

## CHANGES IN CYTOCHROME P450 GENE EXPRESSION AND ENZYME ACTIVITY INDUCED BY XENOBIOTICS IN RABBITS *IN VIVO* AND *IN VITRO*

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**Abstract:** As considerable inter-species differences exist in xenobiotic metabolism, developing new pharmaceutical therapies for use in different species is fraught with difficulties. For this reason, very few medicines have been registered for use in rabbits, despite their importance in inter alia meat and fur production. We have developed a rapid and sensitive screening system for drug safety in rabbits based on cytochrome P450 enzyme assays, specifically CYP1A1, CYP1A2 and CYP3A6, employing an adaptation of the luciferin-based clinical assay currently used in human drug screening. Short-term (4-h) cultured rabbit primary hepatocytes were treated with a cytochrome inducer (phenobarbital) and 2 inhibitors (alpha-naphthoflavone and ketoconazole). In parallel, and to provide verification, New Zealand white rabbits were dosed with 80 mg/kg phenobarbital or 40 mg/kg ketoconazole for 3 d. Ketoconazole significantly increased CYP3A6 gene expression and decreased CYP3A6 activity both *in vitro* and *in vivo*. CYP1A1 activity was decreased by ketoconazole *in vitro* and increased *in vivo*. This is the first report of the inducer effect of ketoconazole on rabbit cytochrome isoenzymes *in vivo*. Our data support the use of a luciferin-based assay in short-term primary hepatocytes as an appropriate tool for xenobiotic metabolism assays and short-term toxicity testing in rabbits.

**Key Words:** CYP450, hepatocyte, ketoconazole, luminescence, rabbit.

### INTRODUCTION

Rabbits are livestock animals in increasing demand for meat and fur production, widely used as laboratory models and a popular pet species. Despite this, only very few medicines have been registered for rabbits. Veterinary practitioners are frequently forced to use medicines registered for dogs or cats, and the high incidence of resulting iatrogenic illness reflects the differences in drug metabolising capacity between species. The investigation of drug metabolism in this species is timely.

Despite the highly conserved functions of cytochrome P450 enzymes, significant inter-species variance exists in the activity and substrate-specificity of individual enzymes. The cytochrome P450 (CYP450) system plays a key role in xenobiotic metabolism, where the CYP enzymes catalyse Phase I reactions. In humans, CYP1A1 is involved in the biotransformation of polycyclic aromatic hydrocarbons, CYP1A2 metabolises phenacetin, theophylline, caffeine, imipramine and propranolol, and CYP3A is the most abundant CYP isoenzyme, metabolising over half of current drugs (Boek-Dohalská *et al.*, 2001).

Valuable information about the safety of drugs, feed additives and other substances can be obtained by measuring their impact on CYP enzyme activities. Animal models are the gold standard but ethical and cost considerations favour *in vitro* systems such as primary hepatocyte cell cultures, which closely approximate to parent cells and provide high initial CYP450 activity (Guillouzo, 1998). A highly sensitive luminescence assay has been developed for assaying the

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human cytochrome system (Cali *et al.*, 2006), and is specific for CYP isoenzyme activity. We tested the luminescent assay's applicability for rabbit CYP detection in rabbit hepatocytes, assuming functional similarity between rabbit CYP3A6 and human CYP3A4, as the 2 enzymes are highly similar (Franklin, 1995). We also measured changes in CYP1A1, CYP1A2 and CYP3A6 gene expression levels, *in vivo* and *in vitro*.

## MATERIALS AND METHODS

**Bioluminescence assay:** The luminogenic compounds are beetle luciferin derivatives that are converted by CYP enzymes to luciferin. D-luciferin is formed and detected in a second reaction with the Luciferin Detection Reagent, generating luminescence proportional to CYP activity.

**Xenobiotics:** Our study utilised phenobarbital and ketoconazole, which are characteristic inducer and inhibitor agents of CYP450 enzymes.

### **Rabbit hepatocyte isolation and plating**

Livers were obtained from female, 6-wk-old New Zealand white rabbits weighing approximately 1.5 kg. The animals were housed in stainless steel cages, 2 rabbits per cage, provided food and water *ad libitum* for 1 wk before use. Rabbits were anaesthetised by the combination of xylazine (5 mg/kg body weight (BW), 2% solution) and ketamine (60 mg/kg BW, 10% solution) administered intramuscularly. After aseptic opening of the abdominal cavity, the portal vein of the liver was cannulated and rabbit liver was perfused with calcium-free Hank's buffer containing 0.5 mM egtazic acid (EGTA) for 10 min at a rate of 30 mL/min. The liver was then removed from the abdominal cavity and perfused with Hank's buffer containing 2.5 mM calcium-chloride and 2.5 mM magnesium-chloride and collagenase type IV (0.5 mg/mL) for 25 min at a flow rate of 30 mL/min. All perfusion buffers were preheated to 37°C and equilibrated with 95% O<sub>2</sub>/5% CO<sub>2</sub> before use.

The next steps were performed under laminar flow. The liver was removed from the Glisson capsule and gently shaken in bovine serum albumin (BSA) buffer (25 mg/mL) to release the hepatocytes. The obtained suspension was filtered through a 125 µm pore size nylon mesh and then centrifuged at 105 g for 4 min., after which the cell pellet was re-suspended in Williams E medium (Sigma-Aldrich, St. Louis, USA) containing 2 mM glutamine and 7.5% NaHCO<sub>3</sub>. The washing procedure was repeated twice. The plating medium was Williams E supplemented with 5% fetal bovine serum (FBS), 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium, 5 ng/mL epidermal growth factor (EGF), 50 IU/mL penicillin, 50 µg/mL streptomycin and 1 µM dexamethasone. The viability of the hepatocytes as assessed by the Trypan blue exclusion test was 88%. Hepatocyte cells (10<sup>6</sup>/0.5 mL/well) were added to 24-well polystyrene plates (Corning, New York, USA) coated with collagen type I (Sigma-Aldrich, St. Louis, USA). After 4 h of incubation at 37°C in 5% CO<sub>2</sub> atmosphere the cells were attached to the surface (Figure 1).

### **Rabbit hepatocyte treatment**

Four hours after isolation, the cells were washed twice with serum-free medium and treated either with inducer or with inhibitors for 2 h. Phenobarbital, ketoconazole and alpha-naphthoflavone were used as model compounds to induce stimulation or inhibition of the CYP enzymes. For general CYP450 enzyme stimulation we used 500 µM phenobarbital, for CYP1A2 inhibition 50 µM alpha-naphthoflavone and for CYP1A1 and CYP3A6 inhibition 25 µM ketoconazole. The inhibitor compounds were added to the cell cultures separately. After treatment, the cells were washed twice in PBS and the P450-Glo™ assays (Luciferin-CEE, Luciferin-1A2, Luciferin-IPA, Luciferin-PFBE; Promega, Madison, USA) were then performed according to the manufacturer's recommendations; the luminogenic substrates were added to each well, respectively. After the required time, the Luciferin Detection Reagents (Promega, Madison, USA) were added to the cells and the luminescence was detected by a luminometer (Victor X2, PerkinElmer, Massachusetts, USA). Hepatocyte cells were collected in Tergitol buffer for protein determination (Bicinchoninic Acid Protein Assay Kit, Thermo Scientific, Rockford, USA).

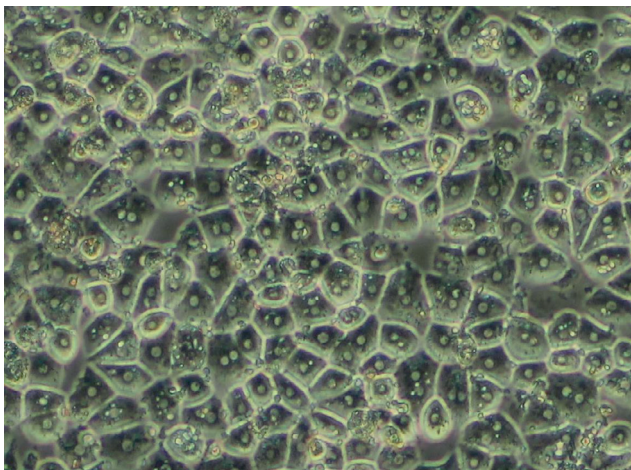


Figure 1: Rabbit primary hepatocyte cell culture after 4 h incubation. Phase-contrast micrograph, magnification: 200×. Primary hepatocytes display the typical cubic cell shape and often contain 2 nuclei.

### Quantitative Real-Time PCR

After treatment, culture medium containing the treatment compounds was removed and changed to serum-free culture medium. Five hours after treatment, 1 mL of ice-cold RNAzol RT reagent (Sigma-Aldrich) was added to the hepatocyte culture and the samples collected and kept at  $-80^{\circ}\text{C}$  until further processing. Total RNA was isolated from the cells according to the manufacturer's instructions. Quantity, A260/A280 and A260/A230 ratios of the extracted RNA were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). Synthesis of the first strand of cDNA from 1000 ng of total RNA was achieved using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Roth, Germany) according to the manufacturer's recommendations, using the random hexamer as a priming method. Quantitative real-time polymerase chain reaction (qRT-PCR) was

Table 1: Sequence of primer sets used for quantitative real-time PCR.

Gene symbol	Accession number	Primer sequences*	Product size (bp)	Efficiency	R <sup>2</sup>
GAPDH	NM_001082253	F 5'-GGCAAAGTGGATGTTGTCGC-3' R 5'-GCCGTGGGTGGAATCATACT-3'	87	0.887	0.988
Beta-actin	NM_001101683	F 5'-GTGCTTCTAGGCGGACTGTT-3' R 5'-CGGCCACATTGCAGAACTTT-3'	240	0.766	0.986
HPRT1	NM_001105671	F 5'-AGCCCCAGCGTTGTGATTAG-3' R 5'-TCGAGCAAGCCTTTCAGTCC-3'	141	0.905	0.982
CYP1A1	NM_001171072	F 5'-CTTCGGAGCTGGGTTTGACA-3' R 5'-AGAGGTGTGTCGGAAGGTCT-3'	199	0.960	0.982
CYP1A2	NM_001171121	F 5'-GTGGCAAATCAACCACGACC-3' R 5'-CGTGACCTTCTCACTCAGGG-3'	115	0.813	0.983
CYP3A6	NM_001171268	F 5'-CAAAAGCGCCGAGTGGATTT-3' R 5'-AAGTCTGCCAGCAATTGGGT-3'	327	0.731	0.990

\*All primers were designed using the Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) (Ye *et al.*, 2012).

HPRT1: hypoxanthine phosphoribosyl transferase-1, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, F: forward; R: reverse.

performed using the iQ SYBR Green Supermix kit (BioRad, Hercules, CA, USA) on the MiniOpticon System (BioRad). The cDNA was diluted 5-fold, before equal amounts were added to duplicate qRT-PCR reactions. The tested genes were CYP1A1, CYP1A2 and CYP3A6. Hypoxanthine phosphoribosyl transferase-1 (HPRT1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin were used as reference genes. Primer sequences are listed in Table 1. For each PCR reaction, 2.5 µL cDNA was added directly to a PCR reaction mixture, set to a final volume of 25 µL, containing 1× concentrated iQ SYBR Green Supermix and 0.2 µM of the appropriate primers. The thermal profile for all reactions was 3 min at 95°C, then 40 cycles of 20 s at 95°C, 30 s at 60°C, and 30 s at 72°C. At the end of each cycle, fluorescence monitoring was set for 10 s.

### ***Rabbits, housing and treatments***

Twelve clinically healthy New Zealand White rabbits (S & K Lap Nyúltenyésztő Kft., Kartal, Hungary) 6 wk old were used in this study. The animals were housed in stainless steel cages (40×60×80 cm), 2 rabbits per cage, at the animal housing facility of the Department of Pharmacology and Toxicology, acclimated at 20±2°C on a 12 h light 12 h dark schedule. One hundred grams of commercial pellet feed was provided daily and water was available *ad libitum*. The rabbits (mean weight 1350±96 g) were randomly divided into 3 groups comprising a control group, a phenobarbital group (80 mg/kg BW) and a ketoconazole group (40 mg/kg BW). The xenobiotics were administered to the rabbits for 3 consecutive days, *per os*. On the third day, the rabbits were anaesthetised by a combination of Zoletil® injection (0.3 mL/kg BW) and Xylavet injection (0.25 mL/kg BW) intramuscularly. After aseptic opening of the abdominal cavity, the portal vein of the liver was cannulated; the liver was perfused with neutral buffered saline and then removed, flash-frozen in liquid nitrogen and stored at -80°C till further processing.

### ***Microsome separation***

The livers were homogenised with 2 volumes of ice-cold buffer (1.15% KCl, 0.1 mM EDTA, pH 7.4) by a Potter-Elvehjem homogeniser. Microsomes were isolated by differential ultracentrifugation (Beckman L7-65 Ultracentrifuge, Beckman-Coulter) according to (Nebbia *et al.*, 2001). Protein concentrations were measured by the BCA protein assay kit.

### ***Statistical analyses***

Relative gene expression levels of the gene of interests were calculated by the Relative Expression Software Tool (REST) 2009 Software. Statistical analyses were performed by Statistica 12 software (Statsoft, Tulsa, USA). Differences between means were evaluated by one-way analysis of variance (ANOVA) followed by a post hoc comparison using Fisher's least significant difference (LSD) test.

### ***Ethics statement***

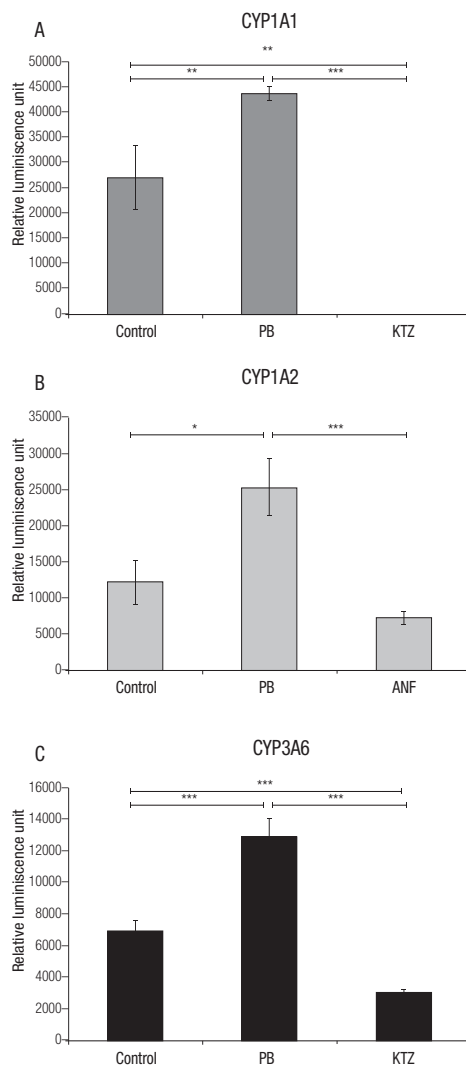
The animal trials were conducted according to approved laboratory animal experimentation ethics under national and European law, compatible with the conditions set out in the Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research. Rabbits were anaesthetised by the combination of xylazine (5 mg/kg BW, 2% solution) and ketamine (60 mg/kg BW, 10% solution) intramuscularly. The study was authorised by the Local Institutional Animal Care Committee (LIACC) of the Faculty of Veterinary Science (Permit number: 3/2015).

## **RESULTS**

### ***Results of the in vitro experiments***

The rabbit primary hepatocyte cell culture protein concentration was effectively standardised (800±30 µg/mL) in each well. By exclusion, any differences in CYP activity were therefore caused by induction or inhibition. Phenobarbital at 500 µM was an effective inducer of all 3 tested isoenzymes. Alpha-naphthoflavone at 50 µM had no effect on any of the 3 examined isoenzymes. Ketoconazole at 25 µM significantly inhibited CYP1A1 and CYP3A6.

In respect to CYP1A1, ketoconazole-evoked inhibition was so powerful that CYP1A1 activity was almost totally suppressed (Figure 2A). The alpha-naphthoflavone did not cause inhibition on the activity of CYP1A2 enzyme ( $P=0.069$ ) (Figure 2B). Regarding CYP3A6, both induction and ketoconazole-mediated inhibition were highly effective (Figure 2C).



**Figure 2:** Changes in cytochrome enzyme activity in rabbit primary cell cultures. Control: non-treated, PB: treated with 500  $\mu$ M phenobarbital for 2 h, ANF: treated with 50  $\mu$ M alpha-naphthoflavone for 2 h, KTZ: treated with 25  $\mu$ M ketoconazole for 2 h. ( $n=4$ /group; \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ ) Data are shown as mean $\pm$ standard error. (A) CYP1A1 activity. (B) CYP1A2 activity. (C) CYP3A6 activity.

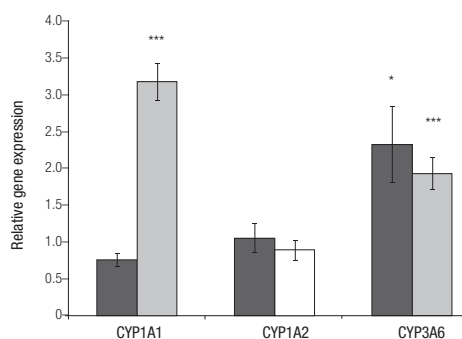
Luciferin-IPA substrate was used to measure CYP3A6 activity, as Luciferin-PFBE did not generate sufficient luminescent signal. The obtained Relative luminescence unit (RLU) was  $261\pm 26$  for the control cells,  $312\pm 77$  for the induced cells and  $212\pm 15$  for the inhibited cells. This substrate did not work well with the rabbit CYP3A6 enzyme.

### Gene expression

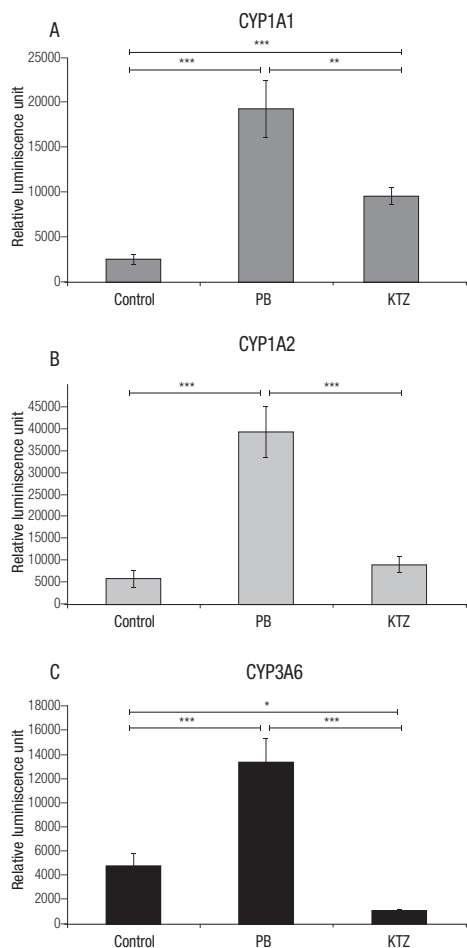
PCR results are shown in Figure 3. Phenobarbital treatment upregulated the CYP3A6 gene, while expression of CYP1A1 and CYP1A2 remained unchanged. CYP1A1 and CYP3A6 genes were upregulated after treatment with ketoconazole.

### Results of the in vivo experiments

Protein concentrations of the microsomes from the different groups of animals were similar ( $23335\pm 2344$   $\mu$ g/mL) in each sample. Microsomes from the phenobarbital treated rabbits showed increased activity of all 3 isoenzymes (CYP1A1, CYP1A2, CYP3A6) (Figure 4). Ketoconazole treatment did increase the activity of CYP1A1, decreased the activity of CYP3A6, and had no effect on CYP1A2 (Figure 4).

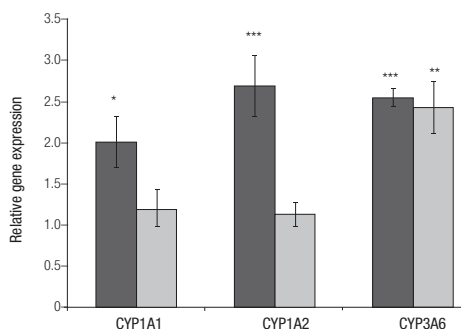


**Figure 3:** Relative gene expression of the CYP genes in rabbit primary cell cultures. ■ PB: phenobarbital, 500  $\mu$ M; ▒ KTZ: ketoconazole, 25  $\mu$ M; □ ANF: alpha-naphthoflavone, 50  $\mu$ M. The cell cultures were treated for 2 h. ( $n=4$ /group; \* $P<0.01$ , \*\*\* $P<0.001$ ) Data are shown as mean $\pm$ standard error.



**Figure 4:** Changes in cytochrome enzyme activity in rabbit microsomes. Control: non-treated, PB: treated with 80 mg/kg body weight (BW) phenobarbital for 3 d, KTZ: treated with 40 mg/kg BW ketoconazole for 3 d. (n=4/group; \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ ) Data are shown as mean $\pm$ standard error. (A) CYP1A1 activity. (B) CYP1A2 activity. (C) CYP3A6 activity.

binding to the enzyme's active site. Our results showed that ketoconazole acts as an inducer at the transcriptional level in rabbit CYP1A1 and CYP3A6, *in vitro*. Our findings are in accordance with Korashy *et al.* (Korashy *et al.*, 2007) where ketoconazole was found to induce CYP1A1 at the transcriptional and protein level in human and murine cell lines. Others have found that ketoconazole is an effective CYP1A1 inhibitor in humans (Paine *et al.*, 1999) and rats (Elsherbiny *et al.*, 2008); our experiments are concordant, showing that ketoconazole is also a highly effective inhibitor of rabbit CYP1A1, *in vitro*. In our study, ketoconazole had opposite effects at the transcriptional and protein levels on CYP450 enzymes, *in vitro*. *In vivo*, however, ketoconazole increased CYP1A1 activity. According to the qRT-PCR results, ketoconazole treatment increased rabbit hepatocyte CYP1A1 and CYP3A6 gene expression of hepatocytes



**Figure 5:** Relative gene expression of the CYP genes in rabbits. ■ PB: phenobarbital, 80 mg/kg body weight (BW), □ KTZ: ketoconazole, 40 mg/kg BW. The rabbits were treated orally for 3 d. (n=4/group; \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ ) Data are shown as mean $\pm$ standard error.

PCR results are shown in Figure 5. After 3 d of phenobarbital treatment, gene expression levels were increased for all 3 isoenzymes. Ketoconazole treatment did not alter the expression of CYP1A1 and CYP1A2, but increased expression of the CYP3A6.

## DISCUSSION

Rabbits provide an excellent model for investigating the CYP450 system, as they have high initial enzyme activity compared to other lab species such as rodents (Nebbia *et al.*, 2003; Fink-Gremmels, 2008). Primary hepatocyte cell cultures are a suitable tool for assaying enzyme induction and inhibition, and the rapid degradation of CYP enzymes in culture (Paine, 1996; Eeckhoutte *et al.*, 2002) can be obviated by using 4-h cultures (Beigel *et al.*, 2008; Noel *et al.*, 2013).

Phenobarbital-mediated induction is regulated at the transcriptional level (Moore *et al.*, 2002), while ketoconazole (Ekroos and Sjögren, 2006) and  $\alpha$ -naphthoflavone (Cho *et al.*, 2003) cause conformational changes in the enzyme structure by



*in vitro*, but the gene expression level of CYP1A1 remained unchanged *in vivo* after 3 d of ketoconazole treatment. Ketoconazole upregulated the gene expression of CYP3A6 both *in vitro* and *in vivo*, in line with previous findings that ketoconazole increased the gene expression of CYP3A4 in human cell lines and human primary hepatocytes (Novotna *et al.*, 2014). This is the first proof of the inducer effect of ketoconazole on CYP1A1 enzyme activity *in vivo* in rabbits.

The inducer effect of phenobarbital was most pronounced in the CYP3A6 enzyme *in vitro*, and is in line with phenobarbital's known stimulating effect on the CYP3A subfamily (Ohno *et al.*, 2009). Phenobarbital treatment caused increased gene expression and increased CYP enzyme activity in all examined isoenzymes both *in vitro* and *in vivo*.

Luciferin-IPA is the most sensitive substrate for CYP3A4 (Cali, Ma *et al.*, 2006). This substrate worked well with the rabbit CYP3A6 enzyme, providing further evidence of the functional similarity of the 2 enzymes. The RLU values obtained for all 3 CYP activities in rabbit hepatocytes are very comparable with RLU values found in human hepatocytes HepG2 (Yueh *et al.*, 2005) and HepaRG (Mueller *et al.*, 2014), and in freshly isolated and cryoplateable hepatocytes (Moeller *et al.*, 2011). The Luciferin-PFBE substrate did not produce detectable signal with the rabbit CYP3A6 enzyme. Others have used the HepG2 human hepatoma cell line with the same cell numbers as used in our study and found RLU of approximately 1000 in 2D cell cultures (Lan *et al.*, 2010). In another study, recombinant (yeast) CYP3A4 at a concentration of  $5 \times 10^7$  cells/mL in 200  $\mu$ L medium was used, producing an RLU value of approximately 300 (Neunzig *et al.*, 2011). According to these and our studies, the Luciferin-PFBE substrate did not produce a strong luminescent signal with either human CYP3A4 or rabbit CYP3A6.

Compared to other cytochrome assays, the P450-Glo™ Assays have key advantages. For example, the Masters method (Masters *et al.*, 1967) is not specific for individual isoenzymes and can only provide indirect measurement of overall cytochrome activity. While the similarly indirect Nash method (Nash, 1953) does provide information regarding specific CYP isoenzymes, it is insufficiently sensitive to register most xenobiotic-induced changes. Indirect but isoenzyme-specific measurement is possible with high-performance liquid chromatography (Lahoz *et al.*, 2008; Lee *et al.*, 2013) and Western blot (LeCluyse, 2001), but these methods are complex and problematic because the amount of CYP protein is not directly proportional to CYP activity. Analytical methods based on  $^3\text{H}$ - or  $^{14}\text{C}$ -labeled drug substrates are accurate but require protective equipment, subsequent decontamination and specialised waste disposal (Zlokarnik *et al.*, 2005). The fluorescence methods suffer from optical interference between test substances and CYP substrates (Cali, Ma *et al.*, 2006). In contrast to these, the P450-Glo™ CYP assay provides a simple and specific method for the detection of cytochrome activity.

In conclusion, the cytochrome P450 luminescent method is a fast, safe, simple and sensitive tool for testing the effect of active substances on the rabbit's cytochrome P450 system. Our *in vitro* findings were largely consistent with the *in vivo* results, validating the short-term primary hepatocyte cell culture method in xenobiotic metabolism investigations and in short-term toxicity screening. This therefore appears to be an effective and cost-effective way to progress much-needed new medicines to registration in rabbits.

Furthermore, the changes in rabbit CYP1A1 and CYP3A6 enzymes after phenobarbital and ketoconazole exposure ran in parallel with those previously described for human CYP1A1 and CYP3A4 enzymes. These results suggest that the rabbit is a good model for preliminary pharmacological investigations.

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