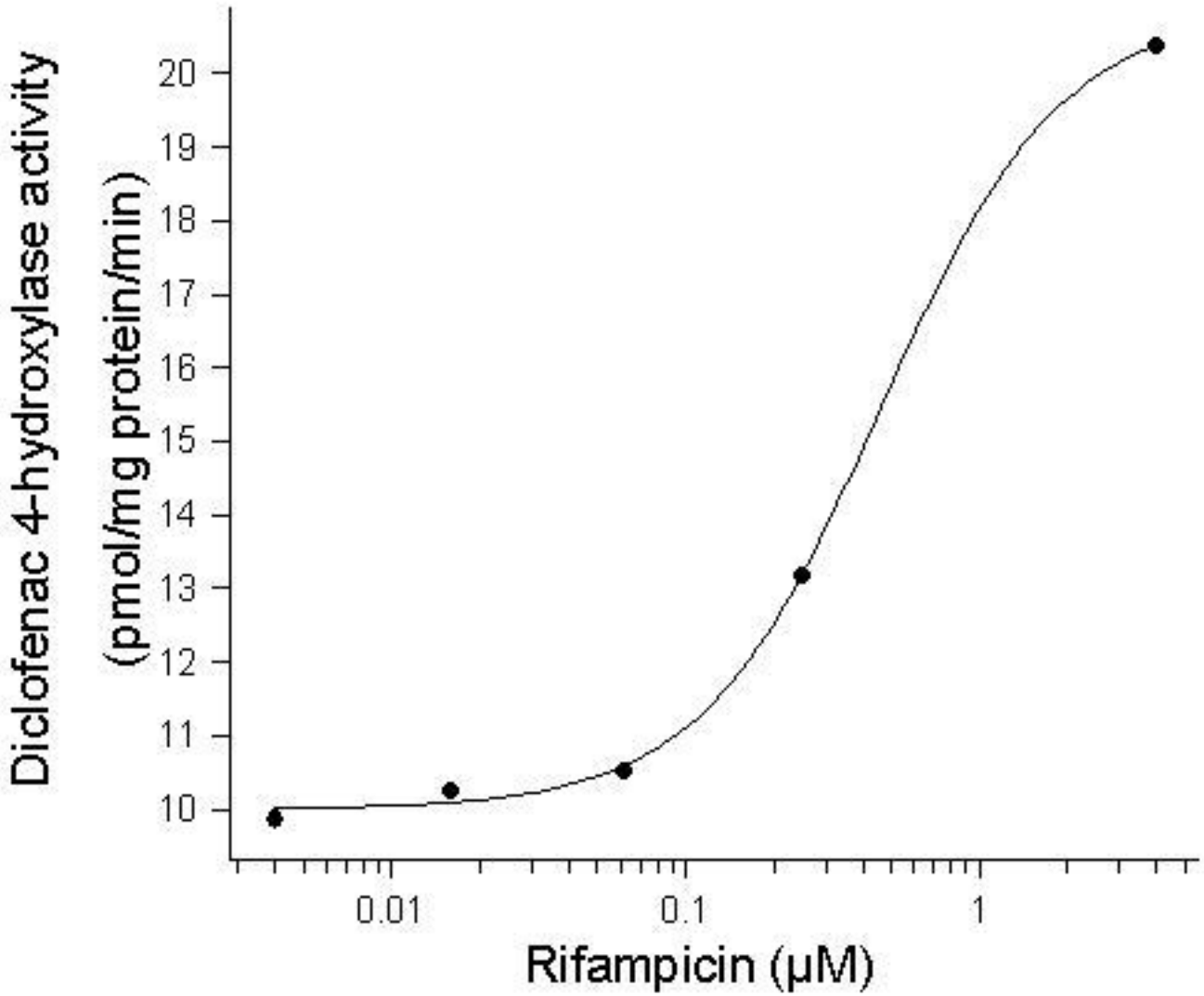

Fig. 2D

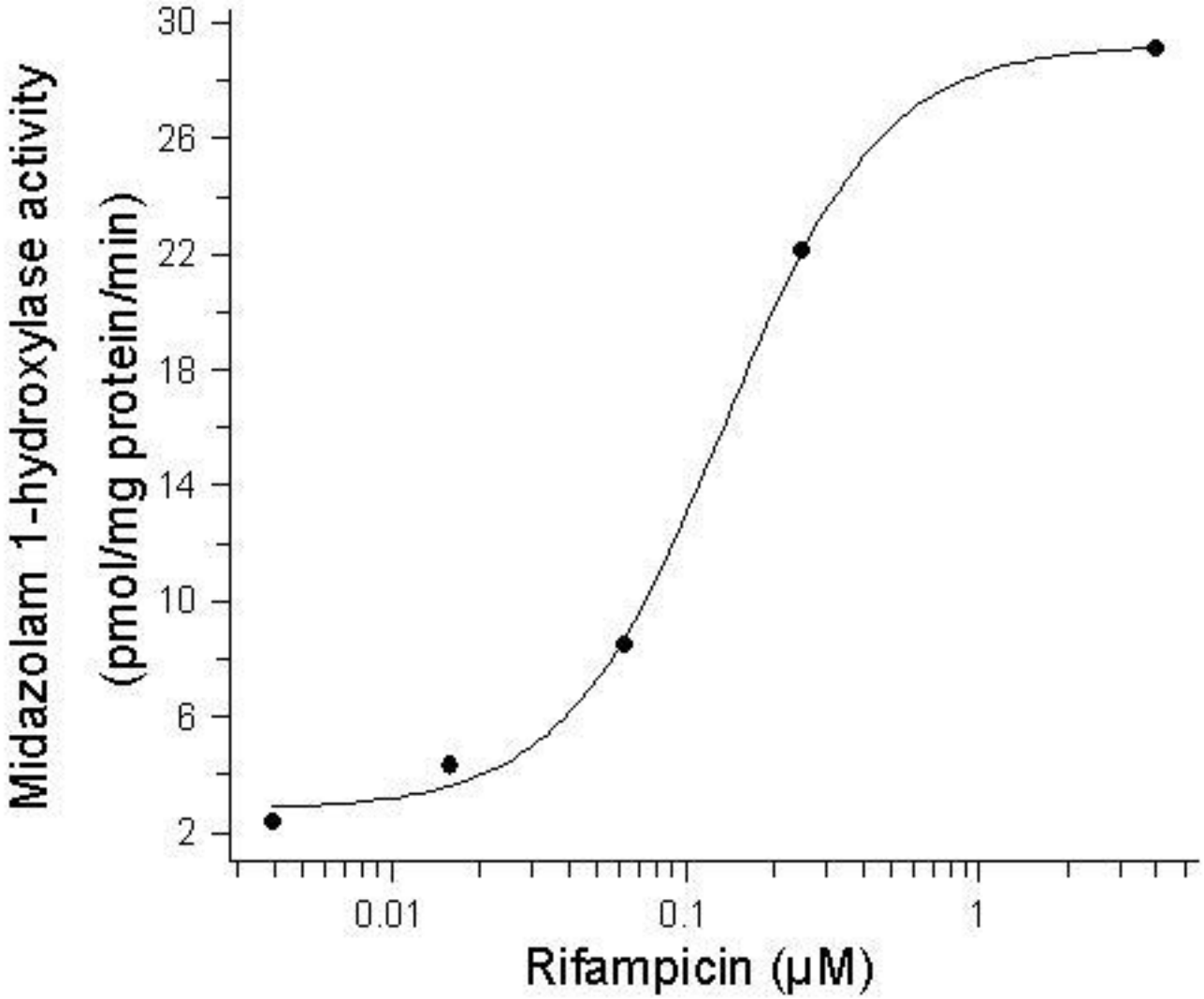

Fig. 3

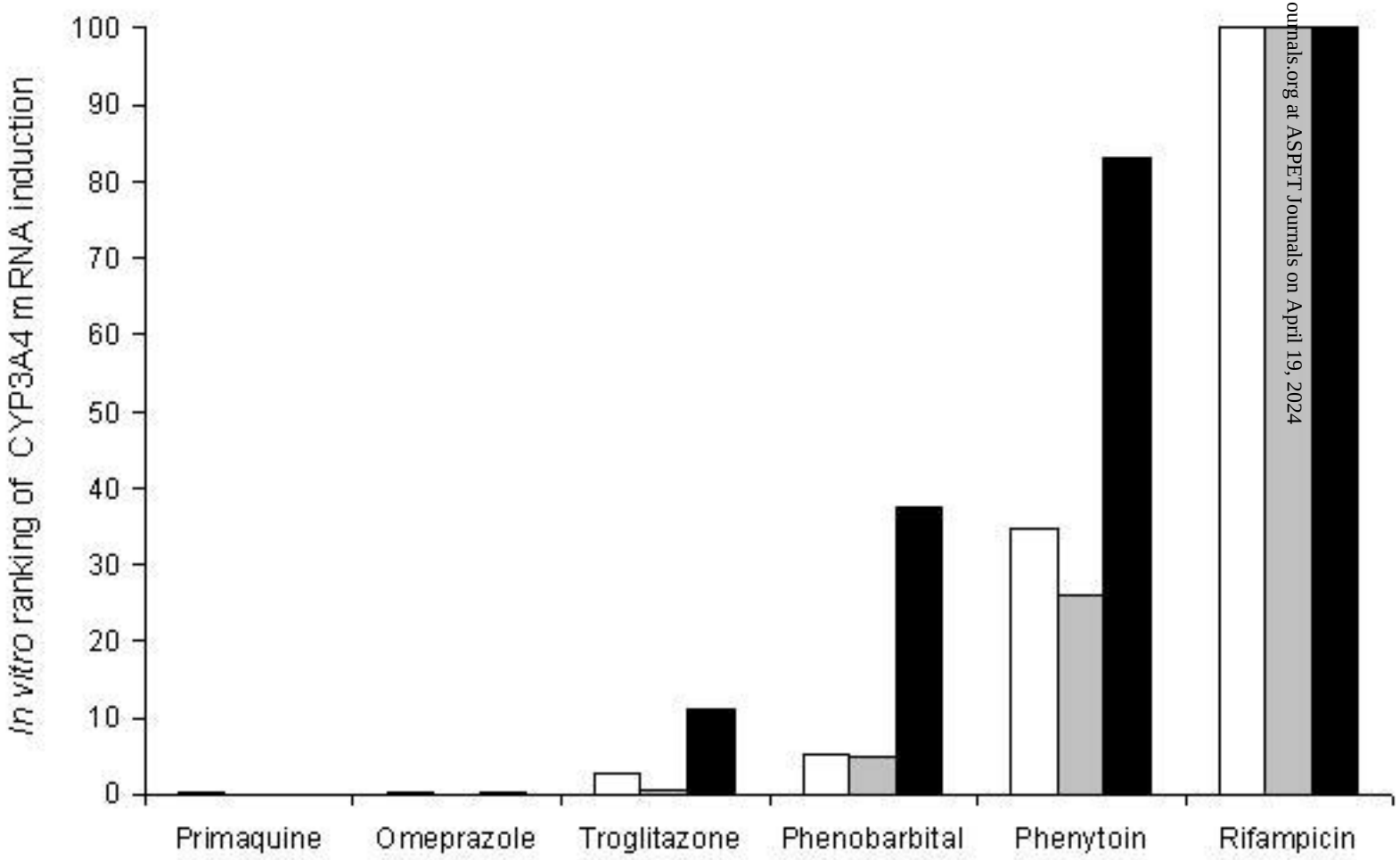

Fig. 4