Follow-up to the pre-validation of a harmonised protocol for assessment of CYP induction responses in freshly isolated and cryopreserved human hepatocytes with respect to culture format, treatment, positive reference inducers and incubation conditions

Catherine Abadie-Viollon a,1, Hélène Martin b,1, Nadège Blanchard a,1, Dumrongsak Pekthong c, Philippe Bachellier d, Georges Mantion e, Bruno Heyd e, Frantz Schuler f, Philippe Coassolo f, Eliane Alexandre a, Lysiane Richert a,b, *

a KaLy-Cell, Bioparc, Boulevard Sébastien Brant, 67400 Illkirch, France
b Faculté de Médecine et de Pharmacie, Laboratoire de Toxicologie Cellulaire, EA 258P, IFR 133, 25030 Besançon, France
c Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000, Thailand
d Centre de Chirurgie Viscérale et de Transplantation, Hôpital de Hautepierre, 67088 Strasbourg, France
e Service de Chirurgie Viscérale et Digestive – Centre de Transplantation Hépatique, Hôpital Jean Minjoz, 25000 Besançon, France
f Drug Metabolism and Pharmacokinetics, Hoffmann-La Roche Ltd., Grenzacherstrasse 124, 4070 Basel, Switzerland

**ABSTRACT**

We have compared induction responses of human hepatocytes to known inducers of CYP1A2, CYP2B6, CYP2C and CYP3A4/5 to determine whether the culture format, treatment regimen and/or substrate incubation conditions affected the outcome. CYP induction responses to prototypical inducers were equivalent regardless of pre-culture time (24 h or 48 h), plate format (60 mm or 24-well plates) used or whether CYP activities were measured in microsomes or whole cell monolayers. Fold-induction of CYP3A4/5 by 1000 μM PB and 10 μM RIF were equivalent. In contrast, the fold-induction of CYP2B6 by PB was 3-fold higher that by 10 μM RIF. In addition to inducing CYP1A2, 50 μM OME also induced CYP3A4/5 in 50% of the donors tested. CYP2B6 was induced in 14 out of 21 donors by BNF; however CYP3A4/5 was unaffected by BNF in these donors. In order to confirm that donor-to-donor variation was not due to inter-laboratory differences, the induction responses of 5 different batches of cryopreserved human hepatocytes were compared in two different laboratories. The induction of CYP1A2, CYP2B6 and CYP3A4 measured in our laboratory were equivalent to those obtained by the commercial companies, proving good between-laboratory reproducibility.

In conclusion, there is some flexibility in the treatment and incubation protocols for classical CYP induction assays on human hepatocytes. Both RIF and PB are suitable positive control inducers of CYP3A4/5 but PB may be more appropriate for CYP2B6 induction. BNF may be more appropriate for CYP1A2 induction than OME since, in contrast to the latter, it does not induce CYP3A4. Induction responses using hepatocytes from the same donor but in different labs can be expected to be similar. The good reproducibility of induction responses between laboratories using cryopreserved hepatocytes underlines the usefulness of these cells for these types of studies.

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1. Introduction

The induction of drug metabolising enzymes (DMEs) can result in deleterious effects such as drug–drug interactions, increased toxicity and failure to reach a therapeutic plasma concentration. Examples of drugs that induce human DMEs include rifampin (RIF), which induces cytochrome P450 (CYP) 3A4/5 (Lin, 2006) and CYP2C9 (Park et al., 2004; Heimark et al., 1987) and omeprazole (OME), which induces CYP1A2 in CYP2C19 polymorphic
patients (Rost et al., 1992; Rost and Roots, 1994). CYP2B6 may also be considered as an important enzyme since it constitutes at least 5% of the total P450, contributes to the metabolism of more than 25% of all pharmaceutical drug metabolism (Wang et al., 2003) and exhibits high inducibility (Drocourt et al., 2001; Xie et al., 2000). Thus, these CYPs account for metabolism of most of the drugs on the market (Zuber et al., 2002). Ideally, drug companies would like to deselect candidate compounds which strongly induce major CYPs before they reach development or clinical trials, therefore, the screening of compounds for their potential to induce CYPs is important. There are significant species-differences in the induction responses of animal and human hepatocytes (see review by Hewitt et al. (2007a)), therefore, the best in vitro model to predict induction in humans is human hepatocytes. There are a number of recommendations set out by the US Food and Drug Administration (FDA) for the conduct of in vitro induction studies and these guidelines allow for a certain amount of flexibility in the methodologies (FDA) for the conduct of in vitro induction studies and these guidelines allow for a certain amount of flexibility in the methodologies (Huang and Stifano, 2006). Our laboratory chaired a European Centre for Validation of Alternative Methods (ECVAM) project to investigate the inter-laboratory and intra-laboratory reproducibility of an induction protocol (Richert et al., 2009b). The results suggested that the harmonized protocols used to study the responses of human hepatocytes to prototypical inducers were transferable and reproducible within a given laboratory and between laboratories. In order to firmly support our hypothesis that minor differences observed between laboratories were due to donor-to-donor variation and not to laboratory practices, we extended our work to investigate the induction responses of hepatocytes from the same donor in different laboratories. The best way to achieve this was to use cryopreserved human hepatocytes which attach in culture and form confluent monolayers. These are now accepted by the FDA as a valid alternative to fresh human hepatocytes and have been shown to be comparable to fresh hepatocytes in terms of their responses to prototypical CYP1A2 and CYP3A4 inducers (Huang and Stifano, 2006; Hewitt et al., 2007b).

In order to use cryopreserved hepatocytes to assess between-laboratory reproducibility, we first had to consider the way in which CYP activities are measured at the end of the induction period. The simplest and less cell-consuming method is to add the CYP substrate to the (washed) hepatocyte monolayers. However, this does not allow for determination of CYP approteins by western blotting due to high nonspecific binding of the antibodies to the hepatocellular proteins (unpublished observation). The preparation of microsomes from cell monolayers allows for aporotein analysis and multiple CYP activity determination using the same samples. In addition, CYP activities in microsomes prepared from freshly isolated rat hepatocytes have been shown to be comparable with those measured in vivo (Guillouzo, 1986; Wortelboer et al., 1990). Possible disadvantages of the monolayer method to assess CYP induction include the sensitivity of the activity measurement and the effect of residual test compound on the measured activity, especially if it is a competitive inhibitor in addition to being an inducer. An advantage of the direct measurement on monolayers is that it gives a global picture of the whole cell system following treatment with an unknown test compound. Therefore, we have compared the CYP induction responses of rat hepatocytes to prototypical positive inducers measured using microsomes prepared from hepatocyte cultures and direct addition of the substrate to whole cell monolayers.

We extended our studies to investigate other practicalities of the induction protocol, such as the pre-incubation time (i.e., the time between cell seeding and addition of inducers) and the treatment duration for determining changes in mRNA expression. The pre-incubation time allows the cultures to establish a confluent monolayer, which is needed for the cells to respond to inducers (Maurel, 1996). Bjornsson et al. (2003) recommended a 48 h to 72 h recovery period, however, the 2004 and 2006 FDA guidelines do not give any recommendations for this pre-treatment recovery period (Huang, 2004; Huang and Stifano, 2006). In a recent survey, the majority of researchers allowed only 24 h to 48 h for the cells to establish a culture and none pre-incubated cultures for 72 h (Hewitt et al., 2007c). We compared the effect of a 24 h and 48 h pre-incubation period on CYP1A2, CYP2C9, CYP2B6 and CYP3A4/5 induction responses of human hepatocytes to prototypical inducers.

The preferred positive control compounds for CYP2B6 and CYP3A4/5 induction in situ studies are phenobarbital (PB) and RIF, respectively (Huang and Stifano, 2006). PB can also be used as a positive control inducer for CYP3A4/5 induction but RIF is not recommended as an alternative for CYP2B6 induction (Huang and Stifano, 2006). Our ECVAM pre-validation study (Richert et al., 2009b) suggested that PB could indeed be used as a positive control inducer for CYP3A4/5 since both 10 μM RIF and 1000 μM PB induced this CYP to the same extent, while the fold-induction response of CYP2B6 by 1000 μM PB was greater than that by 10 μM RIF. We also found that β-naphthoflavone (BNF) induced CYP2B6 in some donors. Likewise, other positive control inducers have been shown to induce other CYPs than the target enzyme, e.g. OME induces CYP3A4/5 and not only CYP1A2 in human hepatocytes (Roumans et al., 2005). With this in mind, we compared the CYP1A1/2, CYP3A4/5 and CYP2B6 induction responses of human hepatocytes from a number of human hepatocyte cultures from different donors to RIF, OME and BNF.

2. Materials and methods

2.1. Chemicals and reagents

Unless specified, chemicals used in this study were obtained from Sigma–Aldrich (St. Quentin-Fallavier, France). Unless specified, reagents for cell culture were from Invitrogen (Cergy Pontoise, France). Cell culture plastics were purchased by Becton Dickinson (Le Pont-De-Clai, France).

2.2. Source of human livers

Human liver biopsies were from patients undergoing liver resection for different pathologies. All experimental procedures were performed in compliance with French laws and regulations after approval by the National Ethics Committee. Informed consent was obtained from all patients for the use of liver tissue for research purposes. Cryopreserved hepatocytes were from CellzDirect Inc, North Carolina, US and KaLy-Cell, Strasbourg, France. Table 1 shows the donor information for the human hepatocytes used in the present study (data from Result section and Figs. 2–4 and 8). All other donors were from historical data of our laboratory: 16 male and 17 female donors (age range between 29 and 85 years) with similar pathologies to those described in Table 1.

2.3. Hepatocyte isolation, culture and treatment with CYP inducers

Male Wistar rat hepatocytes and human hepatocytes were isolated as described previously by Richert et al. (2002) and Alexandre et al. (2002). All animal experiments were approved by the local authorities and were conducted in compliance with the local animal welfare regulations. Cells with a viability higher than 85% were seeded in 60 mm Biocoat® dishes at a cell density of 4 × 10⁶ cells/dish, 12-well plates at a cell density of 0.75 × 10⁶ cells/dish, or in 24-well Biocoat® multi-well plates at a cell density of 0.25 to 0.35 × 10⁶ cells/well in attachment medium (DMEM containing 5% FCS, 1 μM dexamethasone (DEX) and 4 μg/ml insulin). Seeding
The cells were harvested and transferred to Eppendorf tubes and kept on water–ice. Total RNA was extracted according to the manufacturer's instructions (Invitrogen, France). RNA was quantified by spectrophotometry. cDNA was synthesized from 1 μg of total RNA using the iScript kit from Biorad (France) at 42 °C for 30 min. An aliquot of 5 μl of diluted RT reaction (1:10) was used for real-time PCR amplification by using SYBR Green kit from Biorad (France). The following program was used: a denaturation step at 95 °C for 3 min, and 40 cycles of PCR (denaturation, 95 °C, 10 s; annealing, 58 °C, 1 min) then one cycle at 55 °C for 1 min. In all cases, the quality of the PCR product was assessed by monitoring of a fusion step at the end of the run.

3. Results

3.1. Comparison of substrate incubation methods

Table 2 shows basal CYP1A, CYP2C and CYP3A activities in rat hepatocytes treated with vehicle control (0.1% DMSO) and using different methods for substrate incubations. The activities in microsomal incubations were also expressed per mg hepatocellular protein (determined for each hepatocyte preparation, data not shown) in order to compare basal activities between microsomal and whole cell incubations. CYP1A and CYP2C activities measured in microsomes were similar to those measured in hepatocyte monolayers. CYP3A activities measured in microsomes were marginally lower than in monolayers. All three CYP activities were equivalent when substrates were added directly to hepatocytes cultured in either 60 mm or 24-well plates.

The induction responses of rat hepatocytes cultured in 60 mm plates and 24-well plates to 50 μM BNF (CYP1A) and 10 μM DEX (CYP2C and CYP3A) are shown in Fig. 1. The CYP1A induction response to BNF was marginally but not statistically (P > 0.23) lower when induction was measured in hepatocyte monolayers than in microsomes (approximately 7.5-fold in monolayers compared to 10.5-fold in microsomal incubations). Likewise, the fold-induction of CYP3A by DEX was marginally but not statistically (P > 0.09) lower in monolayers than in microsomal incubations. CYP2C induction by DEX was the same when activities were determined in microsomes and monolayers (both from cells cultured in 60 mm plates). The induction responses of all three CYPs in 60 mm plates and 24-well plates were equivalent.

We also performed the activity measurements in both cell microsomes and hepatocyte monolayers for human hepatocyte cultures of one donor (donor 9) cultured in 60 mm plates. The activities expressed per min and per mg hepatocellular protein, were 3.98 for CYP1A when determined in microsomal incubations and 0.720 when determined directly on monolayers and respectively 14.7 and 15.6 for CYP3A. The induction response of CYP1A to 50 μM BNF was 7-fold when inductions were measured in

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**Table 1**

Donor demographics.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Medical history</th>
<th>Fresh (FR) or cryopreserved (CR)</th>
<th>Viability upon thawing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>79</td>
<td>Male</td>
<td>Metastasis (rectal)</td>
<td>(FR)</td>
<td>84</td>
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<tr>
<td>2</td>
<td>67</td>
<td>Male</td>
<td>Colic adenocarcinoma</td>
<td>(FR)</td>
<td>90</td>
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<tr>
<td>3</td>
<td>75</td>
<td>Male</td>
<td>Metastasis (hepatic)</td>
<td>(FR)</td>
<td>87</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>Female</td>
<td>NA</td>
<td>CR</td>
<td>97</td>
</tr>
<tr>
<td>5</td>
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<td>NA</td>
<td>CR</td>
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<td>NA</td>
<td>CR</td>
<td>92</td>
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<td>7</td>
<td>55</td>
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<td>NA</td>
<td>CR</td>
<td>88</td>
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<tr>
<td>8</td>
<td>34</td>
<td>Female</td>
<td>NA</td>
<td>CR</td>
<td>89</td>
</tr>
<tr>
<td>9</td>
<td>75</td>
<td>Male</td>
<td>NA</td>
<td>FR</td>
<td>87</td>
</tr>
<tr>
<td>10</td>
<td>56</td>
<td>Male</td>
<td>Colic adenocarcinoma</td>
<td>FR</td>
<td>82</td>
</tr>
<tr>
<td>11</td>
<td>53</td>
<td>Male</td>
<td>Colic adenocarcinoma</td>
<td>FR and CR</td>
<td>90%</td>
</tr>
<tr>
<td>12</td>
<td>64</td>
<td>Female</td>
<td>Colic adenocarcinoma</td>
<td>FR and CR</td>
<td>80%</td>
</tr>
</tbody>
</table>

NA = not available.
CYP substrates were incubated directly on the monolayers ([43x358]) cultured on 60 mm plates, and, after 72 h treatment with inducers, microsomes prepared from hepatocytes cultured on 60 mm plates, (b) hepatocyte monolayers cultured on 60 mm plates and (c) hepatocytes monolayers cultured on 24-well plates. Mean of 3 to 6 hepatocyte preparations (±s.d.).

### Table 2

<table>
<thead>
<tr>
<th>Substrate incubation condition</th>
<th>CYP1A</th>
<th>CYP2C</th>
<th>CYP3A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes</td>
<td>34.8 ± 7.9</td>
<td>10.6 ± 2.7</td>
<td>142.4 ± 78.0</td>
</tr>
<tr>
<td>Expressed per mg microsomal protein</td>
<td>3.3 ± 0.2</td>
<td>0.96 ± 0.1</td>
<td>15.0 ± 2.6</td>
</tr>
<tr>
<td>Monolayers</td>
<td>5.0 ± 1.2</td>
<td>1.4 ± 0.2</td>
<td>21.4 ± 5.9</td>
</tr>
<tr>
<td>60 mm plate (expressed per mg hepatocellular protein)</td>
<td>4.2 ± 0.6</td>
<td>1.2 ± 0.2</td>
<td>20.8 ± 6.7</td>
</tr>
<tr>
<td>24-well plate (expressed per mg hepatocellular protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Image 1](C. Abadie-Violon et al. / Toxicology in Vitro 24 (2010) 346–356 349)

**Fig. 1.** CYP1A, CYP2C and CYP3A induction responses of fresh rat hepatocytes cultured and incubated under different conditions. Rat hepatocytes were either cultured on 60 mm plates and, after 72 h treatment with inducers, microsomes were prepared from the cultures and then incubated with CYP substrates (■); cultured on 60 mm plates and, after 72 h treatment with inducers, CYP substrates were incubated directly on the monolayers (□) or cultured on 24-well plates and, after 72 h treatment with inducers, CYP substrates were incubated directly on the monolayers (□). The induction responses to 50 μM BNF (CYP1A), 10 μM DEX (CYP2C and CYP3A) are expressed as a fold induction of activities in treated hepatocytes over activities in control treated hepatocytes (mean ± s.d., n = 3). The CYP1A, CYP2C and CYP3A substrates were 10 μM ethoxyresorufin, 250 μM tolbutamide and 250 μM testosterone, respectively.

Microsomes and 8 fold when measured in hepatocyte monolayers; that of CYP3A to 10 μM RIF was respectively 7.5- and 6-fold. We also confirmed with human hepatocyte cultures from donor 10 that the induction responses of CYPs were not affected by the culture format: the induction response to 50 μM BNF (CYP1A) was respectively 73-fold in 12-well plates and 36-fold in 24-well plates, the response to 20 μM RIF (CYP3A and CYP2C) being respectively 43- and 55-fold and 2.7- and 2.2-fold.

### 3.2. Effect of pre-culture time

The effect of the duration between plating and addition of the inducer was compared in fresh human hepatocyte cultures. The induction responses were determined with respect to mRNA expression (Fig. 2) and CYP activities (Fig. 3). The concentration induction response profiles of CYP1A2 (to BNF), CYP2C9 (to RIF) and CYP3A4 (to RIF) were measured by changes in mRNA expression, were comparable between the two pre-culture conditions (Fig. 2). Data for donor 2 are shown as an example but the comparison was similar for donors 1 and 3. There was a difference in the maximum induction of CYP1A2 mRNA by 50 μM BNF using a 24 h pre-culture period (112-fold) and a 48 h pre-culture period (56-fold) (Fig. 2A) but this was not evident in other donors tested (data not shown). Maximal induction of mRNA for CYP2C9 and CYP3A4/5 were similar after a 24 h pre-incubation period (3-fold and 33-fold, respectively) and after a 48 h pre-culture period (3-fold and 32-fold, respectively). The CYP1A2, CYP2C9 and CYP3A4/5 induction responses, measured using CYP activities in monolayers of hepatocytes from three donors to BNF and RIF are shown in Fig. 3. As with mRNA expression, there was no marked effect of pre-culture time on any of the CYP induction responses.

### 3.3. Effect of treatment time on mRNA expression

The effect of the time at which mRNA analysis is carried out is shown in Fig. 4. The increase in CYP1A2 mRNA in response to 50 μM BNF was variable between the 3 donors tested, especially at 24 h, at which time CYP1A2 was induced by 51-fold, 1010-fold and 107-fold in donors 1, 2 and 3, respectively (Fig. 4A). The expression of CYP1A2 mRNA in donor 2 decreased over the treatment time but was still 112-fold higher than control hepatocyte expression after 72 h. CYP1A2 mRNA expression in donor 1 peaked at 48 h but was still 197-fold higher than control cells after 72 h. The expression of CYP1A2 mRNA in donor 3 was similar throughout the incubation (106-fold, 94-fold and 143-fold after 24 h, 48 h and 72 h, respectively).

The increase in CYP2B6 expression in response to 1000 μM PB was also variable between donors and, with the exception of donor 1, increased with increasing treatment times (Fig. 4B). The magnitude of the fold induction of CYP2C9 mRNA in response to 10 μM RIF (Fig. 4C) reflected the fold increases of the corresponding CYP activities in these cells (Fig. 3B). There was either little change in the fold induction of this CYP over treatment time (donor 2 and 3) or a small decrease (donor 1). By 72 h, the fold induction of this CYP was 1.5-fold, 3.1-fold and 2.0-fold in donor 1, 2 and 3, respectively.

The increase in CYP3A4 mRNA expression in response to 10 μM RIF peaked at 48 h in hepatocytes from donor 1 and 2 but the fold induction in these hepatocytes after 72 h was still 15.4-fold and 32.7-fold higher than control cells, respectively (Fig. 4D). By contrast, CYP3A4 mRNA expression in hepatocytes from donor 3 increased from 48 h to 72 h treatment.

### 3.4. CYP3A4 and CYP2B6 induction by PB, RIF, OME and BNF

Fig. 5 compares CYP3A4 mRNA expression (Fig. 5A) and CYP2B6 mRNA expression (Fig. 5B) in response to 0.1 μM (low), 1 μM (mid) and 10 (high) RIF and to 50 μM (low), 250 μM (mid) and 1000 μM (high) PB treatment of the 3 fresh donors tested. Maximum responses for each concentration were taken. Although there was a donor-to-donor variation in the responses, RIF and PB caused similar fold-inductions of CYP3A4 in these donors at their low, mid and high concentrations (1/1 ratio). In contrast CYP2B6 mRNA was not induced to the same extent by RIF and PB (Fig. 5B). Induction responses to PB were on an average 2-fold higher than the corresponding responses to RIF.

Fig. 6 compares the CYP3A4/5 activity (Fig. 6A) and CYP2B6 activity (Fig. 6B) induction responses to 10 μM RIF and 1000 μM testosterone, respectively.
Fig. 2. The effect of the pre-culture period on the changes in mRNA expression in fresh human hepatocytes treated with CYP inducers. Human hepatocytes were cultured in 12-well plates and pre-incubated for 24 h or 48 h before the addition of CYP inducers for 72 h (2 wells per treatment condition). Inducers were (A) 2 μM, 10 μM and 50 μM BNF (CYP1A2), (B) 0.1 μM, 1 μM and 10 μM RIF (CYP2C9) and (C) 0.1 μM, 1 μM and 10 μM RIF (CYP3A4/5). The induction responses are expressed as a fold induction of mRNA in treated hepatocytes over control hepatocytes (results from donor 2). ND = not done. □ = lowest concentration, ■ = medium concentration, ▶ = highest concentration. Results are from donors 1, 2 and 3.

Fig. 3. The effect of the pre-culture period on the induction of CYP activities in fresh human hepatocytes treated with inducers. Human hepatocytes were cultured in 24-well plates and pre-cultured for 24 h (2 wells per treatment condition). Inducers were (A) 50 μM BNF (CYP1A2), (B) 10 μM RIF (CYP2C9) and (C) 10 μM RIF (CYP3A4/5). The induction responses are expressed as a fold induction of CYP activities in treated hepatocytes over control hepatocytes BLQ = below quantitative limits. The CYP1A, CYP2C and CYP3A substrates were 10 μM ethoxyresorufin, 250 μM tolbutamide and 250 μM testosterone, respectively. Results are from donors 1, 2 and 3.
PB in fresh human hepatocytes from the same donors (historical data of our laboratory). Although there was marked donor-to-donor variation in the responses, RIF and PB caused similar fold inductions of CYP3A4/5 in the same donors. The maximum CYP3A4/5 induction by RIF and PB was 18.5-fold and 18.2-fold, respectively. On average ($n = 21$ donors), RIF responses were 1.2-fold higher than the corresponding responses to PB in the same donor. In contrast to CYP3A4/5, CYP2B6 was not induced by the same extent by RIF and PB (Fig. 6B). The maximum CYP2B6 induction by PB and RIF was 39-fold and 21-fold, respectively. On average ($n = 22$ donors), induction responses to PB were 3.4-fold higher than the corresponding responses to RIF in the same donor.

OME (50 $\mu$M) increased CYP3A4/5 activities in hepatocytes from 8 of the 13 donors tested (Fig. 7). In contrast, OME decreased activities in hepatocytes from 3 of the donors (down to as low as 6% of the control activities) and activities in hepatocytes from two other donors were unaffected. The CYP3A4 induction response to OME was not related to the overall ability of hepatocytes to respond to inducers since CYP1A2 induction did not correlate with CYP2B6 responses. In contrast to CYP1A2 and CYP2B6, OME did not affect the CYP3A4 activity of hepatocytes from any of the donors tested (data not shown).

3.5. Between-laboratory reproducibility of induction responses

We first confirmed with two batches of freshly isolated human hepatocytes that appeared plateable after cryopreservation/thawing that basal activity of CYP1A2 and CYP3A4/5 and their response to inducers of were comparable. Basal activity, 24 h after plating of fresh and cryopreserved/thawed human hepatocytes were for donor 1: 5.36 and 2.14 (CYP1A1/2), 236 and 46.5 (CYP3A4/5), for donor 2: 5.21 and 3.02 (CYP1A1/2), 92.4 and 44.9 (CYP3A4/5). The induction response of CYP1A2 was respectively 23- and 29-fold for fresh and cryopreserved/thawed human hepatocytes from donor 11, 15.5- and 16-fold from fresh and cryopreserved/thawed human hepatocytes from donor 12. The induction response of CYP1A2 was respectively 23- and 29-fold for fresh and cryopreserved/thawed human hepatocytes from donor 11, 15.5- and 16-fold from fresh and cryopreserved/thawed human hepatocytes from donor 12.
Second, the induction responses of five different batches of cryopreserved human hepatocytes to CYP inducers were compared in different laboratories, namely the commercial company which supplied the hepatocytes and our laboratory. The CYP1A2, CYP2B6 and CYP3A4/5 induction responses to 3-MC, PB and RIF, respectively, were measured using an induction protocol that included a 24 h pre-culture period, a 72 h induction treatment period and assessment of CYP activities in hepatocytes monolayers. There was a good correlation between the CYP1A2, CYP2B6 and CYP3A4 induction responses between the laboratories (Fig. 9). The reported CYP1A2 induction responses (by the supplier) to 2 μM 3-MC treatment were between 36-fold and 73-fold and the CYP responses measured in the same hepatocytes in our laboratory were between 31-fold and 90-fold. The reported CYP2B6 induction responses by 1000 μM PB were between 8-fold and 32-fold and the CYP responses measured in our laboratory were between 8-fold and 21-fold. The reported CYP3A4/5 induction responses by 10 μM RIF were between 6-fold and 20-fold and the CYP responses measured in our laboratory were between 3-fold and 14-fold. The overall correlation coefficient ($R^2$) between the responses obtained between laboratories was 0.85.

4. Discussion

Induction studies using human hepatocytes are considered to be the most relevant model for predicting in vivo induction potential of drug candidates in humans (Hewitt et al., 2007a, b; LeCluyse et al., 2000; Parkinson et al., 2004). Until recent years, there has been little guidance on how to perform such studies and even since
the 2004 and 2006 FDA guidelines were published, there has been no standardization of the protocol (Hewitt et al. 2007c). The aim of an ECVAM Project (Contract Number 19471-2002-05) was to evaluate the prediction of CYP induction using harmonised protocols. Two of the requirements for the validation of a method are that it is transferrable and reproducible. In a pre-validation study in which three laboratories took part, it was shown that induction data were comparable across laboratories but that there were some differences which were most likely due to differences in the responses in the individual donors tested (Richert et al., 2009b). In order to firmly confirm that variation in responses were not due to inter-laboratory differences, five different batches of cryopreserved human hepatocytes from commercial companies, for which induction responses were known, were incubated with 3-MC, PB and RIF and the induction responses determined. The results confirmed that there were donor-to-donor variations in CYP1A2, CYP2B6 and CYP3A4/5 induction responses but that responses obtained by the commercial supplier were reproducible in our laboratory. It is well-established that inter-individual variations in DMEs (Hewitt et al., 2007a; Shimada et al., 1994) and in induction responses (LeCluyse, 2001; Madan et al., 2003) occur but few, if any, have reported on the reproducibility of data across laboratories using hepatocytes from the same donor.

A comparison of CYP activities in rat hepatocyte monolayers and microsomes prepared from the same monolayers has been reported previously (Vind et al., 1989; Wortelboer et al., 1990; LeCluyse, 2001), however, the method of microsomal preparation can result in a poor recovery of microsomes (Wortelboer et al., 1990). Moreover, when activities were compared as “per mg microsomal protein” activities in microsomes have been shown

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**Fig. 7.** CYP3A4/5 induction responses to 50 μM OME in human fresh hepatocytes from different donors (historical data). The induction responses are expressed as a fold induction of CYP activities in treated hepatocytes over CYP activities in control hepatocytes. Values in brackets are the fold induction of CYP1A2 by OME in hepatocytes from the same donor.

**Fig. 8.** CYP2B6 induction responses to 50 μM BNF in fresh human hepatocytes from different donors (historical data). The induction responses are expressed as a fold induction of CYP activities in treated hepatocytes over CYP activities in control hepatocytes. Values in brackets are the fold induction of CYP1A2 by BNF in hepatocytes from the same donor.
to be three times lower than in corresponding monolayers (Wortelboer et al., 1990). In contrast to these findings, our results show that, on a “per mg cellular protein” basis, basal CYP1A2, CYP2C and CYP3A activities in microsomes and monolayers of rat and human hepatocytes were equivalent or only marginally lower. This suggests that the method by which the microsomes were prepared in our laboratory did not compromise the endoplasmic membrane. Similar to the findings of others (Vind et al., 1989; Wortelboer et al., 1990, 1996; Hamilton et al., 2001). Another possible reason could be that this is easier to perform and uses fewer cells.

There is little information in the literature on the effect of pre-culture time on the induction response, although most use between 24 h to 48 h after plating (Hewitt et al., 2007c). Maurel (1996) found that hepatocytes treated with BNF or RIF did not respond in the first 24 h to 48 h of treatment. In contrast, one participant of a survey reported by Hewitt et al. (2007c) obtained significant induction responses in human hepatocytes that had been plated for only 3 h. Our data show that the induction of CYP1A2, CYP2C9 and CYP3A4, measured by mRNA expression or CYP activities, is comparable between human hepatocytes which have been pre-cultured for 24 h and 48 h prior to addition of the respective inducers. This is of practical importance for researchers wishing to carry out a 72 h inducer treatment regimen within a 5-day window. The difference between our findings and those of Maurel (1996) may be due to differences in the time required for the cultures to form sufficient cell–cell contact, which is needed for cells to be responsive to CYP inducers (Richert et al., 2004; LeCluyse, 2001).


In the pre-validation study, it was noted that 0.1, 1 and 10 μM RIF and 50, 250 and 1000 μM PB induced CYP3A4 to the same extent (Richert et al., 2009b). We recently found that there was a good correlation between enzyme activities and mRNA expression in hepatocytes treated with inducers (Richert et al., 2009a). In the present study, we confirmed this observation at the level of mRNA expression of CYP3A4 mRNA in response to RIF (0.1, 1 and 10 μM) and to PB (50, 250 and 1000 μM). We extended the data set to a further 22 donors (historical data of the laboratory) to confirm that this was reproducible. Indeed, the maximum induction responses by RIF and PB were similar (20-fold and 27-fold, respectively) and there was a good correlation between the fold-induction CYP3A4/5 responses by 1000 μM PB and 10 μM RIF. It is likely that a maximum CYP3A4/5 is reached with 10 μM RIF, since in our hands 20 μM RIF, which can be toxic at this concentration, did not result in a significantly higher induction of this CYP (data not shown). This supports the recommendation that either RIF or PB can be used as positive controls for CYP3A4/5 induction (Huang and Stifano, 2006; Richert et al., 2009b). In contrast, the fold-induction of CYP3A4/5 by PB was twice that of RIF at the level of mRNA expression in the three tested donors and when extending the data set to the 22 donors, a three times higher activity was found with RIF than with PB. This finding gives weight to the recommendation that PB but not RIF should be used as a positive control for CYP3A4/5 induction, as this compound would result in maximal induction of this CYP and, therefore, a larger dynamic range in which to compare the induction potential of unknown compounds.

There are reports that OME, in addition to its well known CYP1A2 inducing property, induces CYP3A4, in some but not all donors, (Roumans et al., 2005; Hewitt et al., 2007b; Curi-Pedrosa et al., 1994). Our studies are in line with these findings and further-
more, we estimated the frequency of this induction effect to be approximately 50%. In addition to CYP3A4/5 induction, 50 μM OME also induced CYP2B6 activities by at least 2-fold in hepatocytes from 3 of the 4 donors tested. We also found that OME induced CYP2C9 in some donors (data not shown). It is suggested that with few exceptions, compounds that induce both CYP3A4/5 and CYP2B6 are likely to be PXR activators rather than CAR activators (Faucette et al., 2004). Indeed, it is known that OME is a selective activator of PXR (Faucette et al., 2006). 50 μM BNF, in addition to its well known CYP1A2 inducing property, induced CYP2B6 in some but not all donors, but never CYP3A4. This is in accordance with the results from the ECVM pre-validation study (Richter et al., submitted for publication). It is noteworthy that donors for which we measured responses to BNF, RIF and PB, either responded to all three compounds or responded only to PB. This suggests there is a clustering of donor responses, namely donors that are responsive to CYP2B6 induction by both RIF and BNF (via PXR) and those that are not (via CAR only).

In conclusion, flexibility in the way in which human hepatocytes can be incubated for classical CYP induction assays. Both RIF and PB are suitable positive control inducers of CYP3A4/5 but PB may be more appropriate for CYP2B6 induction. None of them induce CYP1A2. BNF may be more appropriate for CYP1A2 induction than OME since, in contrast to the latter, it does not induce CYP3A4 nor CYP2C9. Induction responses using hepatocytes from the same donor but in different labs can be expected to be similar. The good reproducibility of induction responses between laboratories using cryopreserved hepatocytes underlines the usefulness of these cells for these types of studies.

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References


