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# Use of human hepatocytes from the second generation of the upcyte® brand in investigations of CYP inhibition and induction

**[1]Dr. B. ARUNA,[2]DR.M.SPURTHIMITHRA,[3] DR C.RENUKATEJASWINI,**
**[4]DR M SRIRAMACHANDRA,[5]DR S.RAJESHRAJA,[6]R.JONA METHUSULA**
**Department of Pharmacology, Dr K.V Subba Reddy Institute of Pharmacy**

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## Abstract
Histologic features shared by primary human hepatocytes are maintained by proliferating hepatocytes from the human upcyte® line. The use of four donors' second-generation upcyte® hepatocytes in inhibition and induction tests with a variety of reference inhibitors and inducers was thoroughly evaluated. Reproducible inhibition of CYP1A2, CYP2B6, CYP2C9, and CYP3A4 occurred at concentrations ranging from very low to very high, and the IC50 values computed for each chemical accurately designated them as powerful inhibitors. Hepatocytes from Upcyte® demonstrated functional AhR-, CAR-, and PXR-mediated CYP regulation as they responded to inducers targeting prototypical CYP1A2, CYP2B6, CYP2C9, and CYP3A4. Eleven inducers were evaluated, divided into three categories: strong, moderate, and noninducers of CYP3A4 and CYP2B6. Data from upcyte® hepatocytes were well-fit by three distinct models for predicting CYP3A4 induction: RIS, AUCu/F2, and Cmax,u/Ind50. Also seen were CAR-selective inducers of CYP2B6 induction (carbamazepine and phenytoin) and PXR (rifampicin) inducers of CYP3A4. The results of this study provide credence to the idea that upcyte® hepatocytes from the second generation are the best choice for CYP inhibition and induction experiments. Prototypical CYP inhibitors and inducers were able to block or stimulate CYP activity in these cells, respectively, to levels comparable to or greater than those seen in primary human hepatocyte cultures. In addition, three prediction models may be used to forecast the in vivo CYP3A4 induction potential utilizing these. Upcyte® hepatocytes are available in bulk from various donors, making them ideal for DDI screening and other in-depth mechanistic studies.

## Introduction
When drugs lower the metabolic clearance of other drugs or themselves, plasma concentrations rise to levels that might be harmful. This is because these drugs block xenobiotic metabolizing enzymes (XMEs), which are responsible for metabolism. According to Zhou et al. (2007), the majority of deadly drug-drug interactions occur as a result of XME inhibition. To further ensure that plasma levels do not fall below the therapeutic threshold, medication developers steer clear of both inhibitory and XME-inducing compounds. This is due to the fact that elevated XME levels enhance the metabolic clearance of medicines. Hence, it is critical to find strong XME inhibitors and inducers during early drug screening so that they may be omitted from development or labeled once they hit the market. When testing the induction potential of novel medicines, the gold standard in vitro model is primary human hepato-cytes (Hewitt et al. 2007). Researchers have looked for other models for studying cytochrome P450 (CYP) induction because of the erratic quality and quantity of these cells. Youdim et al. (2007) and Kanebratt and Andersson

(2008) both looked at different cell types, and among them were HepG2, HepaRG, and Fa2N-4 cells. Nevertheless, each of these donor cells only comes from one source; HepG2 cells do not respond well to CYP3A4 inducers (Westerink and Schoonen 2007); and Fa2N-4 cells do not express the androstane receptor (CAR) constitutively (Hariparsad et al. 2008). Hence, a predictive hepatic model is still required for early drug screening to determine metabolism and DDI potential.

The product known as human upcyte® hepatocytes (www.upcyte.technolo gies.com) is made from primary human hepatocytes that have been transduced with proliferating genes. This process causes the cells to divide a finite number of times, but they do not become immortalized or lose any of the characteristics of adult primary cells, such as adult cell markers, albumin production, or XMEs (Burkard et al. 2012). Various cell kinds and batches may be treated with this approach, allowing a variety of primary cells from diverse sources to have a longer lifetime (Scheller et al., 2013). One example is microvascular endothelial cells. To satisfy the huge demand for predictive human-based cell screening investigations, one vial of primary human hepatocytes may be transformed into over 12 billion upcyte® hepatocytes. According to Burkard et al. (2012), the first generation of upcyte® hepatocytes exhibited CYP inducer responsiveness. Specifically, when it came to CYP3A4, the level of induction in up-cyte® hepatocytes was found to be similar to that in the matched primary cells. Although prototypical inducers were successful in inducing CYP1A2, CYP2B6, and CYP3A4 at the mRNA level, phenobarbital failed to significantly induce CYP2B6 activity, indicating the need for more optimization. This is the upcyte'

Levy et al. (2015) state that as a result, the technology was upgraded, and second-generation upcyte® hepatocytes were created. In addition to displaying a differentiated phenotype, these cells are able to generate polarized cultures that are metabolically active and have functioning bile canaliculi. It was similarly similar to primary human hematopoietic cells in terms of nuclear receptor expression, phase 1 and 2 enzyme expression, and drug transporter gene expression. The second generation of upcyte® hepatocytes share many traits with the first generation, including the absence of fetal markers, expression of cytokeratin 8 and 18, storage of glycogen, and human serum albumin (Levy et al. 2015).

High CYP activi-ties are required for the use of upcyte® hepatocytes in research involving metabolism or inhibition. We detail here the steps used to improve the culture conditions for CYP inhibition and metabolism experiments, with the goal of eliciting increased CYP activity. To establish the viability of upcyte® hepatocytes, we tested them in traditional CYP inhibition experiments using well-established, powerful CYP inhibitors. Following previous research by Mao et al. (2012) and Moeller et al. (2013), we chose a-naphthoflavone, miconazole, and ketoconazole as competitive inhibitors of CYP1A2, CYP2C9, and CYP3A4, respectively. An inhibitor of CYP2B6 activities, ticlopidine was used (Turpeinen et al. 2004). The enzyme CYP2B6 is the only one that can metabolize ticlopidine to its inactive byproduct, a reactive metabolite that acts as an inhibitor of ticlopidine (Richter et al. 2004). For the purpose of comparing baseline CYP activity and their suppression, another laboratory examined upcyte® hepatocytes.

In addition, we have looked at the possibility of using upcyte® hepatocytes of the second generation in CYP induction screening experiments. To measure the induction of aryl hydrocarbon receptor (AhR), central activation receptor (CAR), and peroxisome proliferator-activated receptor (PXR) genes, we used the following prototypical inducers: omeprazole for AhR-mediated CYP1A2 induction, phenobarbital for CAR-mediated CYP2B6 induction, and rifampicin for PXR- and CAR-mediated CYP2C9 and CYP3A4 induction (FDA, 2012). To confirm that these cells had a fully working induction route leading to active enzyme proteins, we measured CYP activities throughout induction instead of changes in mRNA expression. Predictions based on the maximum fold induction (Indmax), the concentration producing 50% maximal induction (Ind50), or the concentration causing a twofold induction (the "F2"). These have been used to simulate the in vitro-in vivo CYP3A4 induction correlation. Each drug's in vivo CYP3A4 induction may be compared to the Indmax and Ind50, which are linked to the unbound drug plasma concentration (Cmax,u) using the Relative Induc-tion Score (RIS). Results from investigations to induce CYP3A4 in human hepatocytes with upcyte® were

applied to three models recommended by PhARMA (Chu et al. 2009) to determine whether they can mimic the correlations already established for human hepatocytes (Fahmi et al. 2008).

## Materials and Methods

### Materials

Hepatocyte growth medium (HGM), high-performance medium (HPM), and hepatocyte thawing medium (HTM) were all obtained from Medicyte GmbH, Heidel- berg, Germany. Ham F12 and Williams E media, L-gluta- mine, HEPES, sodium pyruvate, penicillin, and streptomycin were purchased from Gibco (Paisley, UK). Trypsin/EDTA was obtained from PAN, and phosphate- buffered saline (PBS) with calcium or magnesium was from GE Healthcare Life Sciences, Freiburg, Germany. The MTS assay was a kit from Promega, Mannheim, Ger- many (CellTiter 96® AQueous Non-Radioactive Cell). All
the inducers and inhibitors and probe substrates and their metabolites (midazolam, 1'-hydroxymidazolam, tolbuta- mide, 4-hydroxytolbutamide, dextromethorphan, dextror- phan, phenacetin, and *O*-deethyl-phenacetin) were from Sigma–Aldrich, Seelze, Germany. The 1'-hydroxymidazo- lam glucuronide was synthesized by the Isotope Chemis- try and Metabolites Synthesis Department of Sanofi (Chilly-Mazarin, France). Stock solutions were prepared using dimethyl sulfoxide (DMSO) as the solvent (Appli- Chem GmbH, Darmstadt, Germany) for all test com- pounds (except for phenobarbital, which was dissolved in PBS) and were stored at —20°C for no longer than 2 months. All other chemicals were of analytical grade, unless otherwise stated, and were of the highest purity possible (obtained from Sigma–Aldrich, Merck GmbH or AppliChem GmbH). All CYP assays were carried out using collagen type I-coated 48-well plates (Corning Life Sciences, Amsterdam, The Netherlands).

### Cells and media

Second-generation upcyte® hepatocytes (Donors 10-03, 151-03, 422A-03 and 653-03) were from Medicyte GmbH, Heidelberg, Germany. All cells used in these studies were quality controlled and shown to lack *a*-fetoprotein but expressed cytokeratin 8 and 18, human serum albumin, and stored glycogen (measured using PAS staining). Pri- mary human hepatocytes were from Invitrogen, North Carolina, or from KaLy–Cell, Strasbourg, France. The cul- ture conditions for all cells and experiments were the same: incubation in a humidified incubator maintained at 37°C and 5% $CO_2$ and 95% air. Upcyte® hepatocytes with a PD of 20–25 were used for all studies. Upcyte®

3 days with 100 *l*mol/L omeprazole to preinduce this CYP. After this time, the cells were used for inhibition assays. The cells were washed twice with PBS (containing $Ca^{2+}$ and $Mg^{2+}$) and preincubated with 0.1 mL of an appropriate CYP inhibitor dissolved in Krebs Henseleit buffer (KHB). *a*-naphthoflavone (0.1–10 *l*mol/L), mico- nazole (0.01–50 *l*mol/L), and ketoconazole (0.01–20 *l*mol/L) were preincubated for 5 min and ticlopidine (0.01–50 *l*mol/L) was preincubated for 30 min (as this is a mechanism-based inhibitor). CYP activities were mea- sured by adding 0.1 mL of the CYP-selective substrate in and primary human hepatocytes were thawed in HTM and Williams Medium E (containing 0.1% BSA, 100 lg/ mL streptomycin and 100 U/mL penicillin), respectively. The initial cell viability and density were determined using Trypan blue exclusion or a Scepter™ Automated Cell Counter from Millipore, Darmstadt, Germany.

### Experiments conducted in Lab 1

The following methods were carried out in Lab 1, Medi- cyte, Heidelberg, Germany.

### Upcyte® hepatocyte preculture and conditioning conditions

Two preculture durations were used: A standard precul- ture of 3 days, with a population doubling (PD) of one, or a longer 6- to 7-day preculture with a PD of 3.5. Stan- dard precultures were used for testing the induction of all four CYPs by a single concentration of inducer (as part of the quality control of the cell batch). All other inhibition and induction assays were conducted using a 6–7 day preculture period.

Standard preculture involved seeding the cells at 75,000 cells/$cm^2$ HPM medium (0.5 mL/well) in collagen type I-coated 48-well plates. The cells were cultured for 3 days without a medium change, after which time, the medium was replaced with fresh HPM with the respective control and inducer compounds. The cells were cultured for a further 3 days during which time the medium was replaced daily with HPM containing the positive control inducers (50 *l*mol/L omeprazole for CYP1A2, 2 mmol/L phenobarbital for CYP2B6 and 20 *l*mol/L rifampicin for CYP2C9 and CYP3A4) or vehicle controls (0.1% PBS as the phenobarbital solvent and 0.1% DMSO for all other compounds).

For the longer preculture period, upcyte® hepatocytes were seeded at 5000 cells/$cm^2$ in collagen type I-coated T150 flasks in HGM and precultured for up to 1 week or until they reached 70–80% confluence. The medium was changed every 2–3 days. The cells were then trypsinized and reseeded into 48-well plates at 150,000 cells/$cm^2$ (confluence) in HPM (0.5 mL per well).

### Upcyte® hepatocyte inhibition assays

For inhibition assays conducted in Lab 1, after the cells had attached (2–4 h), the medium was replaced with either HGM or HPM containing either 0.1% or 0.5% DMSO. The cells were cultured for a further 3 days dur- ing which time the medium was replaced daily with the appropriate medium. Cultures which were subsequently used for CYP1A2 inhibition assays were treated daily for

KHB and incubating for 1 h (final concentrations were: 26 *l*mol/L phenacetin, 500 *l*mol/L bupropion, 75 *l*mol/ L tolbutamide, and 250 *l*mol/L testosterone).

CYP inducer was tested to determine CYP1A2, CYP2B6, CYP2C9, and CYP3A4 induction and
(2) calibration induction assays in which a range of con- centrations of test compounds were incubated to deter- mine their potential to induce CYP2B6 and CYP3A4 only. For both assays, after attachment of upcyte® hepatocytes in 48-

well plates, the medium was replaced with HPM and the cells were cultured for 24 h. After this time, the cells were treated daily for 3 days with the test compound/prototypical inducer (required for induction assays measuring activities, as opposed to mRNA mea- surements, which only require 2 days).

For standard induction assays, the prototypical induc- ers were 50 *l*mol/L omeprazole (CYP1A2 inducer), 1 mmol/L phenobarbital (CYP2B6 inducer), and 20 *l*mol/L rifampicin (CYP2C9 and CYP3A4 inducer). At the end of the induction period, the cells were washed with PBS and CYP activities were measured by adding
0.2 mL KHB containing final concentrations of CYP sub- strate: 26 *l*mol/L phenacetin, 500 *l*mol/L bupropion, 75 *l*mol/L tolbutamide, and 250 *l*mol/L testosterone and incubating for 30 min (testosterone) or 1 h (phenacetin, bupropion, and tolbutamide). After incubation, the supernatant was transferred to a fresh 96-well plate and processed for HPLC analysis.

For induction assays generating dose–response curves for CYP3A4 and CYP2B6 and subsequent calibration curves relating to in vivo induction, the test compounds were rif- ampicin (0.05–40 *l*mol/L), phenobarbital (20–2000 *l*mol/ L), phenytoin (1–1000 *l*mol/L), carbamazepine (1– 100 *l*mol/L), troglitazone (0.5–50 *l*mol/L), pioglitazone (0.5–40 *l*mol/L), dexamethasone (0.1–500 *l*mol/L), nifed- ipine (0.05–100 *l*mol/L), omeprazole (0.5–200 *l*mol/L), flumazenil (0.05–100 *l*mol/L), and quinidine (0.1–
250 *l*mol/L). At the end of the induction period, the cells were washed with PBS and 0.2 mL 250 *l*mol/L testosterone in KHB was added to each well and incubated for 30 min. The supernatant was transferred to a fresh 96-well plate and processed for HPLC analysis. The remaining cell cul- tures were again washed twice with PBS and then incubated with 500 *l*mol/L bupropion in KHB for 1 h. The superna- tant was transferred to a fresh 96-well plate and processed for HPLC analysis.

### Metabolite analysis by HPLC

At Medicyte, all metabolites were analyzed using UV-HPLC. All metabolites and their respective internal stan- dards (chlorpropamide for phenacetin, bupropion, and tolbutamide and cortexolone for testosterone) were sepa- rated on a SunFire C18 2.5 *l*m 2.1 9 20 mm column (Waters, Munich, Germany). The mobile phases for phenacetin metabolites were 17:1:1000 isopropanol:formic acid:distilled water (A) and 100% methanol (B). The peaks were detected on a UV detector set at 240 nm. The mobile phases for bupropion and tolbutamide and their hydroxy- metabolites were (a) 10 mmol/L $KH_2PO_4$ +5% acetonitrile, pH 4.6 and (B) 50:50 acetonitrile:water. The metabolites were detected on a UV detector set at 200 nm. The mobile phases for testosterone and its metabolites were (A) 390:600:10 methanol:water: acetoni- trile and (B) 800:180:20 methanol:water:acetonitrile. The peaks were detected on a UV detector set at 252 nm.

### Upcyte® hepatocyte culture viability and protein content

The viability of upcyte® hepatocyte cultures was measured using the MTS assay (CellTiter 96® AQueous Non-Radioac- tive Cell kit), according to the supplier's protocol. Briefly, the stock MTS solution was thawed and diluted in Krebs Henseleit buffer (fivefold dilution) and a volume of 0.2 mL MTS was added per well. The cultures were incubated with MTS for 1 h in a humidified incubator at 37°C, under an atmosphere of 5% $CO_2$/95% air. The absorbance was read at 490 nm against a background absorbance of 620 nm.

After the MTS incubation, the cultures were washed twice with PBS and the proteins dissolved in 0.2 mL lysis buffer (8.76 mg/mL NaCl; 0.2 mg $MgCl_2.6H_2O$; 1% (v/v) NP40; 50 mmol/L Tris-HCl). The protein content was measured using the Pierce assay.

### Calculation and curve fitting

Experiments were carried out in duplicate and each compound was tested in at least two different experi- ments. All curve fitting was carried out using Prism

$$RIS = \frac{C_{max;u}}{} + Ind_{50} \quad (1)$$

The $C_{max,u}$ value for omeprazole was taken from Mostafavi et al., Mostafavi and Tavakoli (2004); the $C_{max,u}$ value for all other compounds used for RIS determinations were taken from Ripp et al. (2006) or Fahmi et al. (2008) (values for quinidine and flumazenil were not needed as they were the negative control compounds).

The relative induction of CYP3A4 and CYP2B6 by dif- ferent compounds compared to the positive control was calculated using equation 2:

$$\frac{Ind_{50;TC}}{} — 1$$

44 mg/L), ascorbic acid (50 mg/L), arginine (104 mg/L), and L-glutamine (0.7 g/L).

Experiments were performed in 48-well plastic plates coated with rat tail collagen type I. Plates were seeded with
0.16 9 $10^5$ upcyte® hepatocytes per well in a final volume of 0.2 mL. After a 3 h attachment period, the medium was renewed with 0.1 mL serum-free medium supplemented with HEPES (3.6 g/L), ethanolamine (4 mg/L), transferrin (10 mg/L), linoleic acid-albumin (1.4 mg/L), glucose (252 mg/L), sodium pyruvate (44 mg/L), ascorbic acid (50 mg/L), arginine (104 mg/L), and L-glutamine (0.7 g/L). The following day, human hepatocytes were incu- bated with FDA-recommended metabolic CYP probe sub-
strates at a starting concentration of 5 *l*mol/L for midazolam, phenacetin, and tolbutamide, 20 *l*mol/L for dextromethorphan, and 100 *l*mol/L for bupropion, in 0.1 mL of 0.1% BSA (v/v), containing incubation medium. Regardless of the final concentration investigated, the final solvent (DMSO) concentration never exceeded 0.2% (v/v).

To determine the metabolism of the different substrate probes, kinetic studies were performed over 0–24 h. For each time point (0, 1, 2, 3, 4, 6, 8, and 24 h), 0.7 mL ace- tonitrile was added to the specific well for protein precip- itation, and both extracellular medium and cell
compartment were harvested and pooled. Cell extracts were transferred to a glass tube and stored at —20°C until analysis by LC/MS-MS. Before analysis, cell homogenates

$$\% \text{ Relative PC induction} = \frac{\text{Ind}}{\text{}}; \quad (2)$$

Culture of primary human hepatocytes for clearance assays

Cryopreserved human hepatocytes were seeded in 48-well collagen-coated plastic plates in a chemically defined medium adapted from Georgoff et al. (1984), consisting in a 50/50 (v/v) mixture of Ham F12/Williams E medium supplemented with 10% decomplemented fetal calf serum, 10 mg/L insulin, 0.8 mg/L glucagon, and antibiotics (100 IU penicillin and 100 *l*g/mL streptomycin). After a 4–6 h attachment period, plating medium was removed and replaced by the same serum-free culture medium supplemented with HEPES (3.6 g/L), ethanolamine (4 mg/L), transferrin (10 mg/L), linoleic acid-albumin (1.4 mg/L), glucose (252 mg/L), sodium pyruvate
then analyzed for the different probe substrates and their specific metabolites.

Shipment and subsequent testing of upcyte® hepatocytes cultures

Upcyte® hepatocytes which were shipped to Lab 2 were seeded in HGM at 75,000 cells per well in a 48-well collagen (type I)-coated plate on a Friday and grown for 3 days without a change in medium. On day 3 (Monday), the medium was replaced with fresh HPM containing 0.1% DMSO, 100 *l*g/mL streptomycin and 100 U/mL penicillin; the plates were then sealed and shipped to Lab
2 by overnight courier. Upon arrival, the medium was replaced with HPM containing 0.1% DMSO, 100 *l*g/mL streptomycin, and 100 U/mL penicillin and allowed to recover overnight.

Upcyte® hepatocyte clearance and inhibition assays

After shipping and the recovery period, upcyte® hepatocytes were cultured in HPM containing 0.1% DMSO, 100 *l*g/mL streptomycin, and 100 U/mL penicillin for a further 2 days before incubation with test compounds. The clearance and inhibition studies were conducted using the same conditions as those for primary human hepatocytes (described above). The effect of inhibitors on the clearance of substrates was determined by incubating them in the presence or absence of specific CYP inhibi- tors (3 *l*mol/L quinidine for CYP2D6 and 3 *l*mol/L ketoconazole for CYP3A) or the nonspecific mechanism- based CYP inhibitor, 1-aminobenzotriazole (ABT (Emoto et al. 2005)), 1 mmol/L).

Metabolite analysis by HPLC

Supernatants were analyzed for unchanged drug and specific metabolites by LC/MS-MS: phenacetin and 4-acetamidophenol for CYP1A2, tolbutamide and 4- hydroxy-tolbutamide for CYP2C9, bupropion and hydroxyl-bupropion for CYP2B6, dextromethorphan, dex- trorphan and dextrorphan glucuronide for CYP2D6, Midazolam, 1'-hydroxymidazolam and 1'-hydroxymidazo- lam glucuronide for CYP3A activity. The data were col-
lected and processed using MassLynx 4.1 Software from

CYP Induction and Inhibition Assays Using Upcyte® Hepatocytes

Waters-Micromass. The chromatograph (Acquity UPLC system I Class) was fitted with an Acquity UPLC BEH C18 column (2.1 mm i.d. 9100 mm length, 1.7 *l*m par- ticle size), coupled to a Xevo TQS mass spectrometer (all from Waters, Milford, MA) and used in electrospray ion positive mode except for tolbutamide and its hydroxyl
metabolite which were analyzed in ion negative mode.

The mobile phase was a mixture of 1.5 g/L ammonium acetate–2 mL/L formic acid (solvent A) and acetonitrile 80%–methanol 20%–0.15 g/L ammonium acetate-formic acid 2 mL/L (solvent B). The solvent programmer was set to deliver a flow rate of 0.35 mL/min. Compounds were eluted in 2 min with a linear gradient from 10 to 100% solvent B over 1 min, followed by an isocratic step at 100% for 0.7 additional minute.

Statistical evaluations

Data were evaluated for statistical differences using the *t*-test in Prism Software version 6.03. A statistical differ- ence was considered when $P < 0.05$.

## Results

### Culture optimization

We investigated whether the culture conditions could be modified to result in cultures with higher CYP activities than were present using standard conditions, that is, seeding at 50% confluence with a 3-day preculture period (allowing for 1 PD) in HGM followed by a 3 day culture at

confluence in HPM with daily refreshment of medium. The main aspects investigated were the length of the preculture period (as previous studies using these cells in the in vitro micronucleus assay showed that there was less DNA damage to the cells when they were precultured for 7 days prior to performing the assay (No€renberg et al. 2013), the type of basal medium used for the culture of cells at confluence (HGM vs. HPM) and supplementing the preculture (dur- ing growth) and "conditioning" (i.e., at confluence) med- ium with DMSO. Initial experiments using Donor 422A-03 showed little difference in CYP activities when the precul- ture medium contained 0.25 or 0.5% DMSO (data not shown); therefore, in order to ensure maximal CYP activi- ties, all subsequent experiments included 0.5% DMSO in the preculture HGM medium. The effect of DMSO on CYP2B6 and CYP3A4 activities in upcyte® hepatocytes from all four donors was measured using a 7-day precul- ture followed by three daily treatments with HPM supple- mented with a range of concentrations of DMSO (Fig. 1). Activities of both CYPs in cells from all four donors were markedly induced in a concentration-dependent manner by DMSO and maximal effects were evident at 0.5–0.75% (v/v) DMSO. At higher concentrations of DMSO, there was a decrease in both CYP activities.

Figure 2 shows how the culture conditions affected CYP2B6 and CYP3A4 activities in upcyte® hepatocytes from four donors. The conditions compared were as follows: (1) standard culture conditions (allowing for 1 PD); (2) seeding at 3% confluence (5000 cells/$cm^2$) in a T-flask with a 6–7-day preculture period (allowing for ~3.5 PDs) in HGM supplemented with 0.5% DMSO followed by trypsinization, seeding at confluence (i.e., 150,000/$cm^2$) and a 3 day culture in HGM with 0.5% DMSO with daily refreshment of medium; and (3) seeding at 3% confluence in a T-flask with a 6–7-day preculture period in HGM supplemented with 0.5% DMSO followed by trypsinization, seeding at confluence (i.e., 150,000/$cm^2$), and a 3 day culture at confluence in HPM with 0.1% DMSO with daily refreshment of medium. Increasing the preculture time from 3 days to 7 day did not increase the CYP3A4 activi- ties but in three of the four donors, CYP2B6 activity was significantly increased. CYP3A4 activities were increased when the conditioning medium (used when the cells were at confluence) was changed from growth medium (HGM) to endpoint medium (HPM) with a lower concentration of DMSO (0.1% v/v). All further experiments were conducted using a 6–7 preculture period using HGM supplemented with 0.5% DMSO, trypsinization, seeding at 5000 cells/ $cm^2$, and conditioning for 3 days with HPM supplemented with 0.1% DMSO.

Table 1 compares the CYP activities in control incubations of upcyte® hepatocytes (using the optimized conditions) and corresponding primary human hepatocyte

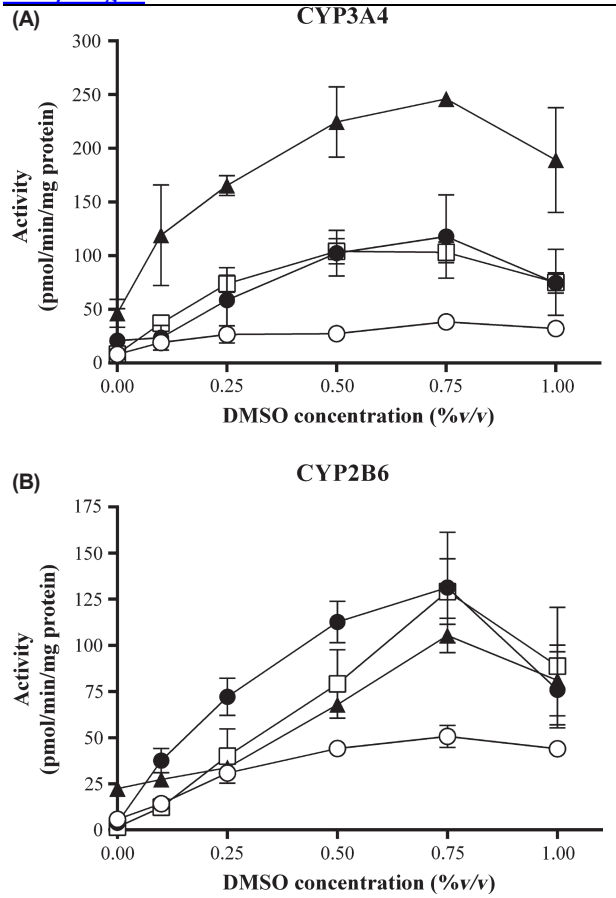


Figure 1. Effect of DMSO on CYP3A4 (A) and CYP2B6 (B) activities in upcyte® hepatocytes from different donors. Donor 10-03 = ○; Donor 151-03 = ●; Donor 422A-03 = ▲; Donor 653-03 = □. Values are a mean of two experiments, each with $n = 2$ wells per treatment.

cultures from which they were derived. Two laboratories (Lab 1 and Lab 2) measured CYP activities to investigate to determine whether the shipment of the cells from Ger- many to France markedly affected the performance of the cells and whether they can be used in metabolism and inhibition assays in a second laboratory. The incubations at Lab 2 were designed for clearance of the parent com- pound and therefore employed lower substrate concentra- tions than those at Lab 1 (see below). For hepatocytes from two of the four donors (151-03 and 422A-03), upcyte® and primary hepatocytes from the same donor were incubated under the same conditions (at Lab 2).

As with primary hepatocytes, the CYP activities in upcyte® hepatocytes varied between donors. CYP1A2 activities were present in upcyte® hepatocytes from all four donors, although this activity in Donors 653-03 and 151-03 was only detected by Lab 2 (using 24 h incuba- tions and analysis by LC-MS), and all were lower than in corresponding paired donor hepatocytes. CYP2B6-mediated bupropion hydroxylation was markedly higher in upcyte® hepatocytes incubated in both laboratories compared to that in the original primary hepatocytes (either measured by the supplier (all donors) or, in Lab 2, incubated under the same conditions as upcyte® hepatocytes (Donor 422A-03)). CYP2C9 activities in upcyte® hepatocytes were higher in cells cultured and incubated at Lab 1 than those shipped and incubated at Lab 2. This could have been due to a number of factors, including the substrate concentration (75 *l*mol/L at Lab 1 and 5 *l*mol/L at Lab 2), the effect of shipment, and/ or the small difference in the preculture methods (which was shorter for Lab 2 studies (see Materials and Meth- ods)). CYP2C9 activities derived from incubations with a high tolbutamide concentration (75 *l*mol/L, Lab 1), were higher in upcyte® hepatocytes from Donor 10-03 than the paired primary cells (also incubated at 75 *l*mol/L, Provider). In Lab 2, in which upcyte® and primary hepatocytes from Donors 151-03 and 442A-03 were incubated under the same conditions – at a low tolbuta- mide concentration (5 *l*mol/L) – CYP2C9 activities were similar. CYP3A4 activities, measured using testosterone and midazolam, were markedly higher in upcyte® hepatocytes than in their paired primary cells. Notably,
phase 2 conjugation of 1'-hydroxymidazolam to its glu- curonide was also detected in upcyte® hepatocytes, the rate of which was comparable to that in primary cells. Dextromethorphan is metabolized by CYP3A4 to 3-methoxymorphinan (Yu and Haining 2001) and, although no comparative data were available, the pres- ence of this pathway was clearly evident in upcyte® hepatocytes. CYP2D6 activities were low in the original primary hepatocytes (2–4 pmol/min/mg, compared to an average of ~20 pmol/min/mg in primary cells (www.bio reclamationivt.com, accessed 20 April 2014); therefore, activities in upcyte® and primary hepatocyte were con- sidered to be equivalent. The CYP2D6 metabolite, dextr- orphan, was further metabolized to its glucuronide in both upcyte® and primary hepatocytes.

## Inhibition studies

Inhibition studies were conducted in two laboratories, one using short-term incubation period of 1 h and higher sub- strate concentrations (Lab 1) and the second incubated at time points over 24 h at substrate concentrations at or below Km (Lab 2). Although the CYP1A2 activities that were present in upcyte® hepatocytes were measurable, cells incubated in Lab 1 were preinduced with 100 *l*mol/L omeprazole in order to obtain consistently high CYP1A2 activities for the inhibition studies using UV-HPLC as the analytical method. Two preinduction regimen were investi- gated, namely a single treatment of 100 *l*mol/L omepra-

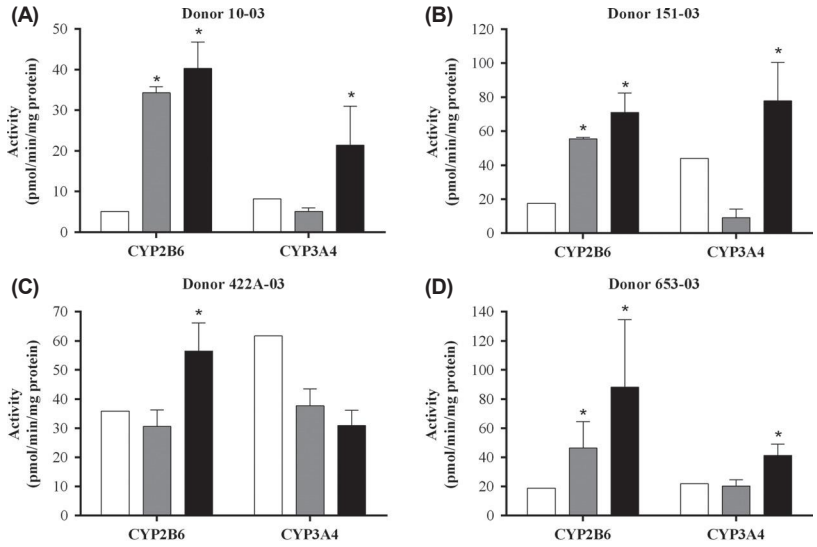


Figure 2. Effect of preculture time and DMSO treatment on CYP2B6 and CYP3A4 activities in upcyte® hepatocytes from different donors. White bars represent cells grown over a 3 day preculture and 3 days conditioning at confluence in GM +0.1% DMSO (standard conditions); gray bars represent cells grown over a 6–7-day preculture period followed by reseeding at confluence and a 3 day conditioning in HGM +0.5% DMSO, and
black bars represent cells grown over a 6–7-day preculture period followed by reseeding at confluence and a 3 day conditioning in HPM +0.1% DMSO. Values are a mean of two experiments, each with $n = 3$ wells per treatment. *Significantly different from standard conditions ($P < 0.05$). DMSO, dimethyl sulfoxide, HGM, hepatocyte growth medium, HPM, high-performance medium.

zole over 3 days and a daily treatment of 100 *l*mol/L omeprazole over the same period. Both induction regimen resulted in high CYP1A2 activities in upcyte® hepatocytes from Donors 422A-03 and 10-03 (>40 pmol/min/mg pro- tein), suggesting both could be employed for CYP1A2 inhi- bition studies. In our studies, we used daily treatments as this resulted in higher CYP1A2 activities than a single treat- ment. The induced activities were 57.1 17.1, 83.8 21.7, 205.0 44.9, and 115.2 30.2 pmol/min/ mg protein in upcyte® hepatocytes from Donors 10-03, 151-03, 422A-03, and 653-03, respectively. CYP2B6, CYP2C9, and CYP3A4 did not require preinduction with an inducer; therefore, conditioning medium included 100 *l*mol/L omeprazole for CYP1A2 assays only.

The results for inhibition studies using upcyte® hepato- cytes from Donor 422A-03 and conducted at Lab 1 are shown in Figure 3 (results for Donors 10-03, 151-03, and 653-03 are shown in Fig. S1). In these studies, CYPs were inhibited using competitive (*a*-naphthoflavone, miconaz- ole, and ketoconazole) and time-dependent (ticlopidine) inhibitors. There was a concentration-dependent inhibi- tion of CYP1A2, CYP2B6, CYP2C9, and CYP3A4, such that at the highest concentration all activities were com- pletely inhibited. The inhibitors did not cause significant cytotoxicity at any concentration tested in upcyte® he- patocytes from all four donors (with the exception of tic- lopidine, which caused ~10% cytotoxicity in upcyte® hepatocytes from Donor 151-03 at the highest concentra- tion only). The inhibition of different CYPs was repro- ducible across experiments (Fig. 3 and Fig. S1) and in upcyte® hepatocytes from all four donors tested (Table 2). The $IC_{50}$ values for each CYP tested also com- pared well with those reported in primary human hepato- cytes or human liver microsomes (Table 2), such that all four CYP inhibitors were classified as potent inhibitors of the respective CYP.

Results for inhibition studies using upcyte® hepato- cytes from three donors and conducted at Lab 2 are shown in Table 3. In these studies, cultures were coincu- bated over 24 h with either the nonspecific mechanism- based CYP inhibitor, ABT, or the CYP-selective inhibi- tors (ketoconazole for CYP3A4 and quinidine for CYP2D6). ABT was a potent inhibitor of all CYPs tested and inhibited between 90% and 100% of activities over 6 h and 24 h. Ketoconazole also inhibited midazolam metabolism by ~90% over 6 h but this effect was lower after 24 h (between 40% and 75%), most likely due to the metabolism of the inhibitor over this time period. CYP2D6-selective inhibition by quinidine was low and did not reach more than 47% inhibition. As with CYP3A4 inhibition by ketoconazole, the inhibitory effect of quinidine on CYP2D6 was lower after 24 h, both observations (low % inhibition and time-dependent effects) possibly due to the metabolism of quinidine. The production of the CYP3A4-mediated metabolite of dextromethorphan, 3-methoxymorphinan, was almost

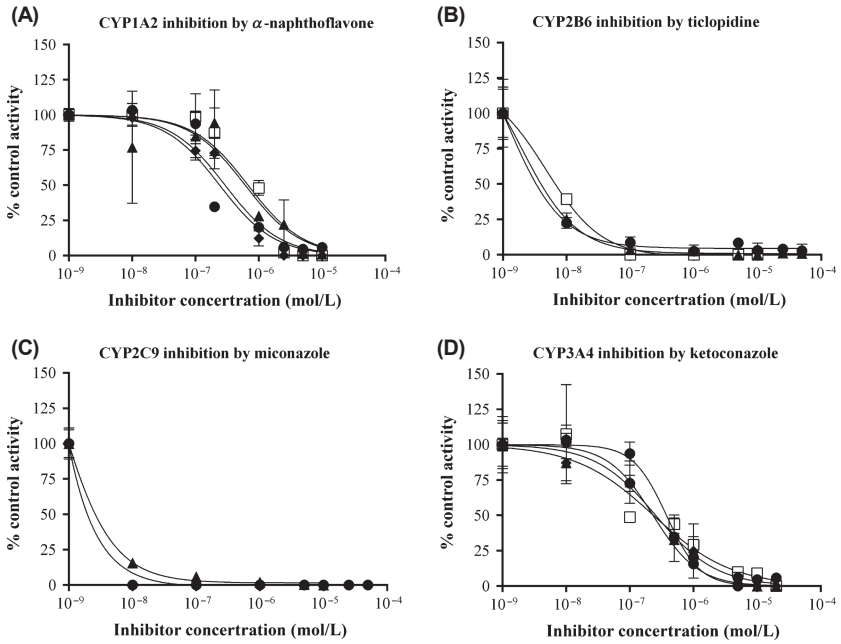


Figure 3. Inhibition of (A) CYP1A2 by *a*–naphthoflavone, (B) CYP2B6 by ticlopidine, (C) CYP2C9 by miconazole, and (D) CYP3A4 by ketoconazole in upcyte® hepatocytes from Donor 422A-03. Values are the mean SD from triplicates in 2–5 experiments (denoted by different symbols).

Table 2. $IC_{50}$ values of CYP inhibitors incubated with upcyte® hepatocytes from different donors. For comparison, literature values for microsomes and/or hepatocytes are also shown. Values for upcyte® hepatocytes are mean SD, $n$ = 6–8 wells from at least two separate experiments.

| | $IC_{50}$ value | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CYP1A2 by *a*-naphthoflavone /mol/L | | CYP2B6 by ticlopidine nmol/L | | | CYP2C9 by miconazole nmol/L | | CYP3A4 by ketoconazole /mol/L | |
| Donor | upcyte® | Primary[1] | upcyte® | | Microsomes[2] | upcyte® | Primary[3] | upcyte® | Primary[3] |
| 10-03 | 0.15, 0.04 ($n$ = 2) | 0.1 | 8.1 | 1.6 ($n$ = 4) | 0.32 | 251, 95.8 ($n$ = 2) | 2.12 | 0.30 0.3 ($n$ = 4) | 0.28 |
| 151-03 | 0.38, 0.19 ($n$ = 2) | | 36.1 | 27.9 ($n$ = 4) | | 12, 6.6 ($n$ = 2) | | 0.15 0.01 ($n$ = 4) | 0.14 |
| 422A-03 | 0.46 0.22 ($n$ = 4) | | 7.3 | 1.6 ($n$ = 4) | | 3.1, 4.3 ($n$ = 2) | | 0.27 0.06 ($n$ = 5) | |
| 653-03 | 0.36, 0.12 ($n$ = 2) | | 27.5 | 19.7 ($n$ = 4) | | 14.7, 4.1 ($n$ = 2) | | 0.23 0.12 ($n$ = 4) | |

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completely inhibited by ABT but, in contrast, quinidine either had little or no inhibitory effect on this pathway, confirming the CYP-selective properties of this inhibitor. Interestingly, in the case of Donor 653-03, quinidine marginally increased the production of the metabolite at both time points, possibly as a result of the diversion of the metabolic pathways towards CYP3A4 as the clearance of the parent compound did not change (data not shown).

These results support the use of upcyte® hepatocytes in inhibition studies incubated in short-term assays to derive an $IC_{50}$ value, or in longer term assays to determine clear- ance in the presence and absence of selected inhibitors.

CYP induction

Induction of CYP1A2, CYP2B6, CYP2C9, and CYP3A4 in upcyte® hepatocytes

Upcyte® hepatocytes from all four donors tested using a 3 day preculture period were responsive to CYP1A2, CYP2B6, CYP2C9, and CYP3A4 induction by prototypical

Table 3. Inhibition of CYP activities in upcyte® hepatocytes from different donors incubated in Lab 2.

| | | | Donor 151-03 | | Donor 422A-03 | | Donor 653-03 | |
|---|---|---|---|---|---|---|---|---|
| CYP | Metabolite(s) | Inhibitor | 0–6 h | 24 h | 0–6 h | 24 h | 0–6 h | 24 h |
| 1A2 | APAP | 1 mmol/L ABT | 100 | 100 | 100 | 98 | 95 | 96 |
| 2B6 | OH-Bup | 1 mmol/L ABT | 85 | 94 | 90 | 94 | 94 | 97 |
| 2C9 | OH-Tolb | 1 mmol/L ABT | 100 | 98 | 100 | 100 | 95 | 99 |
| 3A4 | 1′OH-MID | 1 mmol/L ABT | 99 | 97 | 98 | 98 | 98 | 98 |
| | +1′OH-MID-G | 3 *l*mol/L ketoconazole | 91 | 40 | 93 | 75 | 87 | 58 |
| 3A4 | MEM | 1 mmol/L ABT | 94 | 96 | 92 | 95 | 91 | 97 |
| | | 3 *l*mol/L Quinidine | 0 | 0 | 10 | 10 | —38 | —38 |
| 2D6 | Dex+Dex-G | 1 mmol/L ABT | 100 | 91 | 100 | 92 | 100 | 91 |
| | | 3 *l*mol/L Quinidine | 47 | 15 | 24 | 16 | 36 | 21 |

Values are expressed as a percentage inhibition (including phase 1 and 2 metabolites). "—38%" denotes the activity was increased by 38% in the presence of the inhibitor.

inducers (Fig. 4). The only exception was CYP2C9 induc- tion in upcyte® hepatocytes from Donor 653-03, in which this activities was already relatively high (51 pmol/min/ mg). The responsiveness to CYP inducers was a signifi- cant finding as induction of CYP2B6 in first-generation upcyte® hepatocytes only evident at the mRNA level (Burkard et al. 2012). This suggests the responsiveness of these cells, especially via CAR, was improved by the refined process used to produce second-generation up- cyte® hepatocytes. Likewise, upcyte® hepatocytes demon- strated functional AhR- and PXR-mediated CYP induction as CYP1A2 and CYP3A4 were also induced by omeprazole and rifampicin, respectively.

In order to rule out false positive results from CYP3A4 induction studies, the FDA recommends including a nega- tive control, that is, a noninducer, in each induction assay. In these assays, two negative controls were included, namely quinidine (0.1–250 *l*mol/L) and flumazenil (0.05– 50 *l*mol/L), both of which did not induce CYP3A4 or CYP2B6 at any concentration tested (data not shown).

## Prediction models for in vivo CYP3A4 induction

There are three main prediction models recommended by the FDA, EMA, and PhARMA for CYP3A4 induction (Chu et al. 2009; EMA, 2012; FDA, 2012), namely, the RIS, $AUC_u/F_2$, and $C_{max,u}/Ind_{50}$. Data from upcyte® he- patocytes from Donor 653 were used to compare the dif- ferent models (Fig. 5). Of the three, the fit was best when the RIS ($R^2 = 0.92$) and $C_{max,u}/Ind_{50}$ ($R^2 = 0.93$) were used; however, the $F_2$ value ($R^2 = 0.89$) may also be used when compounds are too toxic or insoluble to reach a maximal induction response. The $Ind_{max}$ and $Ind_{50}$ values from all four donors were applied to the RIS model (values shown in Table 4) and, although the calibration curves were different across donors, they all exhibited a good fit of the data ($R^2 = 0.87–0.94$, Figs. 5C, 6).

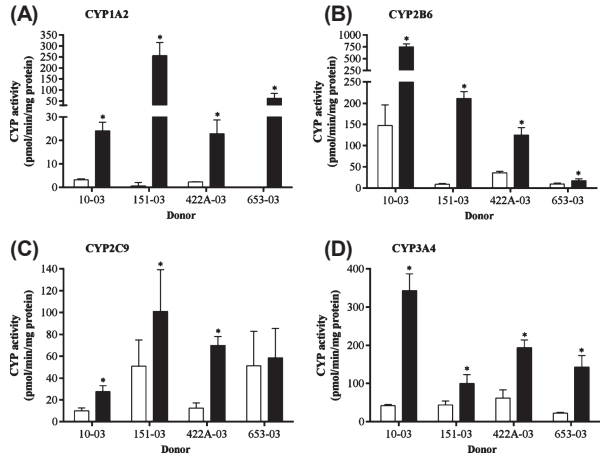


Figure 4. Induction of (A) CYP1A2 (by 50 *l*mol/L omeprazole), (B) CYP2B6 (by 2 mmol/L phenobarbital), (C) CYP2C9 (by 20 *l*mol/L rifampicin), and (D) CYP3A4 (by 20 *l*mol/L rifampicin) in upcyte®
hepatocytes from Donors 10-03, 151-03, 422A–03, and 653-03. White bars indicate control values and black bars indicate values for the prototypical inducers. Values are the mean SD from triplicates.

## CAR and PXR selective induction of CYP3A4 and CYP2B6 in upcyte® hepatocytes

The relative induction of CYP2B6 and CYP3A4 is known to be a result of selective activation of either

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the PXR or CAR receptors (Faucette et al. 2007); therefore, we inves- tigated this attribute by treating upcyte® hepatocytes with different inducers and measuring CYP3A4 and CYP2B6 activities in the same wells. Table 5 summarizes the CYP3A4 and CYP2B6 induction responses of upcyte he- patocytes from all donors to the same compounds tested for CYP3A4 induction. Figure 7 compares the relative induction of both CYPs (compared to the maximal fold induction by the positive controls, according to equation 2 in the Materials and Methods) CYP Induction and Inhibition Assays Using Upcyte® Hepatocytes S. D. Ramachandran *et al.*

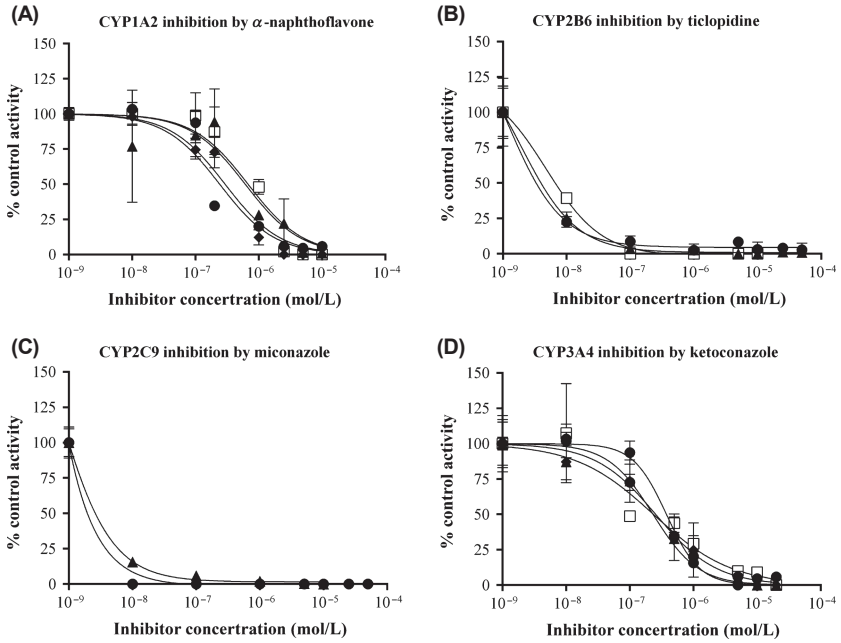


Figure 3. Inhibition of (A) CYP1A2 by *a*–naphthoflavone, (B) CYP2B6 by ticlopidine, (C) CYP2C9 by miconazole, and (D) CYP3A4 by ketoconazole in upcyte® hepatocytes from Donor 422A-03. Values are the mean SD from triplicates in 2–5 experiments (denoted by different symbols).

Table 2. $IC_{50}$ values of CYP inhibitors incubated with upcyte® hepatocytes from different donors. For comparison, literature values for microsomes and/or hepatocytes are also shown. Values for upcyte® hepatocytes are mean SD, $n$ = 6–8 wells from at least two separate experiments.

| | $IC_{50}$ value | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CYP1A2 by *a*-naphthoflavone /mol/L | | CYP2B6 by ticlopidine nmol/L | | | CYP2C9 by miconazole nmol/L | | CYP3A4 by ketoconazole /mol/L | |
| Donor | upcyte® | Primary[1] | upcyte® | | Microsomes[2] | upcyte® | Primary[3] | upcyte® | Primary[3] |
| 10-03 | 0.15, 0.04 ($n$ = 2) | 0.1 | 8.1 | 1.6 ($n$ = 4) | 0.32 | 251, 95.8 ($n$ = 2) | 2.12 | 0.30 0.3 ($n$ = 4) | 0.28 |
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[3]Mao et al. (2012) and Moeller et al. (2013).

Table 3. Inhibition of CYP activities in upcyte® hepatocytes from different donors incubated in Lab 2.

| | | | Donor 151-03 | | Donor 422A-03 | | Donor 653-03 | |
|---|---|---|---|---|---|---|---|---|
| CYP | Metabolite(s) | Inhibitor | 0–6 h | 24 h | 0–6 h | 24 h | 0–6 h | 24 h |
| 1A2 | APAP | 1 mmol/L ABT | 100 | 100 | 100 | 98 | 95 | 96 |
| 2B6 | OH-Bup | 1 mmol/L ABT | 85 | 94 | 90 | 94 | 94 | 97 |
| 2C9 | OH-Tolb | 1 mmol/L ABT | 100 | 98 | 100 | 100 | 95 | 99 |
| 3A4 | 1′OH-MID | 1 mmol/L ABT | 99 | 97 | 98 | 98 | 98 | 98 |
| | +1′OH-MID-G | 3 *l*mol/L ketoconazole | 91 | 40 | 93 | 75 | 87 | 58 |
| 3A4 | MEM | 1 mmol/L ABT | 94 | 96 | 92 | 95 | 91 | 97 |
| | | 3 *l*mol/L Quinidine | 0 | 0 | 10 | 10 | —38 | —38 |
| 2D6 | Dex+Dex-G | 1 mmol/L ABT | 100 | 91 | 100 | 92 | 100 | 91 |
| | | 3 *l*mol/L Quinidine | 47 | 15 | 24 | 16 | 36 | 21 |

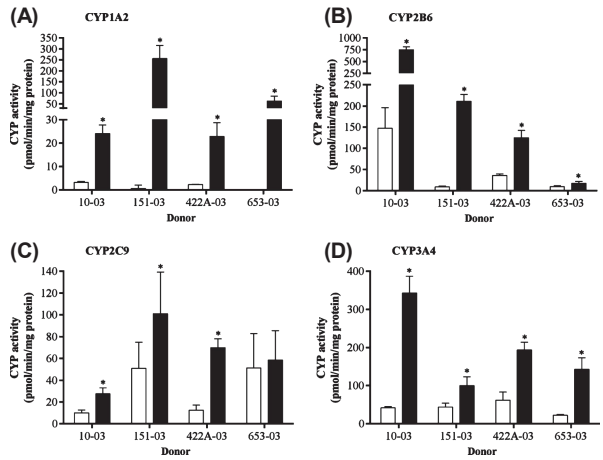
Values are expressed as a percentage inhibition (including phase 1 and 2 metabolites). "—38%" denotes the activity was increased by 38% in the presence of the inhibitor.

inducers (Fig. 4). The only exception was CYP2C9 induc- tion in upcyte® hepatocytes from Donor 653-03, in which this activities was already relatively high (51 pmol/min/ mg). The responsiveness to CYP inducers was a signifi- cant finding as induction of CYP2B6 in first-generation upcyte® hepatocytes only evident at the mRNA level (Burkard et al. 2012). This suggests the responsiveness of these cells, especially via CAR, was improved by the refined process used to produce second-generation up- cyte® hepatocytes. Likewise, upcyte® hepatocytes demon- strated functional AhR- and PXR-mediated CYP induction as CYP1A2 and CYP3A4 were also induced by omeprazole and rifampicin, respectively.

In order to rule out false positive results from CYP3A4 induction studies, the FDA recommends including a nega- tive control, that is, a noninducer, in each induction assay. In these assays, two negative controls were included, namely quinidine (0.1–250 *l*mol/L) and flumazenil (0.05– 50 *l*mol/L), both of which did not induce CYP3A4 or CYP2B6 at any concentration tested (data not shown).

## Prediction models for in vivo CYP3A4 induction

There are three main prediction models recommended by the FDA, EMA, and PhARMA for CYP3A4 induction (Chu et al. 2009; EMA, 2012; FDA, 2012), namely, the RIS, $AUC_u/F_2$, and $C_{max,u}/Ind_{50}$. Data from upcyte® he- patocytes from Donor 653 were used to compare the dif- ferent models (Fig. 5). Of the three, the fit was best when the RIS ($R^2 = 0.92$) and $C_{max,u}/Ind_{50}$ ($R^2 = 0.93$) were used; however, the $F_2$ value ($R^2 = 0.89$) may also be used when compounds are too toxic or insoluble to reach a maximal induction response. The $Ind_{max}$ and $Ind_{50}$ values from all four donors were applied to the RIS model (values shown in Table 4) and, although the calibration curves were different across donors, they all exhibited a good fit of the data ($R^2 = 0.87–0.94$, Figs. 5C, 6).

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hepatocytes from Donors 10-03, 151-03, 422A–03, and 653-03. White bars indicate control values and black bars indicate values for the prototypical inducers. Values are the mean SD from triplicates.

The relative induction of CYP2B6 and CYP3A4 is known to be a result of selective activation of either the PXR or CAR receptors (Faucette et al. 2007); therefore, we inves- tigated this attribute by treating upcyte® hepatocytes with different inducers and measuring CYP3A4 and CYP2B6 activities in the same wells. Table 5 summarizes the CYP3A4 and CYP2B6 induction responses of upcyte® he- patocytes from all donors to the same compounds tested for CYP3A4 induction. Figure 7 compares the relative induction of both CYPs (compared to the maximal fold induction by the positive controls, according to equation 2 in the Materials and Methods) CYP Induction and Inhibition Assays Using Upcyte® Hepatocytes S. D. Ramachandran *et al.*

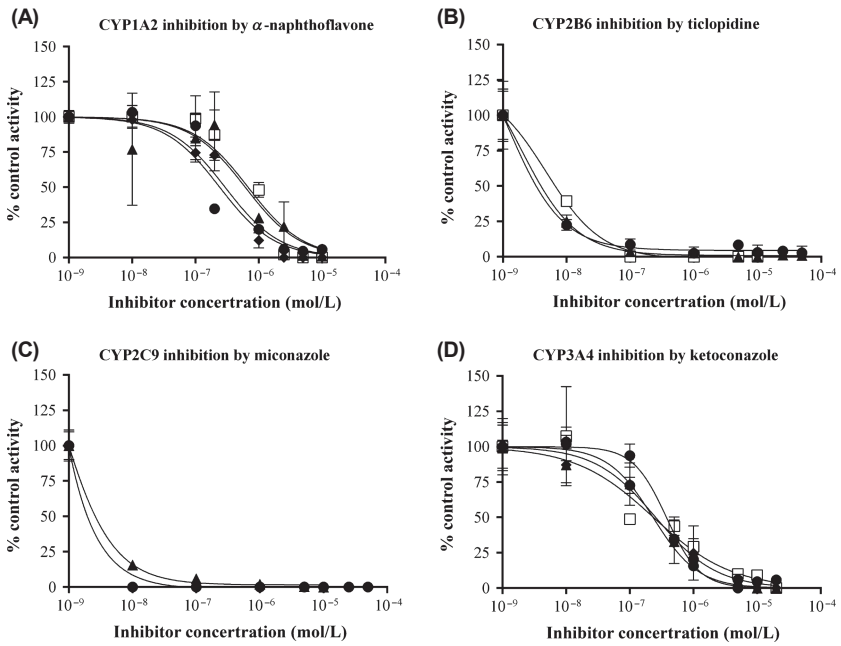


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Table 2. $IC_{50}$ values of CYP inhibitors incubated with upcyte® hepatocytes from different donors. For comparison, literature values for microsomes and/or hepatocytes are also shown. Values for upcyte® hepatocytes are mean SD, $n$ = 6–8 wells from at least two separate experiments.

| | $IC_{50}$ value | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
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## CYP induction

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Upcyte® hepatocytes from all four donors tested using a 3 day preculture period were responsive to CYP1A2, CYP2B6, CYP2C9, and CYP3A4 induction by prototypical



Table 3. Inhibition of CYP activities in upcyte® hepatocytes from different donors incubated in Lab 2.

| | | | Donor 151-03 | | Donor 422A-03 | | Donor 653-03 | |
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| 3A4 | 1'OH-MID | 1 mmol/L ABT | 99 | 97 | 98 | 98 | 98 | 98 |
| | +1'OH-MID-G | 3 *l*mol/L ketoconazole | 91 | 40 | 93 | 75 | 87 | 58 |
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### Prediction models for in vivo CYP3A4 induction

There are three main prediction models recommended by the FDA, EMA, and PhARMA for CYP3A4 induction (Chu et al. 2009; EMA, 2012; FDA, 2012), namely, the RIS, $AUC_u/F_2$, and $C_{max,u}/Ind_{50}$. Data from upcyte® he- patocytes from Donor 653 were used to compare the dif- ferent models (Fig. 5). Of the three, the fit was best when the RIS ($R^2$ = 0.92) and $C_{max,u}/Ind_{50}$ ($R^2$ = 0.93) were used; however, the $F_2$ value ($R^2$ = 0.89) may also be used when compounds are too toxic or insoluble to reach a maximal induction response. The $Ind_{max}$ and $Ind_{50}$ values from all four donors were applied to the RIS model (values shown in Table 4) and, although the calibration curves were different across donors, they all exhibited a good fit of the data ($R^2$ = 0.87–0.94, Figs. 5C, 6).

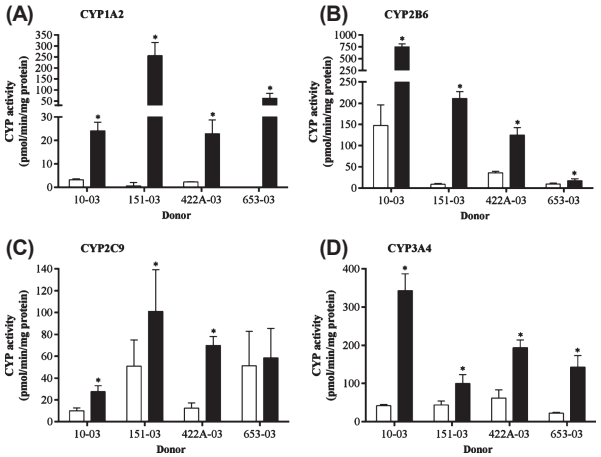


Figure 4. Induction of (A) CYP1A2 (by 50 *l*mol/L omeprazole), (B) CYP2B6 (by 2 mmol/L phenobarbital), (C) CYP2C9 (by 20 *l*mol/L rifampicin), and (D) CYP3A4 (by 20 *l*mol/L rifampicin) in upcyte® hepatocytes from Donors 10-03, 151-03, 422A–03, and 653-03. White bars indicate control values and black bars indicate values for the prototypical inducers. Values are the mean SD from triplicates.

CAR and PXR selective induction of CYP3A4 and CYP2B6 in upcyte® hepatocytes

The relative induction of CYP2B6 and CYP3A4 is known to be a result of selective activation of either the PXR or CAR receptors (Faucette et al. 2007); therefore, we inves- tigated this attribute by treating upcyte® hepatocytes with different inducers and measuring CYP3A4 and CYP2B6 activities in the same wells. Table 5 summarizes the CYP3A4 and CYP2B6 induction responses of upcyte® hepatocytes from all donors to the same compounds tested for CYP3A4 induction. Figure 7 compares the relative induction of both CYPs (compared to the maximal fold induction by the positive controls, according to equation 2 in the Materials and Methods)

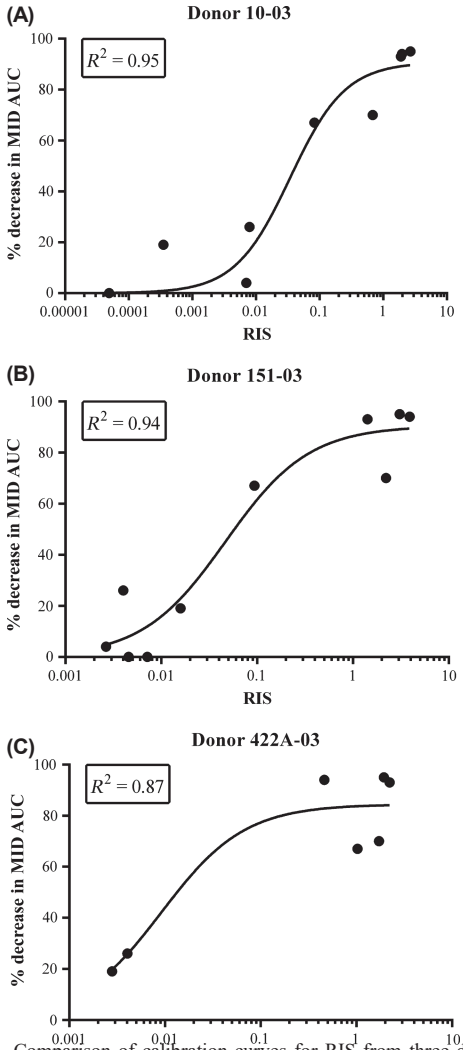


Figure 6. Comparison of calibration curves for RIS from three donors of upcyte® hepatocytes (Donor 10-03 (A); Donor 151-03 (B) and Donor 422A-03 (C)). Values are the mean from duplicate values of two experiments. RIS, relative induction score.

hepatocytes from Donors 10-03, 151-03, and 653-03 (between 14% and 34% of the maximal PB response). Phenobarbital was a strong inducer of both CYP3A4 and CYP2B6 and resulted in maximal induction of both CYPs in upcyte® hepatocytes from all four donors. Phenytoin was also a strong inducer of CYP3A4, in keeping with the findings of Raucy (Raucy 2003) who showed that phenyt- oin increased CYP3A4 mRNA markedly and the response was equivalent to that seen with rifampicin. Phenytoin is a weak PXR activator (Raucy 2003) and was subsequently shown to be a selective activator of CAR (Wang et al. 2004). This was reflected in these studies by the predomi- nance for CYP2B6 over CYP3A4 induction by this compound in three of the four donors (e.g., the induction of CYP2B6 and CYP3A4 in upcyte® hepatocytes from Donor 151-03 was 130% and 60% of the positive con- trols, respectively). Although carbamazepine was a moder- ate inducer of CY3A4 and CYP2B6, the relative predominance for CYP2B6 induction was also evident for this CAR-selective compound in three of the four Donors.

## Discussion

We have investigated the applicability of second-generation upcyte® hepatocytes to metabolism-related assays. In order to carry out these assays, the cells should express sufficient levels of CYP activities; therefore, our initial studies explored whether the inclusion of DMSO in the media could increase CYP activities. DMSO is known to induce CYP3A4 activities in primary human hepatocytes (LeC- luyse 2001) and CYP3A4 and CYP2B6 activities in HepaRG cells (Antherieu et al. 2010) by activating PXR and/or CAR, although, CYP1A2 is not induced over the same con- centration of DMSO (LeCluyse 2001). Our investigations confirmed that supplementing the medium with DMSO could be beneficial to the overall XME properties of the cells. During the preculture, HGM can be supplemented with 0.5% DMSO but during the conditioning period, HPM and not HGM should be used as the basal medium and optimally supplemented with 0.1% DMSO. The reason for lower XME activities cells conditioned in DMSO-sup- plemented HGM may be due to a downregulation of CYPs when the DMSO concentration is maintained at the higher concentration and/or that factors in the growth medium may not be suitable for differentiating the cells once they reach confluence. The concentration of 0.1% DMSO in the conditioning medium was considered acceptable as it is the standard solvent and concentration for many test com- pound control incubations.

The metabolism studies conducted in two laboratories demonstrated that second-generation upcyte® hepatocytes maintained in optimized culture conditions expressed good levels of CYP activities equivalent to or higher than those in paired primary cell cultures from the same donor. With the exception of CYP1A2, the CYP activities were generally higher than in their corresponding primary human hepatocyte cultures. Despite the low CYP1A2 activities demonstrated in short-term incubations of 1 h, longer incubations of 24 h showed that CYP1A2 sub- strates were also metabolized. Moreover, in the case of CYP2B6 and CYP3A4, activities in upcyte® hepatocytes were 5- to 10-fold higher then paired

primary cultures. Although different substrate concentrations were used, the relative levels of activities measured in upcyte® hepatocytes from different donors was comparable in the

Table 5. CYP3A4 induction responses of upcyte® hepatocytes from different donors to known inducers and noninducers of CYP3A4 and CYP2B6.

| Compound | Donor 10-03 | | | | Donor 151-03 | | | | Donor 422A-03 | | | | Donor 653-03 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CYP3A4 | | CYP2B6 | | CYP3A4 | | CYP2B6 | | CYP3A4 | | CYP2B6 | | CYP3A4 | | CYP2B6 | |
| | $Ind_{max}$ | % Rif | $Ind_{max}$ | % PB | $Ind_{max}$ | % Rif | $Ind_{max}$ | % PB | $Ind_{max}$ | % Rif | $Ind_{max}$ | % PB | $Ind_{max}$ | $Ind_{50}$ | $Ind_{max}$ | % PB |
| Rifampicin | 31.1 | 100 | 2.0 | 34 | 7.2 | 100 | 1.0 | 14 | 11.9 | 100 | 7.8 | 108 | 10.0 | 100 | 1.6 | 29 |
| Phenobarbital | 23.3 | 74 | 3.9 | 100 | 5.2 | 68 | 2.8 | 100 | 11.3 | 95 | 7.3 | 100 | 11.8 | 120 | 3.1 | 100 |
| Phenytoin | 14.6 | 45 | 3.6 | 88 | 4.7 | 60 | 2.0 | 89 | 15.8 | 137 | 4.0 | 48 | 6.3 | 59 | 2.8 | 85 |
| Carbamazepine | 4.8 | 13 | 2.0 | 33 | 3.8 | 45 | 2.5 | 68 | 4.0 | 28 | 4.3 | 52 | 5.7 | 52 | 1.4 | 17 |
| Troglitazone | 4.7 | 12 | 5.4 | 150 | 1.8 | 23 | 2.1 | 57 | 2.7 | 15 | 5.0 | 63 | 2.2 | 14 | 2.3 | 63 |
| Pioglitazone | 4.3 | 11 | 2.6 | 55 | 4.0 | 48 | 1.9 | 49 | 2.6 | 14 | 3.6 | 42 | 9.2 | 91 | 2.1 | 54 |
| Dexamethasone | 2.5 | 5 | No induction | | 3.5 | 39 | No induction | | 3.4 | 22 | No induction | | 2.7 | 19 | No induction | |
| Nifedipine | 2.1 | 4 | 3.9 | 98 | 4.6 | 58 | No induction | | Not determined | | No induction | | 7.2 | 69 | No induction | |
| Omeprazole | 2.3 | 4 | 2.0 | 31 | 3.1 | 34 | No induction | | Not determined | | Not determined | | 1.5 | 5 | 2.2 | 56 |
| Flumazenil | No induction | | No induction | | No induction | | No induction | | No induction | | No induction | | No induction | | No induction | |
| Quinidine | No induction | | No induction | | No induction | | No induction | | No induction | | No induction | | No induction | | No induction | |

$Ind_{max}$ is the maximum fold induction of CYP activity induced by the compound. The relative % of the positive control for CYP3A4 ("%Rif") and CYP2B6 ("%PB") was calculated according to equation 2, as described in the Materials and Methods. Values are means from two experiments.

CYP Induction and Inhibition Assays Using Upcyte® Hepatocytes two laboratories, confirming that shipment of live cells does not markedly compromise the cells. The good levels of CYP activities in these cells makes them a promising model for long-term (>24 h) incubations for generating and identifying metabolites, as well as clearance and inhi- bition studies, which all require XMEs higher than that present in short-term cultures of primary human hepato- cytes. Upcyte® hepatocytes therefore offer an advantage over liver microsomes and hepatocytes, which generally do not metabolize test compounds sufficiently in short- term assays (<24 h) to determine an area under the curve measurement (Di et al. 2012). The studies carried out in Lab 2 also support the use of upcyte® hepatocytes in clearance assays for stable compounds, as these were con- ducted over 24 h and demonstrated good metabolic func- tion over the entire incubation period. In additional to metabolism studies, we have shown that second-genera- tion upcyte® hepatocytes can also be used in CYP inhibition studies, which also require sufficient metabolic activities to ensure a good dynamic range. For CYP1A2, inhibition assays can be conducted using a 24 h incuba- tion period to ensure higher control activities; whereas, incubations of 1 h require boosting of CYP activity by preinducing CYP1A2 (e.g., with omeprazole). All four CYPs were inhibited in a concentration-dependent man- ner and the calculated $IC_{50}$ values confirmed that all ref- erence compounds tested were potent inhibitors of the respective CYPs.

Second-generation upcyte® hepatocytes were responsive to prototypical CYP1A2, CYP2B6, CYP2C9, and CYP3A4 inducers, confirming that they have functional AhR-, CAR- , and PXR-mediated CYP regulation. Additional studies carried out at Lab 2 investigated the effect of prototypical inducers (omeprazole, phenobarbital, and rifampicin) on a panel of CYP, UDP-glucuronosyltransferase (UGT), and transporter mRNAs and showed that the induction pattern was similar to that in human hepatocytes (data not shown). These findings support the use of second-generation upcyte® hepatocytes for investigating the induction of multiple genes using mRNA as the endpoint measurement. Like primary human hepatocytes, there were differences in the responsiveness of upcyte® hepatocytes to CYP inducers. There were also differences in the CYP3A4 and CYP2B6 induction responses in the same cultures of upcyte® he- patocytes to different inducers. For example, phenytoin preferentially activates CAR over PXR, evident in these cells as a more potent induction of CYP2B6 than CYP3A4 at the same concentration. By contrast, rifampicin was a more potent inducer of CYP3A4 than phenytoin, indicative of the preferential activation of PXR by this potent inducer. The CAR and PXR selective induction of CYP3A4 and CYP2B6 in upcyte® hepatocyte correspond to the findings of Faucette et al. (2004) who reported phenytoin, pheno-

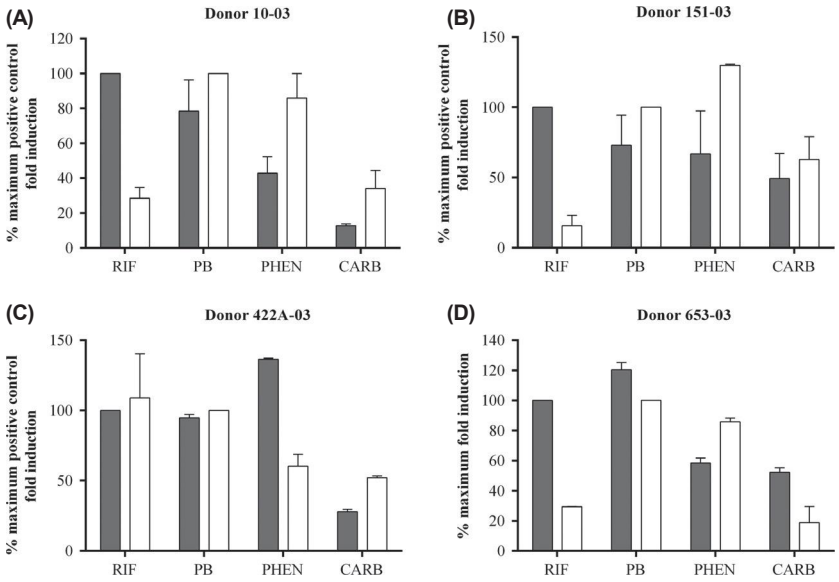


Figure 7. A comparison of % maximum fold induction by rifampicin, phenobarbital, phenytoin, and carbamazepine in upcyte® hepatocytes from different donors (Donor 10-03 (A); Donor 151-03 (B); Donor 422A-03 (C); Donor 653-03 (D)). Black bars indicate values for CYP3A4 and white bars indicate values for CYP2B6. Values are the mean from duplicate values of 2 experiments.

barbital, and rifampicin to be classified as strong inducers, and carbamazepine as a moderate inducer of CYP2B6 (based on mean fold induction values). By contrast, dexa- methasone – a known PXR selective activator – did not induce CYP2B6 in upcyte® hepatocytes from any of the donors but it was a weak inducer of CYP3A4 (Table 5). Troglitazone is a mixed PXR/CAR activator and induced both CYP3A4 and CYP2B6. This drug is known to cause in vivo CYP3A4 induction; whereas, pioglitazone is a weak inducer (Sahi et al. 2003; Ripp et al. 2006). In upcyte® he- patocytes, troglitazone was a more potent inducer of both CYP3A4 and CYP2B6 than pioglitazone, either due to a higher fold induction or a lower $Ind_{50}$ (i.e., the efficiency ratio was higher).

There was a good fit of data from these studies when they were applied to three different predictive models for CYP3A4 induction, namely the RIS, $AUC_u/F_2$, and $C_{max,u}/Ind_{50}$. Importantly, there was a very good intra- and interexperimental reproducibility of the measurements for all end points measured in these studies. Induction studies on one donor of first-generation upcyte® hepatocytes showed that the $Ind_{max}$ and $Ind_{50}$ values, and consequently the RIS values, were similar at each growth stage (spanning 15 PDs) (Burkard et al. 2012). Additional investigations on second-generation upcyte® hepatocytes showed that the fold induction of CYP3A4 by rifampicin was also consistent at different stages of growth (between a PD of 21 and 42 (Levy et al. 2015)). Therefore, as the PD of all the cells used in these studies was between 20 and 25, we did not expect marked variation in the results between batches from the same donor. For researchers employing the RIS calibration curve as part of their screening process, the robust nature of these cells means that the calibration curve would not need to be repeated once established (although a yearly check would be advisable). By contrast, those who employ cryopreserved human hepatocytes for the same assay need to re-establish a new calibration curve for each batch once the previous batch is depleted. Since billions of upcyte® he- patocytes from a number of donors are available results over a period of years can be compared.

In conclusion, second-generation upcyte® hepatocytes are a promising model with which to test the metabolism and drug interaction potential of novel compounds prior to being tested in regulatory submission studies using pri- mary human hepatocytes. Upcyte® hepatocytes represent a more predictive and relevant tool for predicting enzyme induction than PXR activation studies or receptor-binding assays. A genome-wide comparison between these cells and primary hepatocytes and/or human liver tissue would be necessary to confirm their use as a surrogate for pri- mary hepatocytes. Under the culture conditions used, these cells expressed good amounts of CYP activities, which could be inhibited by CYP-selective inhibitors. Upcyte® hepatocytes are responsive to CYP1A2, CYP2B6, CYP2C9, and CYP3A4 induction and can be used to pre- dict in vivo CYP3A4 induction potential using three com- mon prediction models. The availability of large quantities of cells from multiple donors makes upcyte® hepatocytes

suitable for drug–drug interaction (DDI) screening, as well as more in-depth mechanistic investigations.

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