Liverbeads™: a practical and relevant in vitro model for gene induction investigations

Ihab Al Khansa, Olivier Blanck, André Guillouzo and Rémi Bars

Bayer Cropscience, Toxicologie Recherche, Sophia-Antipolis, France (IAK, RB, OB); Inserm UMR 991, Faculté des Sciences Pharmaceutiques et Biologiques, Rennes, France (IAK, AG); Université Rennes 1, Rennes, France (IAK, AG).

Copyright 2010 by the American Society for Pharmacology and Experimental Therapeutics.
Running title: CYP and UGT genes inducibility in rat Liverbeads™

Corresponding author:

Olivier Blanck, BayerCropscience, Toxicologie Recherche, B.P. 153, 06903 Sophia-Antipolis, France. Tel.: +33-(0)492943441, Fax: +33-(0)493958454.

E-mail: olivier.blanck@bayercropscience.com

Number of text pages: 24
Number of tables: 2
Number of figures: 3
Number of references: 40
Number of words in Abstract: 239
Number of words in Introduction: 518
Number of words in Discussion: 1137

Abbreviations used are: AhR, aryl hydrocarbon receptor; BNF, beta-naphthoflavone; CAR, constitutive androstane receptor; CLO, clofibrac acid; CTM, clotrimazole; P450, cytochrome P450; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; PB, phenobarbital; PCN, pregnenolone 16α-carbonitrile; PHE, phenytoin; PPARα, peroxisome proliferator-activated receptor alpha; PXR, pregnane X receptor; UGT, UDP-glucuronosyltransferase.
Abstract

Cryopreserved rat hepatocytes entrapped within an alginate matrix commercially available as Liverbeads™, were evaluated for their relevance as a screening tool for gene induction in vitro, using qRT-PCR. They were treated with the reference compounds beta-naphthoflavone (BNF), phenobarbital (PB), pregnenolone 16α-carbonitrile (PCN) and clofibric acid (CLO) and analyzed for mRNA levels of Cyp1a1, Cyp2b1, Cyp3a1, Cyp4a1, Ugt1a6 and Ugt2b1. In addition, for PB and PCN, the results were compared with those obtained in rat liver in vivo. For each inducer the gene induction profiles obtained with the Liverbeads™ in vitro model were time- and dose- dependent. The in vitro gene expression profiles confirmed the corresponding known P450 and UGT induction by each reference compound. Specifically, the most strongly induced genes were Cyp1a1 by BNF, Cyp2b1 by PB, Cyp3a1 and Ugt2b1 by PCN and Cyp4a1 and Cyp2b1 by CLO. Other genes investigated were also induced by the reference compounds but the expression levels were lower and increases were seen only after prolonged treatment. Specifically, Ugt1a6 and Cyp2b1 were increased by BNF, Cyp1a1, Cyp3a1 and Ugt2b1 by PB and Cyp3a1 and Ugt2b1 by CLO. All these results correlated well with published in vitro data and our in vivo data. In conclusion, our results suggest that Liverbeads™ is a relevant and useful in vitro screening tool for determining gene induction profiles of new molecules. In addition, as Liverbeads™ from different species are available, this tool offers the possibility to conduct interspecies comparisons.
Introduction

Research and development of new chemicals is costly and time-consuming, which makes the identification of potential toxicity for new chemical entities critical during the early phase of their development. Hepatotoxicity is a very important cause of drug development arrest. Chemical-induced changes in the level of expression of cytochrome P450 (P450) and other drug metabolizing genes can be indicators of liver tumor formation in rodents (Whysner et al., 1996) and are a key cause of drug-drug interactions. Hepatocyte-based screening in toxicology is largely used in order to reduce animal use and compounds development costs. Fresh and cryopreserved hepatocytes have long been considered as the most suitable in vitro model system for metabolism and toxicity studies. However, since rat primary hepatocyte monolayers exhibit a rapid decline in their P450 activities, various 2D (sandwich configuration for example) and 3D culture conditions have been proposed to improve both cell survival and metabolic competence (Guillouzo, 1998; Chia et al., 2000; Hewitt et al., 2007). 3D models are receiving growing interest; among them gel entrapment offers the advantage of being highly protective during the freeze/thaw process (Guyomard et al., 1996; Rialland et al., 2000). Indeed, after thawing hepatocyte viability is only slightly decreased and P450 activities remain expressed at levels comparable to those measured in primary hepatocyte monolayer cultures. Commercially available as Liverbeads™, alginate-entrapped hepatocytes from various species are a ready to use laboratory reagent, immediately available to laboratories without requiring any particular expertise in hepatocytes isolation.

Liverbeads™ have been used for interspecies xenobiotic metabolism investigations (identification of metabolite structures and analysis of metabolic clearance) (Guyomard et al., 1996), determination of drug-metabolizing enzyme activities after xenobiotic exposure (Guyomard et al., 1996; Rialland et al., 2000) and evaluation of cytotoxic (Biagini et al., 2006)
and genotoxic (Vian et al., 2002) effects of chemicals. However, their relevance as a screening tool for xenobiotic metabolism gene induction following treatment with chemicals has not yet been evaluated. The objective of the present study was, therefore, to evaluate the effects of four reference inducers phenobarbital (PB), beta-naphthoflavone (BNF), clofibric acid (CLO) and pregnenolone 16α-carbonitrile (PCN) on four major CYPs and two UGTs in cryopreserved rat Liverbeads™ by studying changes in mRNA levels using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). This method is now recognized as being reproducible and sensitive for identifying compounds with P450 induction ability in the early phase of drug discovery (Goodsaid et al., 2003; Baldwin et al., 2006). The four P450s investigated, Cyp1a1, Cyp2b1, Cyp3a1 and Cyp4a1, are known to be mainly induced by BNF, PB, PCN and CLO respectively. These four inducers are ligands of the major nuclear receptors AhR, CAR, PXR and PPARα respectively (Lindros et al., 1998; Madan et al., 1999; Kretschmer and Baldwin, 2005; Tamura et al., 2006; Nishimura et al., 2007). The two UGTs, Ugt1a6 and Ugt2b1, are considered as the founding members of the current two evolutionarily conserved UGT families and are induced by AhR and CAR ligands respectively. Finally, to confirm our in vitro findings, rats were treated with either PB or PCN and expression profiles for the same P450s and UGTs were established using liver samples.
Materials and Methods

Chemicals

Phenobarbital (PB), beta-naphthoflavone (BNF), clofibric acid (CLO), pregnenolone 16α-carbonitrile (PCN), phenytoin (PHE), clotrimazole (CTM) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France). Leibovitz-L15 (L15), HepatoZYME-SFM media and fetal calf serum (FCS) were supplied by Invitrogen (Cergy Pontoise, France). All chemicals used were of the highest quality available.

Cell culture and treatments

Cryopreserved male Wistar Rat Liverbeads™ were purchased from Biopredic International (Rennes, France) and cultured according to the manufacturer’s instructions. Briefly, Liverbeads™ were rapidly thawed at 37°C and then put in L15 medium containing 0.6M glucose, 10mM Hepes, 100 IU/ml penicillin and 100µg/ml streptomycin and then distributed in 12-well plates (0.2 ml beads in 1 ml medium per well) for 3 hours in HepatoZYME-SFM medium containing 10% FCS, 4 µg/ml insulin, 2mM L-glutamine, 100 IU/ml penicillin and 100µg/ml streptomycin, at 37°C in 5% CO2/95% air. This medium was then replaced with the same medium supplemented or not with 1µM hydrocortisone hemisuccinate, deprived of FCS and containing the different test compounds or the vehicle. Liverbeads™ were thus exposed to 4 different concentrations of the test compounds PB (100, 300, 1000 and 3000 µM), BNF (1, 5, 10 and 20 µM), CLO (100, 200, 400 and 1000 µM) and PCN (1, 5, 10 and 30 µM) or to the vehicle control DMSO (0.2% final concentration) for 24, 48 or 72 h. Additional cultures were exposed to two concentrations of phenytoin (PHE) (10 and 200 µM) and clotrimazole (CTM) (2.5 and 10 µM) for 24 and 48 h. The test compound concentrations were selected from preliminary cytotoxicity experiments.
**In vivo dosing and experimental design**

Male Wistar Rj:WI (IOPS HAN) rats, 6 week-old, were purchased from R. Janvier (Le Genest St Isle, France). Animal handling and experiments were performed in accordance with The Guide for the Care and Use of Laboratory Animals (Council, 1996). Each animal was individually housed in a stainless steel wire mesh cage under controlled environmental conditions and received drinking water and pelleted rodent diet (Scientific Animal Food and Engineering, Augy, France) *ad libitum*. After a 5 days acclimation and one day prior to treatment, each rat was assigned to a group by the stratified randomization method based upon the body weight. Animals (5 per group) were treated with a low (15 and 25 mg/kg/day) and a high (75 and 125 mg/kg/day) dose of PB and PCN respectively, by daily gavage for 7 days. Doses were chosen from literature (Guzelian et al., 2006; Nie et al., 2006; Slatter et al., 2006; Nioi et al., 2008). Corn oil was used as a vehicle for all the compounds. Clinical observations were performed daily and body weights and physical examinations were recorded weekly. Twenty-four hours after the last dose, all animals were sacrificed by isoflurane (Baxter, Maurepas, France) inhalation followed by exsanguination under deep anesthesia. At necropsy, small pieces of the median and the left lobes of the liver of each animal were collected and flash-frozen in liquid nitrogen prior to storage at –80°C for the purpose of these investigations.

**Total RNA isolation and quality control**

Total RNA was isolated from individual control and treated liver samples or from Liverbeads™ cell lysates samples using RNeasy Mini kits (Qiagen, Valencia, CA) in accordance with manufacturer’s instructions. Resulting total RNA was quantified using a NanoDrop ND-1000 (NanoDrop Technologies, Inc., Montchanin, DE). RNA integrity (28S/18S ratio) and purity were assessed using RNA 6000 nano assay LabChips® (Agilent Technologies, Santa Clara, CA) and analyzed on a 2100 Bioanalyzer (Agilent Technologies).
Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis

One (liverbeads) or ten (in vivo samples) micrograms of total RNA from control and treated samples were used for reverse transcription with random hexamer primers using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. Each sample was assayed in duplicate. qPCR reactions were performed using an ABI prism 7900 HT apparatus (Applied Biosystems). 1/15 (in vitro) or 1/50 (in vivo) diluted first-strand complementary DNA was mixed with Taqman probes (Assay on demand, Applied Biosystems: Cyp1a1, NM_012540.2; Cyp2b1, NM_001134844.1; Cyp3a1, NM_013105.1; Cyp4a1, NM_175837.1; Ugt1a6, NM_057105.3; Ugt2b1 or Udpgtr2, NM_173295.1) and with AmpliTaq Gold PCR Master Mix. \( \beta \)2 microglobulin (B2m, NM_012512.2) was selected as the housekeeping gene for quantitative calculations.

For sample distribution evaluation, relative gene expression was calculated following the Livak and Schmittgen (2001) \( \Delta \Delta C_t \) method. Briefly, mRNA was normalized to a calibrator; in each case, the calibrator was a basal sample (corresponding to one animal from the vehicle-treated group or one control well). Final results were expressed as the \( n \)-fold difference (or RQ, Relative Quantity) in gene expression relative to B2m mRNA and calibrator as follows:

\[ n\text{-fold} = 2^{-\left(\Delta C_t \text{ sample} - \Delta C_t \text{ basal}\right)} \]

where \( \Delta C_t \) values of the sample and calibrator were determined by subtracting the average \( C_t \) value of the transcripts from the average \( C_t \) value of B2m mRNAs for each sample. RQ of the control sample was always equal to one. A change was considered as significant when RQ was above 1.5 or below 0.5.

Statistical analysis

Statistical analysis was performed using a two-way ANOVA test with a Bonferroni post-hoc test. The values were expressed as mean ± SD and a p-value ≤0.05 was considered as statistically significant.
Results

Steady-state RNA levels in Liverbeads during culture

mRNA levels of Cyp1a1, Cyp2b1, Cyp3a1, Cyp4a1, Ugt1a6 and Ugt2b1 were measured in rat Liverbeads™ after 0, 24, 48 and 72 h of culture in a medium containing 0.2% DMSO and 1µM hydrocortisone hemisuccinate (Fig. 1). Compared to values found just after thawing (0 h), those measured in 24 h cultures were either unchanged (Cyp3a1), significantly increased (Cyp1a1, Ugt1a6) or decreased (Cyp2b1, Cyp4a1, Ugt2b1). At 48 h, with the exception of Cyp4a1 mRNA which was slightly more decreased, transcript levels of all other genes were enhanced (Cyp2b1: 10 to 20-fold and Ugt2b1: 15 to 30-fold) and Cyp3a1 transcript levels were around 6-fold increased, compared to 24 h. At 72 h, for all the genes tested, the transcript levels were relatively unchanged (Cyp4a1) or still higher (all other genes) compared to the 48 h time point. Either 1µM hydrocortisone (data not shown) or 0.2% DMSO (supplemental Table 1S) had no influence on the levels of the transcripts whatever the gene tested and the time of culture.

Time- and concentration-dependent responsiveness of Liverbeads™ following exposure to reference compounds

The effects of BNF, PB, CLO and PCN on the 6 drug metabolizing genes of interest were studied in Liverbeads™ after 24, 48 and 72 h of exposure. Several concentrations of each compound were selected from preliminary experiments. The highest concentration used was based on either the maximum non-toxic concentration after 24 and 72 h treatment using the Gerlier and Thomasset (1986) XTT cytotoxicity assay (data not shown) or the maximum soluble concentration. One batch of Liverbeads™ was used in triplicate. The maximum inductions of various CYP and UGT transcript levels observed as a function of time or as a function of concentration (at a chosen time point where the highest inductions were observed) are
summarized in Table 1A and Table 1B, respectively. All corresponding data with means and SDs are displayed in the supplemental Table 2S.

BNF strongly induced Cyp1a1 mRNA levels (Table 1A and 1B) in a concentration-dependent manner and maximum induction was observed after a 24h exposure with 10µM. In addition Ugt1a6 and Cyp2b1 transcripts were also moderately increased, not exceeding 3-fold, with the exception of Cyp2b1 which was increased 7-fold after 72h with the highest BNF concentration (20µM). No noticeable changes were observed for the three other genes.

PB induced a strong increase in Cyp2b1 mRNA levels (Table 1A and 1B). This increase was already high after a 24h treatment with the lowest concentration (100 µM) and the highest fold-change reached 200 at 48 hr with 3000µM. PB also induced, but to a lower extent, Ugt2b1, Cyp1a1 and Cyp3a1 mRNA levels, mainly after 48 and 72h. Other genes were either unchanged (Ugt1a6) or even decreased (Cyp4a1 at 72 h).

CLO mainly induced Cyp4a1 gene (Table 1A and 1B). A concentration- and time-dependent increase in mRNA levels was observed, with the highest values reached at 48 hr (65-fold with 400µM). CLO also strongly increased Cyp2b1 transcripts in a concentration-dependent manner, changes reaching around 12-fold after 24 or 48h and 23-fold after 72 h in the presence of 400µM. In addition Cyp3a1 was slightly increased (2 to 4-fold) dose-dependently after 72 h treatment. Cyp1a1 and Ugt1a6 mRNA levels were unchanged and Ugt2b1 expression was decreased starting 48 h.

PCN mainly induced Cyp3a1 and Ugt2b1 in a concentration- and time-dependent manner (Table 1A and 1B). The highest values were obtained with 10µM after 72 h and did not exceed 4-fold. No obvious changes were recorded for the transcript levels of Cyp1a1, Cyp2b1 and Ugt1a6 and those of Cyp4a1 were slightly decreased after 48 and 72 h treatment.
The reproducibility of the responsiveness of Liverbeads™ batches to reference inducers was confirmed by treating 3 other different Liverbeads™ batches with two concentrations of PB (100 and 1000 µM) and PCN (2 and 10 µM) for 24, 48 and 72h. The data displayed in Figure 2 mostly confirmed those obtained with the original batch (Table 1A and 1B). Indeed, Cyp2b1 transcripts were strongly induced by PB in a time-dependent manner with the levels being slightly higher with 1000µM versus 100µM. Cyp1a1, Cyp3a1 and Ugt2b1 were also induced but to a much lower extent than Cyp2b1 and usually only after 48 and 72h of treatment with the 1000µM PB concentration. The initial results obtained with PCN (Table 1) were confirmed (Fig 2B). A limited but significant increase in Cyp3a1 and Ugt2b1 was observed as early as 24 h after exposure to 2 and 10µM PCN. In addition, Cyp2b1 was also increased but only after 24 h treatment. Ugt1a6 remained unchanged and Cyp4a1 expression was decreased after exposure to 10µM PCN for 48 and 72h. No change in the fold-inductions was observed in Liverbeads™ cultured in hydrocortisone-free medium compared to in the presence of hydrocortisone with the exception of Cyp3a1 which was slightly more increased (2- versus 1.5-fold) in the presence of hormone (data not shown).

The responsiveness of Liverbeads™ to known inducers which are modulators of CAR and PXR was confirmed by treatment with PHE and CTM for 24 and 48 h. PHE was used at 10 and 200µM and CTM at 2.5 and 10µM. The data, displayed in Table 2, showed that the effects of these two inducers were very close to those obtained with their corresponding CAR and PXR ligands, PB and PCN, respectively. They induced the same P450s and UGT to a similar extent in a dose-dependent manner.
Finally, to determine whether Liverbeads™ are representative of the *in vivo* liver for xenobiotic gene induction screening, rats were also treated with two doses of PB and PCN by daily gavage for 7 days (Fig. 3). As shown in Figure 3A, at the low dose of 15mg/kg, PB significantly induced only the two most sensitive genes, Cyp2b1 and Ugt2b1, while at 75mg/kg the expression of three additional genes, Cyp1a1, Cyp3a1 and Ugt1a6, was increased. These *in vivo* results correlated well with the *in vitro* findings (Table 1A and 1B and Fig. 2A) as the most sensitive genes *in vitro* were also Cyp2b1 and Ugt2b1. Moreover the induction was dose-dependent as the fold-inductions of Cyp2b1 and Ugt2b1 reached 180 and 85 with the highest dose versus 75 and 30 with the lower dose, respectively. At the lowest dose of 25mg/kg, PCN significantly increased both Cyp3a1 and Ugt2b1 (Fig. 3B), as was seen *in vitro*. Only a slight additional increase was observed with the dose of 125mg/kg. A 4- and 2-fold increase of Cyp1a1 and Cyp2b1 respectively was also observed while the 2 other genes (Cyp4a1 and Ugt1a6) remained unchanged.
Discussion

It is well established that P450 expression and activity rapidly decline in conventional hepatocyte monolayers, resulting in a low biotransformation of xenobiotics and in the loss of responsiveness to some metabolic enzyme inducers, especially PB (Su and Waxman, 2004; Nishimura et al., 2007). The loss of P450 expression and activity does not affect all P450s in the same way and even a transient increase of Cyp1a1 has been observed (Wortelboer et al., 1990). Phase 2 enzymes undergo a similar loss (Kern et al., 1997). Although a less rapid decline in P450 performance is observed in rat hepatocytes maintained in the 2D sandwich configuration (Schuetz et al., 1988; De Smet et al., 2001; Sidhu et al., 2004), there are limitations to such extracellular matrix-based sandwich cultures. These limitations are due to mass transfer barriers and the fact that protocols for RNA and protein extraction from such cultures are either time-consuming or contain steps that may skew the results. We show here that, contrary to rat hepatocytes in monolayers or in a sandwich configuration, alginate-entrapped rat hepatocytes exhibited a slight or marked increase in transcript levels for most CYP (with the exception of Cyp4a1 which showed a decrease then stabilized) and UGT genes tested between 24 and 72 h in control culture conditions, indicating that in this configuration cryopreserved rat hepatocytes maintained a good expression of genes involved in xenobiotic metabolism.

Several studies have shown that cryopreservation does not alter levels of total cytochrome P450 and phase 1 enzyme activities when measured shortly after thawing in hepatocytes plated in conventional monolayer or in a sandwich configuration (Chesne et al., 1993; Madan et al., 1999) or immobilized in alginate gels (Rialland et al., 2000). However changes in the levels of corresponding mRNAs usually occur early and are much more drastic in conventional monolayer compared to Liverbeads™. Therefore the fact that cryopreserved alginate-entrapped hepatocytes showed increased mRNA levels between 24 and 72 h of culture and were strongly responsive to
most of the reference inducers tested is of major importance for metabolism gene induction investigations.

Indeed, consistent with previous studies in vivo (Heuman et al., 1982; Wrighton et al., 1985; Baldwin et al., 2006) and in vitro (Kocarek et al., 1995; Meredith et al., 2003), a marked increase was observed in mRNA levels of induced P450s after treatment with corresponding reference inducers. BNF was an effective inducer of Cyp1a1 and Ugt1a6; PB of Cyp2b1, Ugt2b1 and Cyp3a1; PCN of Cyp3a1 and Ugt2b1 and CLO of Cyp4a1. Induction of Cyp3a1 by PB and Cyp2b1 by PCN can be explained by the crosstalk between the nuclear receptors CAR and PXR (Moore et al., 2000; Pascussi et al., 2008). In addition, in agreement with other reports, transcripts of additional CYPs were found to be increased in Liverbeads™ but usually only after prolonged treatment and at a lower extent compared to the major P450s. For example, we observed an increase in mRNA levels of Cyp2b1 with BNF and CLO which was coherent with previously published data (Richert et al., 2003; Richert et al., 2009). The slight increase of Cyp1a1 by PHE could be related to its PB-like properties. Some discrepancies were also noticed. Thus, Cyp1a1 was induced by PCN in rat liver but the fold induction was low and observed after 7 days of treatment with the highest dose (125mg/kg). Likely, small fold-inductions of drug metabolizing enzymes, occurring in vitro or in vivo after several days of exposure and usually with high chemical doses, involved different mechanisms than those implicated in the induction of early responsive genes.

The maximum fold induction of individual P450 genes varied greatly reaching for example 200-fold for Cyp2b1 with PB, 65-fold for Cyp4a1 with CLO, 14-fold for Cyp1a1 with BNF and 2-fold for Cyp3a1 with PCN. However it must be borne in mind that Cyp1a1, for example, is constitutively expressed at extremely low levels in the rat liver and that among the various parameters that can modulate the responsiveness of P450s (Burczynski et al., 2001), their
basal level is a critical parameter. The highest fold inductions are usually observed when basal values are low (Guillouzo and Guguen-Guillouzo, 2008). Only Cyp3a1 exhibited a limited fold induction level, not exceeding 2-fold and lower than the values found in other hepatocyte culture models (Kocarek et al., 1995; Meredith et al., 2003). However the increase was statistically significant after treatment with both PCN and CTM. It is interesting to note that this P450 was the most stable during the first 24h. The slightly higher fold-induction of Cyp3a1 in hydrocortisone-free medium could reflect a slight induction effect of this hormone on this P450 without any chemical treatment. An important finding was the very good reproducibility of the data between the different batches of Liverbeads making this model an appropriate in vitro model for xenobiotic gene induction studies.

Several studies have reported induction by DMSO of several P450s, especially the CYP3A subfamily, in both primary hepatocytes (LeCluyse et al., 2000; Su and Waxman, 2004; Lin, 2006) and hepatoma cell lines (Aninat et al., 2006). Although most effects were obtained with a 2% DMSO concentration, some increase was also evidenced with concentrations as low as 0.1% in primary human hepatocytes (LeCluyse et al., 2000). In the present study we did not observe any significant effect of DMSO in the transcript levels of all P450s tested in the presence of 0.2% DMSO. A likely explanation is that we used rat hepatocytes and that the basal P450 levels are better preserved in Liverbeads™ than in primary hepatocyte cultures.

Our in vitro results with the Liverbeads™ model were in line with our in vivo studies with PB and PCN which are in agreement with previous in vivo studies in which similar dose levels were used and similar P450 induction profiles obtained (Wyde et al., 2003; Guzelian et al., 2006; Slatter et al., 2006). Indeed, PB and PCN significantly induced Cyp2b1 and Ugt2b1, and Cyp3a1 and Ugt2b1 respectively, in both situations. Consequently, although it would be of interest to analyze more P450s, such as the main constitutively expressed Cyp2c11 and Cyp3a2 and phase II
enzymes, it may be concluded that our results exhibit striking similarity in the expression profiles of genes encoding for the major inducible xenobiotic metabolizing enzymes between the Liverbeads™ model and the liver in vivo.

In conclusion our results show that Liverbeads™ represent a suitable model system for investigating P450 and UGT induction by xenobiotics. This model presents several major advantages over other hepatocyte culture models. It is an attractive procedure for banking hepatocytes in large yields and for their transportation. Liverbeads™ are easy to handle and respond to reference inducers after long-term storage in liquid nitrogen. Since hepatocytes from various species can be entrapped in alginate gels, this model offers a unique approach for interspecies comparison of gene expression changes induced by drugs, pesticides and other chemicals.
Acknowledgments:

This work was partially financed by “Association Nationale de la Recherche et de la Technologie” (ANRT) which granted a PhD scholarship to Ihab Al Khansa. The authors thank Dr. Helen Tinwell for helpful comments and critical reading of the manuscript, and Muriel Totis, Agnès Arnaud, Marie-Pierre Côme and Claire Guyomard for their help and useful advice.
References


metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab Rev 39:159-234.


Figure Legends

Figure 1: mRNA levels of Cyp1a1, Cyp2b1, Cyp3a1, Cyp4a1, Ugt1a6 and Ugt2b1 in rat Liverbeads™ after 0, 24, 48 and 72 h in culture in a medium containing 0.2% DMSO. Experiments were performed in triplicate using three different batches of Liverbeads™ except for 0 h where only one batch was used. The values are mean ± SD; 24 h values were set up arbitrarily to 1 and were used as the reference group was used for comparisons. *: p ≤ 0.05; **: p ≤ 0.01.

Figure 2: Dose and time effects of PB (A) and PCN (B) exposure on the mRNA levels of Cyp1a1, Cyp2b1, Cyp3a1, Cyp4a1, Ugt1a6 and Ugt2b1 in rat Liverbeads™ cultures. Experiments were performed in triplicates using three different batches of rat Liverbeads™, and the values are as mean ± SD (n=3). For each time-point, comparisons were made to the corresponding untreated cultures. *: p ≤ 0.05; **: p ≤ 0.01; ***: p ≤ 0.005.

Figure 3: Dose effects of PB (A) and PCN (B) exposure on the mRNA levels of Cyp1a1, Cyp2b1, Cyp3a1, Cyp4a1, Ugt1a6 and Ugt2b1 in rat livers. Gene expression was analyzed from 5 different rat livers per treatment group, and the values are shown as mean ± SD (n=5). Doses are in mg/kg/day. Comparisons were made to the corresponding untreated livers. *: p ≤ 0.01; **: p ≤ 0.005; ***: p ≤ 0.001.
Tables

Table 1A: Maximum induction of various CYPs and UGTs by prototypical inducers in Liverbeads™ as a function of time in culture. Four concentrations were investigated for each compound: PB (100, 300, 1000 and 3000 µM), BNF (1, 5, 10 and 20 µM), CLO (100, 200, 400 and 1000 µM) and PCN (1, 5, 10 and 30 µM). Maximum fold inductions, obtained with the highest concentrations used, are given for each time-point. Fold inductions were arbitrarily set as follows: between 2 and 5: +; 5 and 10: ++; > 10: +++; *: decreased values (< 1) and -: no change.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time exposure (h)</th>
<th>P450</th>
<th>Ugt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1a1</td>
<td>2b1</td>
</tr>
<tr>
<td>BNF</td>
<td>24</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>PB</td>
<td>24</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>CLO</td>
<td>24</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>PCN</td>
<td>24</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 1B: Induction of various P450s and UGTs by prototypical inducers in Liverbeads™ as a function of concentration at a selected time point where the highest inductions were obtained (marked in grey in table 1A). The following concentrations were investigated for each compound: PB (100, 300, 1000 and 3000 µM), BNF (1, 5, 10 and 20 µM), CLO (100, 200, 400 and 1000 µM) and PCN (1, 5 and 10 µM). Fold inductions were arbitrarily set as follows: between 1.5 and 2: -/+; 2 and 5: +; 5 and 10: ++; 10 and 30: +++; > 30: ++++; *: decreased values (< 1) and -: no change.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µM)</th>
<th>P450</th>
<th>Ugt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1a1</td>
<td>2b1</td>
</tr>
<tr>
<td>BNF</td>
<td>1</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+++</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>PB</td>
<td>100</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>+/-</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>+/-</td>
<td>++++</td>
</tr>
<tr>
<td>CLO</td>
<td>100</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>PCN</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2: Dose and time effects of PHE and CTM exposure on mRNA levels of Cyp1a1, Cyp2b1, Cyp3a1 and Ugt2b1 in rat Liverbeads™ cultures. Experiments were performed in triplicates using three different batches of rat Liverbeads™, and the values are shown as fold induction means ± SD (n=3). Corresponding control values were set up arbitrarily to 1. *: p ≤0.05.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (µM)</th>
<th>Time exposure (h)</th>
<th>P450</th>
<th>Ugt2b1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1a1</td>
<td>2b1</td>
</tr>
<tr>
<td>PHE</td>
<td>10</td>
<td>24</td>
<td>1.2 ±0.4</td>
<td>3.7 ±1.6</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td></td>
<td>3.6* ±0.8</td>
<td>22.1* ±7.8</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>24</td>
<td>2.4* ±0.8</td>
<td>4.6* ±1.4</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td></td>
<td>6.9* ±0.6</td>
<td>35.2* ±9.4</td>
</tr>
<tr>
<td>CTM</td>
<td>2.5</td>
<td>24</td>
<td>1.2 ±0.07</td>
<td>31.8* ±10.4</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td></td>
<td>2.2* ±0.3</td>
<td>86.9* ±20.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>24</td>
<td>2.4* ±0.7</td>
<td>56.3* ±9.4</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td></td>
<td>3.7* ±0.7</td>
<td>189.8* ±31.6</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2A
**Figure 2B**

### CYP1A1

- **24h:** Bar heights vary with PCN concentration.
- **48h:** Bar heights vary with PCN concentration.
- **72h:** Bar heights vary with PCN concentration.

### CYP2B1

- **24h:** Bar heights vary with PCN concentration.
- **48h:** Bar heights vary with PCN concentration.
- **72h:** Bar heights vary with PCN concentration.

### CYP3A1

- **24h:** Bars show a significant increase with PCN concentration.
- **48h:** Bars show a significant increase with PCN concentration.
- **72h:** Bars show a significant increase with PCN concentration.

### CYP4A1

- **24h:** Bars show a significant increase with PCN concentration.
- **48h:** Bars show a significant increase with PCN concentration.
- **72h:** Bars show a significant increase with PCN concentration.

### UGT1A6

- **24h:** Bars show a significant increase with PCN concentration.
- **48h:** Bars show a significant increase with PCN concentration.
- **72h:** Bars show a significant increase with PCN concentration.

### UGT2B1

- **24h:** Bars show a significant increase with PCN concentration.
- **48h:** Bars show a significant increase with PCN concentration.
- **72h:** Bars show a significant increase with PCN concentration.
Figure 3A
Figure 3B