Expression and induction potential of cytochromes P450 in human cryopreserved hepatocytes.

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Running title: Induction of CYPs in human cryopreserved hepatocytes.

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Number of text pages: 35.
Number of tables: 2.
Number of figures: 7.
Number of references: 49.
Words in abstract: 221.
Words in introduction: 569.
Words in discussion: 1682.

Abbreviations: CYP, cytochrome P450; NCE, new chemical entity; PhRMA, pharmaceutical research & manufacturers of America; QRT-PCR, quantitative reverse-transcriptase polymerase chain reaction.
Abstract

Fresh human hepatocytes are still considered as the ‘gold standard’ to screen in vitro for cytochrome P450 (CYP) induction. However, sparse availability of good quality human liver tissue for research purposes, the demand for standardized cell populations together with the need for proper storage of the cells not immediately required, have resulted in the development of cryopreservation techniques that provide adequate viability and plateability of hepatocytes after thawing. This study aimed at validating cryopreserved human hepatocytes as a model to investigate CYP induction. Cryopreserved cells from four different donors were plated and cultured for 48 h followed by incubation in the presence of typical CYP inducers. During the experiments, quality of the cultured cells was monitored both physiologically and morphologically. Concomitantly, the activity of CYPs 1A2, 2B6, 2C9, 2E1 and 3A4 was measured together with their mRNA and protein expression. Determination of CYP1A2, 2B6, 2C9, 2E1 and 3A4 activity in control versus prototypical inducer-treated hepatocytes revealed a maximal significant mean 11.6-, 2.8-, 1.9-, 1.5-, and 9.0-fold induction over their basal expression, respectively. Protein expression analysis of these CYPs confirmed these results. Moreover, a mean 44.9-, 3.5-, 3.2-, and 13.8-fold induction of CYP1A2, 2B6, 2C9, and 3A4 mRNA was observed. Our data demonstrate that cryopreserved human hepatocytes are a valuable tool to study the induction of CYP1A2, 2B6, 2C9, 2E1 and 3A4.
Cytochromes P450 (CYPs) are a superfamily of heme-thiolate-containing mixed function monooxygenases involved in the metabolism of steroid hormones, bile and fatty acids, and prostaglandins. Besides their role in the metabolism of endogenous molecules, they are also the most important enzymes involved in the phase I biotransformation of xenobiotics like food compounds, pollutants, and drugs (Parkinson, 2001). Some of these CYPs are induced several fold by specific drugs, leading to an increase in their enzymatic activity. As a consequence, the disposition of a drug may be altered, possibly leading to the accumulation of toxic metabolites or therapeutic failure of the drug. Therefore, drugs and new chemical entities (NCEs) are often screened for their ability to induce CYPs or other drug-metabolizing enzymes with the aim of predicting or explaining pharmacokinetic tolerance or drug-drug interactions.

In the past, many CYP induction studies, performed in various animal species, have proven to be beneficial. However, species differences in the induction of CYPs make the extrapolation from animals to humans very difficult or even impossible in some cases. Therefore, simple, robust, and reproducible in vitro models to study CYP induction would greatly facilitate the ability to develop drugs devoid of these possible negative traits (Silva et al, 1998).

An extensive number of studies have been reported demonstrating the soundness of primary cultures of human hepatocytes for toxicological, metabolic and pharmacological experiments (Donato et al, 1995; Maurel, 1996; Ferrini et al, 1997; Guillouzo et al, 1997; Kern et al, 1997). In addition, these human hepatocyte cultures have proven also to be a reliable in vitro model for evaluating NCEs as inducers of CYPs (Kostrubsky et al, 1999; LeCluse et al, 2000; Silva and Nicoll-Griffith, 2002). When in vitro CYP induction experiments are conducted at therapeutically relevant concentrations of the NCEs, in most cases, the in vitro data correlate well with clinical observations (Dilger, 1999; LeCluse et al, 2000; Madan et al, 2003).

For research purposes however, human liver tissue is only sparsely available and the number of sources of healthy tissue limited. Besides the erratic availability of human liver tissue, the supply of these cells is also unpredictable, and limited by legal and ethical issues (Skett et al, 1995). Moreover, the large amount of hepatocytes isolated from liver may not necessarily be required for an immediate use.
The last couple of years, cryopreservation techniques have been improved allowing a high percentage of viable and plateable hepatocytes after thawing (Hengstler et al, 2000a; Hengstler et al, 2000b; Alexandre et al, 2002). Despite this technical progress, limited information on the induction of CYPs in cryopreserved human hepatocytes is available in the current literature. Moreover, the available studies mainly focus only on CYP1A and 3A induction (Reinach et al, 1999; Silva et al, 1999; Skett et al, 1999; Hengstler et al, 2000b; Roymans et al, 2004).

In 1999 an international expert panel advised against the use of cryopreserved human hepatocytes for long-term in vitro CYP induction studies (Li et al, 1999). However, the demand of the pharmaceutical industry for human hepatocytes is immense. They are also in need of standardized hepatocyte populations since different sets of experiments, required to develop a NCE into a marketed drug, are spreaded both geographically and in time. In addition, cryopreserved human hepatocytes would allow a better planning of the different studies.

This study summarizes the validation of different batches of commercially available cryopreserved human hepatocytes as an in vitro model to study CYP induction.
Materials and methods

Materials

Hepatocytes from four separate human donors were obtained from the cryopreserved hepatocyte bank maintained at In Vitro Technologies (Baltimore, MD, USA). Hepatocytes from lots 059, 082, NLR, and BDF, selected for high plating efficiency, were used in this study (Table 1). Hepatocyte thawing and plating media were obtained from In Vitro Technologies. All other cell culture media and additives were from Gibco (Invitrogen, Merelbeke, Belgium). Trypan blue, DMSO, omeprazole, rifampicin, phenobarbital, 7-ethoxyresorufin, resorufin, 4-hydroxymethyl tolbutamide, tolbutamide and testosterone were obtained from Sigma (St. Louis, MO, USA). S-mephenytoin, N-demethylated S-mephenytoin and 6-hydroxy chlorozoxazone were from Ultrafine Chemicals (Manchester, UK). Dexamethasone was obtained from Acros Chimica (New Brunswick, NJ, USA). Clofibric acid and chlorozoxazone were from Serva (10P’s, Zandhoven, Belgium) and Ortho-McNeil (Raritan, NJ, USA) respectively. 6β-hydroxytestosterone was obtained from Steraloids Inc. (Eilton, NH, USA). β-glucuronidase and β-glucuronidase/arylsulfatase were from Roche Diagnostics (Vilvoorde, Belgium). Polyclonal antibodies against CYP1A2 (cat. no 458124), CYP2B6 (cat. no 458226), CYP2C9 (cat. no 458209), CYP2E1 (cat. no 458219), and CYP3A4 (cat. no 458234) were from Gentest (Woburn, MA, USA). Horseradisch peroxidase-conjugated donkey-anti-goat (cat. no 705-035-147) and donkey-anti-rabbit (cat. no 712-035-153) were purchased from Jackson Immunoresearch (West-Grove, PA, USA). The β-actin rabbit-anti-human antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Establishment of hepatocyte cultures

Cryopreserved hepatocytes were thawed in hepatocyte thawing medium and counted to determine yield. Viability was measured using Trypan blue exclusion; only hepatocyte batches with ≥ 85 % viability were used in this study. Isolated hepatocytes were transferred to collagen I precoated 24-well plates, each well containing a cell density of 0.3 × 10^6 viable cells in 0.5 ml of hepatocyte plating medium. After 24 h, hepatocyte plating medium was removed and replaced with incubation medium [Williams E + Glutamax, 10 % FBS, 1 % penicillin/streptomycin, 7 µM insulin and 1 µM dexamethasone]. After an additional 24 h of
culturing, the confluence of the hepatocytes was visually assessed using phase contrast microscopy. At the time of dosing, hepatocytes were ~90% confluent. Following the initial 2-day recovery period, the incubation medium was removed and the hepatocytes were treated daily with induction medium [Williams E + Glutamax, 1 % ITS+ premix solution and 0.1 µM dexamethasone] containing either vehicle (0.1 % DMSO), 25 µM omeprazole, 25 µM rifampicin, 10 µM dexamethasone, 100 µM phenobarbital, 100 µM clofibric acid or 40 mM ethanol for two consecutive days. All incubations were conducted at 37 ± 1 °C, 95 % air/5 % CO₂, and saturating humidity. For each lot of hepatocytes, 3 separate wells per treatment condition were used.

Human albumin ELISA

Human albumin secretion was measured with an ELISA quantitation kit from Bethyl Laboratories (Montgomery, TX, USA) according to the manufacturer’s instructions. Briefly, cells were cultured as described above and human albumin determined just before every medium change. Wells were coated with a goat-anti-human albumin antibody (1:100) diluted in coating buffer [0.05 M sodium carbonate, pH 9.6] for 60 min at RT. After washing the wells 3 x with wash solution [50 mM Tris, 0.14 M NaCl, 0.05 % Tween 20, pH 8.0], wells were blocked for 30 min at RT with post-coat solution [50 mM Tris, 0.14 M NaCl, 1 % BSA, pH 8.0]. Wells were washed again 3 x with wash solution and incubated for another 60 min with 100 µl standard, sample or blank. Subsequently, wells were washed 5 x with wash solution and incubated for 60 min at RT with a goat-anti-human albumin-HRP conjugate antibody (1:100000) diluted in conjugate diluent [50 mM Tris, 0.14 M NaCl, 1 % BSA, 0.05 % Tween 20, pH 8.0]. Wells were washed again 5 x with wash solution, and colorimetric reactions initiated in the wells using TMB as a substrate. Finally, reactions were stopped after 5 min by administration of 2 M H₂SO₄ and absorbance measured at 450 nm using a Safire spectrophotometer (Tecan, Grödig, Austria). To allow the albumin secretion to be expressed as pg/cell.hour, cells were counted at the time of measurement in three identically-treated parallel wells.

Cell viability assay.

Cell viability was determined based on the quantitation of intracellular ATP with the CellTiter-Glo™ assay from Promega (Leiden, The Netherlands) according to the manufacturer’s
instructions. Briefly, hepatocytes were equilibrated to RT for 30 min. CellTiter-Glo™ reagent (1:1) was added to the culture medium and cell lysis was induced by shaking the cells for 2 min at RT. Subsequently, the supernatant of the different wells was transferred to opaque-walled 24-well plates and the luminescent signal stabilized for 10 min at RT. Luminescence was recorded on a Victor² 1420 Multilabel counter (Perkin Elmer, Boston, MA, USA). To allow the ATP content to be expressed as fmol/cell, cells were counted at the time of measurement in three identically-treated parallel wells.

**Cytochrome P450 activity assays**

Prior to the conduct of all enzymatic assays, hepatocytes were rinsed twice with 0.5 ml/well Hank’s Balanced Salt Solution (HBSS) and subsequently incubated for 60 min at 37 °C with 0.5 ml/well HBSS to wash out residual amounts of inducers. After treatment, hepatic CYP1A2 activity was measured by incubating the cells in HBSS containing 2 µM 7-ethoxyresorufin and 10 µM dicoumarol for 60 min at 37 °C. Subsequently, the supernatant was removed from the wells, incubated at 37 °C for 2 h in the presence of 4 x 10-2 U/ml β-glucuronidase, and the reaction was stopped by addition of methanol (1:1). Samples were centrifuged at 1700 g for 30 min at RT and liberated resorufin in the supernatant fluorimetrically analysed (excitation: 550 nm; emission: 585 nm) in a Safire spectrophotometer. CYP2B6 activity was determined by N-demethylation of S-mephenytoin. Briefly, cells were incubated for 2 h at 37 °C in the presence of 200 µM S-mephenytoin in HBSS and the reaction stopped by probe sonication with a Viborcell sonicator (Sonics & Materials, Danbury, CT, USA) at 40 W for 10 sec. At the end of the incubation, 80 µl of culture medium was transferred to a 96-well plate containing an equal volume of methanol. After the addition of 5 µM propanolol as internal standard, samples were centrifuged and the supernatant transferred to an evaporation plate. Once the samples were dried, they were reconstituted in 150 µl of mobile phase (3:1 methanol:water, 0.1 % acetic acid) and 25 µl were injected onto the column for LC/MS/MS analysis. Standards used for analytical linearity were prepared by spiking a 50:50 mixture of HBSS and methanol with standard mixtures of the probe metabolite, nirvanol. Standard curves for this metabolite ranged between 3 and 350 nM. Chromatographic separation of the analytes was achieved by using a Luna C18 (2 x 50mm) column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.1 % acetic acid in water (A) and 0.1 % acetic acid in methanol (B), with a
gradient profile starting at 10 % B then increasing to 80 % B from 0.2 to 1 min, further increasing to 92 % B at 1.5 min, and remaining at 92 % B until 3 min, before returning back to 10 % B at 4 min. The total run time was 5 min and the flow rate 0.3 ml/min. Metabolites were detected on an API2000 triple quadrupole mass spectrometer (Sciex, Concord, Canada). The mass spectrometer was equipped with an electrospray ionisation source and operated in the positive ion mode. Turbo gas temperature was 425 °C and the ionspray needle kept at 5500 V. For quantitation, the mass spectrometer was operated in the multiple reaction monitoring mode to monitor for the metabolite of the substrate. For nirvanol, the precursor-to-product ion reaction monitored was m/z 205 → 134.

CYP2C9 and CYP3A4 activity was analysed with LC-MS/MS by determining the 4-methylhydroxylation and the 6β-hydroxylation of tolbutamide and testosterone respectively. Hepatocytes were incubated at 37 °C with 150 µM tolbutamide in HBSS for 12 h or with 50 µM testosterone in HBSS for 2 h. Subsequently, the reactions were terminated by probe sonication, the cell homogenates deconjugated with β-glucuronidase/arylsulfatase as described above and finally mixed with DMSO (1:1) (tolbutamide) or methanol (1:1) (testosterone). The samples were analyzed on a Surveyor liquid chromatograph interfaced to a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, San Jose, CA, USA). An aliquot (50 µl for tolbutamide and 40 µl for testosterone) from each sample was injected onto the HPLC-column (500 mm x 4.6 mm id) packed with Kromasil C-18 (5 µm).

The flow rate was 1 ml/min. Mobile phase A consisted of 0.1 % HCOOH, 95 % water, 5 % methanol and mobile phase B consisted of 0.1 % HCOOH, 95 % methanol, 5 % water. Mobile phase A was linearly ramped from 100 to 0 % in 5 min, held at 0 % for an additional 1 min, and then brought back to 100 % mobile phase A in 2 min for re-equilibration. After 2 min, the HPLC eluent was diverted from waste to the mass spectrometer fitted with an electrospray ionization source and operated in the negative ion mode or positive ion mode for the analysis of tolbutamide or testosterone respectively. The settings (lens voltages, quadrupole and octapole voltage offsets, etc.) were optimized for maximum intensity for tolbutamide or testosterone by using the auto-tune function within the LCQ Tune program. Following the instrument tune, the needle voltage was set to 4.5 kV (tolbutamide) and 5.2 kV (testosterone).

For quantitation, the mass spectrometer was operated in the multiple reaction monitoring
mode to monitor for metabolites of each substrate. For 4-methylhydroxy tolbutamide and 6β-hydroxytestosterone, the precursor-to-product ion reactions monitored were m/z 285 → 186 and 305 → 269, respectively. The activity of CYP2E1 was measured by incubating the cells for 12 h at 37 °C with HBSS containing 100 µM chlorzoxazone. The reactions were stopped by probe sonication, the cell homogenates deconjugated with β-glucuronidase/arylsulfatase as described above and finally mixed with DMSO (1:1). After centrifugation of the cell homogenates at 1700 g for 30 min, samples were analyzed as described above and formation of 6-hydroxy chlorzoxazone detected by UV-detection at 296 nm. Activity was determined for each separate well (3 wells/treatment).

RNA isolation

After 24 hours of incubation with compounds, total cellular RNA was prepared using the RNeasy method (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions and included an on-column DNase I digestion to minimize genomic DNA contamination. RNA quantitation was determined fluorometrically using Ribogreen RNA quantitation reagent (Molecular Probes, Eugene, OR, USA). Fluorescence was measured on a Safire spectrophotometer (Tecan).

cDNA synthesis.

cDNA was synthesized from total RNA using the SuperScript First-strand cDNA synthesis kit from Invitrogen according to the manufacturer’s instructions. Briefly, 2 µg total RNA from each sample was added to a reaction mixture containing 100 ng random hexamer primers, 0.5 mM dNTP’s, RT buffer [20 mM Tris.HCl, pH 8.4; 50 mM KCl], 5 mM MgCl₂, 10 mM DTT, 40 U ribonuclease inhibitor and incubated at 25 °C for 2 min. Subsequently, 50 U reverse transcriptase was added to the samples and incubated for another 45 min at 25 °C. After terminating the reaction at 70 °C for 15 min, the samples were cooled on ice. Finally, 4 U E. coli RNase H was added and incubated at 37 °C for 20 min.

Real-time QRT-PCR

Cytochrome P450 1A2, 2B6, 2C9, 2E1, and 3A4 mRNA levels were determined using standard TaqMan real-time QRT-PCR methods. Sequence specific primers and TaqMan probes for the different CYPs (Table 2) were designed with Primer Express software (Applied
Biosystems, Foster City, CA, USA) and synthesized at Eurogentec (Luik, Belgium). TaqMan RT-PCR samples were composed in 96-well plates with 0.4 ng/µl of each cDNA sample in a reaction volume of 80 µl prepared from a TaqMan 1000Rxn Gold/Buffer A Reagents Kit (Applied Biosystems). The assays contained 1 µM each of sequence specific forward and reverse primer and 0.5 µM TaqMan probe. Subsequently, triplicate reactions were pipetted into 384-well plates to a final volume of 20 µl with a Biomek 2000 robot (Beckman Coulter, Fullerton, CA, USA). Assays were performed using an Applied Biosystems ABI Prism 7900HT sequence detection system. An initial heating step to 95 °C for 10 minutes was followed by 50 cycles of 95 °C for 15 seconds, 60 °C for one minute. Relative quantitation of gene expression levels was determined by interpolation of threshold cycle (Ct) values to a standard curve generated from a dilution series of human liver total RNA and normalized by the expression of β-actin. Messenger RNA expression was determined for each separate well (3 wells/treatment).

**Protein isolation**

After 48 hours of incubation with compounds, the cell-culture medium was completely aspirated and the cells were washed 2 x with ice-cold PBS. The cells were placed on ice, 100 µl of ice-cold homogenisation buffer [50 mM Tris-HCl, pH 7.0; 150 mM KCl; 2 mM EDTA] was added and the cells were scraped with a rubber policeman. After the cell suspension was transferred to an eppendorf tube, the cells were sonicated with a Vibracell sonicator at 40 W for 10 seconds. The cell homogenate was snap frozen in liquid nitrogen. The samples were stored at –70 °C until use.

**SDS-PAGE gelelectrophoresis**

The protein concentration of the samples was determined with the Coomassie Plus assay from Pierce (Rockford, IL, USA), according to the manufacturer’s instructions. Briefly, 300 µl of Coomassie Plus-reagent was added to 10 µl of sample, mixed for 1 min and incubated at room temperature for 10 min before spectrophotometric measurement at 595 nm in a Safire spectrophotometer. The protein concentration of the different samples was determined by comparing the A595-values against a BSA standard curve, ranging from 0-1000 µg/ml.
SDS-PAGE samples were prepared by mixing 2 µg of total protein of each sample with 4 x LDS sample loading buffer (Invitrogen) and 10 x reducing agent (Invitrogen). The samples were boiled for 5 min and loaded onto a 4-12 % Bis-Tris Gel from Invitrogen and electrophoresed in MOPS SDS running buffer [50 mM MOPS, 50 mM Tris, 3.5 mM SDS and 1 mM EDTA] in a Xcell SureLock™ Electrophoresis Cell from Invitrogen according to the manufacturer's instructions.

**Western blot analysis**

After electrophoresis, proteins were electroblotted for 2 h at 36 V onto a PVDF membrane in NuPAGE transfer buffer (25 mM Bis-Tris, 25 mM Bicine, 1 mM EDTA, 1mM Chlorobutanol, 10 % methanol and 0.001 % antioxidant)(Invitrogen). Membranes were blocked for 1 h at RT in 5 % non-fat milk in TBS-T [25 mM Tris.HCl pH 7.5, 150 mM NaCl, 0.1 % Tween20]. Blots were incubated with a goat-anti-rat CYP1A1/1A2 (1:10000), a rabbit-anti-human CYP2B6 (1:5000); a rabbit-anti-human CYP2C9 (1:5000), a goat-anti-rat CYP2E1 (1:10000), a rabbit-anti-human CYP3A4 (1:5000), or a rabbit-anti-human β-actin (1:2000) antibody in 0.5 % non-fat milk in TBS-T for 1 h at RT. After washing the membranes for 3 x 5 min in TBS-T, they were incubated with a secondary HRP-conjugated donkey-anti-goat (1:60000) or a HRP-conjugated donkey-anti-rabbit (1:100000) antibody in 0.5 % non-fat milk in TBS-T for 1 h at RT. After 4 final washes of the membranes for 5 min in TBS-T, protein expression was detected with a SuperSignal® West Femto ECL kit from Pierce. Relative quantitation of the target signals was performed with a Lumi-Imager from Roche (Basel, Switzerland). β-actin expression was used to normalize relative CYP protein expression. Protein expression was determined for each separate well (3 wells/treatment).

**Statistical analysis.**

Differences were evaluated using a Student’s t-test and considered statistically significant when p < 0.05.
Results

Quality assessment of hepatocyte cultures

The quality of the cultured cells was assessed both by measurement of their ATP content and albumin secretion rate. In a first set of experiments ATP content and albumin secretion rate were determined as a function of culturing time. Both parameters were measured just before every daily medium change. The mean human albumin secretion rate increased from 0.12 ± 0.02 pg/cell.h at 20 h of incubation time to 0.20 ± 0.01 pg/cell.h after 87 h of incubation (Fig 1A). The mean ATP content increased from 16.6 ± 0.1 fmol/cell to 28.6 ± 1.6 fmol/cell (Fig 1B). Starting from 1.3 x 10^4 cells/well, cell number was decreased approximately 25 % after day 4. In addition, after 48 h of incubation of the cells in the presence of different prototypical inducers, relative cell viability of the cells under the different incubation conditions was investigated also by measurement of the intracellular ATP concentration. The relative ATP content in the different lots of hepatocytes ranged between 86 % and 137 % (Fig 1C). No statistically significant differences in mean ATP content were observed under the different incubation conditions. The relative mean ATP content varied between 98 % and 120 % (Fig 1D).

After 96 h in culture, hepatocytes from all 4 lots further demonstrated a three-dimensional cuboidal shape. Hepatocytes from lot 059 appeared to have a higher tendency to accumulate in three-dimensional cell clusters (Fig 2). Moreover, the cells demonstrated a nuclear morphology identical to that observed in cultures of freshly prepared cells (LeCluyse, 2001).

CYP activity and expression profiling

Cells, originating from 4 different donors, were plated, allowed to recover for 48 h and then incubated in the presence of 0.1 % DMSO (control cells), 25 µM omeprazole, 25 µM rifampicin, 10 µM dexamethasone, 100 µM phenobarbital, 100 µM clofibrate acid or 40 mM ethanol. After another 48 h of culturing, the activity of CYP1A2, 2B6, 2C9, 2E1, and 3A4 was determined by measurement of the 7-ethoxyresorufin O-deethylation, S-mephenytoin N-demethylation, tolbutamide 4-methyl hydroxylation, chlorzoxazone 6-hydroxylation, and testosterone 6β-hydroxylation, respectively. Concomitantly, mRNA and protein expression were determined by real-time QRT-PCR and immunoblotting.
CYP1A2 activity in cryopreserved human hepatocytes incubated in the presence of vehicle alone was 2.8 ± 0.4, 6.8 ± 0.9, 7.0 ± 2.2, and 1.7 ± 0.1 pmol/min.mg protein in cells from lots NLR, BDF, 059, and 082, respectively (Fig 3A). This resulted in a mean activity of 4.6 ± 1.4 pmol/min.mg protein (Fig 3B). Incubation of the cells in the presence of the typical CYP1A2 inducer omeprazole increased the mean activity to 53.5 ± 24.7 pmol/min.mg protein, corresponding to a significant 11.6-fold induction of CYP1A2 activity. A high variation in the induction response of the cells from different donors was observed. Induced CYP1A2 activity ranged between 21.2 ± 3.9 pmol/min.mg protein (lot 082) and 126.8 ± 19.9 pmol/min.mg protein (lot BDF). As a result a 5.5- to 18.5-fold increase of the O-deethylation of 7-ethoxyresorufin was observed. When the cells were incubated in the presence of omeprazole, CYP1A2 protein expression was also upregulated in cells from the different lots (Fig 3E).

Cells from lots NLR, BDF, 059, and 082 upregulated CYP1A2 mRNA 30.3-, 29.7-, 9.5-, and 78.4-fold, respectively in the presence of omeprazole (Fig 3C). This resulted in a significant 37.0-fold mean induction of CYP1A2 mRNA (Fig 3D). To investigate if measurement of the CYP mRNA expression can be predictive for the assessment of CYP activity induction, the correlation between CYP mRNA expression and activity was scored. In all of the experimental conditions where CYP1A2 activity was significantly increased, also a significant increase in CYP1A2 mRNA expression was detected (Fig 3F). Moreover, no discrepancies between fold induction of CYP1A2 activity or mRNA expression were observed, indicating that measurement of CYP1A2 mRNA expression may be an excellent parameter to predict induction of CYP1A2 activity.

The basal CYP2B6 activity in hepatocytes originating from the 4 different donors varied between 1.91 ± 0.03 and 3.6 ± 0.6 pmol/min.mg protein, resulting in a mean CYP2B6 activity of 2.7 ± 0.7 pmol/min.mg protein (Fig 4A,B). Incubation of the cells with rifampicin significantly induced CYP2B6 activity 3.5-, 3.0-, 2.0-, and 2.7-fold in cells from lot NLR, BDF, 059, and 082, respectively. This resulted in a mean 2.8-fold induction of the CYP2B6 activity. Treatment of the cells with dexamethasone and phenobarbital tended to increase the mean CYP2B6 activity 1.4-, and 1.8-fold, although changes were not statistically significant. Accordingly, CYP2B6 mRNA expression was significantly induced when cells were incubated in the presence of rifampicin or phenobarbital (Fig 4C,D). Upon rifampicin-treatment, the
mRNA expression increased 3.4-, 4.2-, 4.5-, and 2.1-fold in cells from lot NLR, BDF, 059, and 082, respectively. As a result, the mean CYP2B6 mRNA expression significantly increased 3.5-fold. Administration of phenobarbital to the culture medium significantly increased the CYP2B6 mRNA expression 3.1-, 5.5-, 3.2-, and 2.1-fold (Fig 4C). The mean CYP2B6 mRNA expression in dexamethasone-treated cells increased 1.5-fold (Fig 4D). Analysis of the CYP2B6 protein expression also revealed increased levels of CYP2B6 in rifampicin-, dexamethasone-, and phenobarbital stimulated cells, confirming the observations in CYP2B6 activity assays and mRNA expression experiments (Fig 4E). In 20 out of 24 experimental conditions (83 %), measurement of the CYP2B6 mRNA expression was predictive for measurement of CYP2B6 activity (Fig 4F).

The basal activity of CYP2C9 in cells from lot 082 was approximately 2- to 3-fold higher than the activity in the other cells (Fig 5A). Incubation of the cells with rifampicin, dexamethasone or phenobarbital resulted in significant increases of CYP2C9 activity compared to the control cells. Activity was upregulated between 1.7- and 2.2-fold, 1.3- and 1.8-fold and 1.0- and 3.3-fold when rifampicin, dexamethasone or phenobarbital was administered to the hepatocytes’ culture medium. Under these conditions, the mean CYP2C9 activity increased 1.9-, 1.4- and 1.8-fold, respectively, elevating the formation of 4-hydroxymethyl tolbutamide from 0.033 ±0.009 pmol/min.mg protein in control cells to 0.062 ± 0.013 pmol/min.mg protein in rifampicin-stimulated cells, to 0.047 ± 0.009 pmol/min.mg protein in dexamethasone-stimulated cells and to 0.059 ± 0.003 pmol/min.mg protein when cells were incubated in the presence of phenobarbital (Fig 5B). Analogous to the induction of CYP2C9 activity, CYP2C9 mRNA expression was increased after incubation of the cells with rifampicin, dexamethasone, or phenobarbital (Fig 5C). In these cells, CYP2C9 mRNA increased between 1.6- and 2.5-fold, 1.1- and 2.0-fold, and 1.2- and 4.9-fold respectively. In addition, clofibric acid-treated cells also increased the mean CYP2C9 mRNA expression by 1.6-fold (Fig 5D). Analysis of the CYP2C9 protein expression also revealed slightly increased levels of CYP2C9 in rifampicin-, dexamethasone-, and phenobarbital-stimulated cells, confirming the observations in CYP2C9 activity assays and mRNA expression experiments (Fig 5E). In 18 out of 24 experimental conditions (75 %), measurement of the CYP2C9 mRNA expression was predictive for measurement of CYP2C9 activity (Fig 5F).
The basal 6-hydroxylation of chlorzoxazone varied approximately 4.5-fold between the 4 different batches of hepatocytes investigated. Basal CYP2E1 activity measured in lots NLR, BDF, 059, and 082 was 26.8 ± 0.7, 19.2 ± 0.9, 40.9 ± 3.4, and 9.1 ± 0.1 pmol/min.mg protein (Fig 6A), respectively, resulting in a mean CYP2E1 activity of 24.0 ± 6.7 pmol/min.mg protein (Fig 6B). Treatment of hepatocytes from lots NLR, BDF and 059 with omeprazole, significantly increased the CYP2E1 activity in these cells by 1.9-, 2.1 and 1.9-fold, respectively. A significant mean 1.9-fold induction of the CYP2E1 activity could be observed under these incubation conditions (Fig 6B). In addition, cells from the same lots significantly increased their CYP2E1 activity by 1.7-, 1.8-, and 1.4-fold when they were incubated in the presence of the typical CYP2E1 inducer, ethanol. Activity changed from 26.8 ± 0.7 pmol/min.mg protein to 46.3 ± 6.4 pmol/min.mg protein, from 19.2 ± 0.9 pmol/min.mg protein to 35.1 ± 2.9 pmol/min.mg protein, and from 40.9 ± 3.4 pmol/min.mg protein to 56.1 ± 5.8 pmol/min.mg protein in lot NLR, BDF, and 059, respectively. However, no induction of CYP2E1 was observed in cells from lot 082. As a consequence, the mean CYP2E1 activity was significantly induced 1.5-fold compared to the activity in the vehicle-treated cells.

Analysis of the CYP2E1 protein expression confirmed these results (Fig 6E). No statistically significant induction of CYP2E1 mRNA was observed (Fig 6C,D). In addition, in all of the experimental conditions where CYP2E1 activity was significantly increased, no increase in CYP2E1 mRNA expression was detected (Fig 6F). This observation is consistent with earlier reports demonstrating that CYP2E1 induction by these compounds seems to be mainly regulated by posttranscriptional mechanisms (Fuhr, 2000; Novak and Woodcroft, 2000).

Basal CYP3A4 activity ranged between 22.7 ± 0.6 pmol/min.mg protein and 63.5 ± 5.6 pmol/min.mg protein (Fig 7A). When cells from lots NLR, BDF, 059 and 082 were incubated in the presence of rifampicin, CYP3A4 activity increased 3.5-, 3.5-, 3.0- and 31.1-fold to 80.5 ± 12.0, 88.0 ± 3.7, 193.2 ± 2.3 and 915.8 ± 323.4 pmol/min. mg protein, respectively. The mean CYP3A4 activity under these incubation conditions was calculated to be 319.4 ± 200.5 pmol/min.mg protein, leading to a mean 9.0-fold increase (Fig 7B). Incubation of the cells in the presence of phenobarbital resulted also in a significant increase in CYP3A4 activity. 6β-hydroxylation of testosterone increased to 46.3 ± 0.5 (NLR), 41.0 ± 5.3 (BDF), 151.5 ± 10.0 (059), and 217.3 ± 87.7 pmol/min.mg protein (082). This corresponded to a significant mean
3.2-fold upregulation. In addition, incubation of cells from lot 082 in the presence of
dexamethasone increased CYP3A4 activity to 112.0 ± 50.6 pmol/min.mg protein, resulting in
a significant 3.8-fold upregulation. In general, analysis of CYP3A4 protein expression
confirmed the CYP3A4 activity measurements (Fig 7E). Hepatocytes from all 4 lots
demonstrated upregulated CYP3A4 protein expression upon incubation of the cells with
rifampicin and phenobarbital. However, treatment of the cells with dexamethasone did not
lead to an increase of CYP3A4 protein. Compared to control cells, a statistically significant
7.4-, 6.7-, 3.0-, and 23.1-fold induction of CYP3A4 mRNA expression was observed in
rifampicin-incubated cells from lots NLR, BDF, 059 and 082, respectively (Fig 7C). Moreover,
CYP3A4 mRNA expressed by these hepatocytes was also significantly increased 1.5- (NLR),
4.2- (BDF), 2.3- (059), and 18.6-fold (082) after incubation with phenobarbital. Calculation of
the mean CYP3A4 mRNA expression in omeprazole-, rifampicin-, and phenobarbital-
stimulated cells resulted in statistically significant increases of 4-, 13.8- and 10.1-fold,
respectively (Fig 7D). As a consequence, in 17 out of 24 experimental conditions (71 %),
measurement of the CYP3A4 mRNA expression was predictive for measurement of CYP3A4
activity (Fig 7F).
Discussion

In 1999, an international expert panel advised against the use of cryopreserved human hepatocytes for long-term CYP induction experiments (Li et al, 1999). Besides a significantly lower CYP enzyme activity after induction in these cells compared to freshly isolated human hepatocytes, their conclusion was also based on the limited availability of CYP induction data in cryopreserved human hepatocytes. Indeed, the amount of studies reported in the literature today is limited and mainly focused on the induction of CYP1A2 and CYP3A4 (Reinach et al, 1999; Silva et al, 1999; Skett et al, 1999; Hengstler et al, 2000b; Roymans et al, 2004). More recently, the use of cryopreserved human hepatocytes for induction experiments was also questioned in a paper from Bjornsson et al (2003), describing the pharmaceutical research and manufacturers of America (PhRMA) standpoint. Their viewpoint is mainly inspired by the fact that, previously, cryopreserved cells not often attached properly to their culture substratum. Especially in the pharmaceutical industry however, there is a high demand for human hepatocytes to test the metabolism and the pharmacological or toxicological behaviour of new drug candidates. Since many of these studies are spread geographically and/or in time, there is also a need for standardized hepatocyte populations. Moreover, the ability to properly store and have access to healthy human hepatocytes whenever required, would greatly improve planning of the different experiments. Therefore, the current study was undertaken.

A panel of CYPs was selected to be included in the study based on their importance in the metabolism of drugs. At present, three categories can be classified: CYPs of major importance, emerging importance, and low importance (Bjornsson et al, 2003). CYP1A2, 2C9, and 3A4 were selected for their established role in drug metabolism. The panel of CYPs was further completed with one representative of the emerging (CYP2B6) and one of the low importance (CYP2E1) class. To further obtain maximal potential mechanistic information, it was decided not only to measure CYP activities but also to determine protein and mRNA expression in the absence or presence of prototypical inducers.

Proton-pump inhibitors have been demonstrated in many studies as effective in vitro inducers of CYP1A2 (Curi-Pedrosa et al, 1994; Jang and Maurel, 2000). We selected omeprazole as a prototypical inducer of CYP1A2 and incubated the cells at a dose level as recommended by...
PhRMA drug metabolism and clinical pharmacology working groups (Bjornsson et al., 2003). The mean basal and induced CYP1A2 activity measured in four batches of cryopreserved human hepatocytes was comparable and in the same order of magnitude than the mean CYP1A2 activity measured in different independent studies including freshly prepared human hepatocytes (Curi-Pedrosa et al., 1994; Donato et al., 1995; LeCluyse et al., 2000; Meunier et al., 2000; Runge et al., 2000; Madan et al., 2003; LeCluyse, 2001). No significant induction of CYP1A2 activity, protein and mRNA expression was observed in cells other than the ones incubated in the presence of omeprazole. This is in agreement with other studies (Curi-Pedrosa, 1994; Donato et al., 1995; Meunier et al., 2000), and demonstrates that the response of CYP1A2 in the examined cryopreserved human hepatocytes to prototypical inducers is specific and identical to the response in fresh human hepatocytes.

Several recent studies have indicated that CYP2B6 is present in most batches of freshly isolated human hepatocytes and inducible by compounds like rifampicin and phenobarbital (Curi-Pedrosa, 1994; Donato et al., 1995; Gervot et al., 1999; Meunier et al., 2000; Madan et al., 2003). Basal CYP2B6 activity in cryopreserved human hepatocytes was found to be comparable to the activity observed in freshly isolated cells (Madan et al., 2003). In addition, CYP2B6 activity and mRNA expression increased when cells were incubated in the presence of the prototypical CYP2B6-inducers rifampicin and phenobarbital, although the difference in CYP2B6 activity in phenobarbital-treated cells was not statistically significant. In addition, administration of dexamethasone to the culture medium of the cells also weakly induced CYP2B6. While several studies did not observe increased CYP2B6 activity or mRNA expression upon incubation of human hepatocytes or liver slices with dexamethasone (Pascussi et al., 2000; Edwards et al., 2003; Wang et al., 2003), occasionally others have (Donato et al., 1995; Chang et al., 1997; Faucette et al., 2004). In general, the results of this study demonstrate that the magnitude and specificity of the CYP2B6-induction response in the cryopreserved human hepatocytes is comparable to the one in fresh hepatocytes.

Several studies have reported the induction of CYP2C9 by rifampicin, phenobarbital and dexamethasone in primary cultures of human hepatocytes (Morel et al., 1990; Gerbal-Chaloin et al., 2001; Rae et al., 2001; Raucy et al., 2002; Madan et al., 2003). Measurement of CYP2C9 activity or mRNA and protein expression in the investigated batches of cryopreserved cells
was in agreement with these studies. It should be noted however that the basal activity was approximately two-orders of magnitude lower than observed in freshly isolated hepatocytes (Cohen et al, 2000; LeCluyse et al, 2000; LeCluyse, 2001).

Induction of CYP2E1 by ethanol both in vivo and in vitro in human hepatocytes is well established (Kostrubsky et al, 1995; Carpenter et al, 1996; Niemela et al, 2000; Madan et al, 2003). Although the mean basal CYP2E1 activity of the cells included in our study was about 2- to 10-fold lower than the activity observed in freshly prepared human hepatocytes (Madan et al, 2003), in both systems an approximately 2-fold induction of CYP2E1 activity was observed. Incubation of the cells with omeprazole also resulted in an increased chlorzoxazone 6-hydroxylation rate. This is in accordance with a study from Ono et al (1995) that has demonstrated that at a chlorzoxazone concentration of 10 µM, CYP1A2 catalyzes the reaction as efficiently as CYP2E1. At 500 µM chlorzoxazone, CYP1A2 catalyzes 6-hydroxy-chlorzoxazone 10-fold less efficient as CYP2E1. However, our results demonstrated that the increased hydroxylation of chlorzoxazone may also be the result of induction of CYP2E1 by omeprazole. CYP2E1 protein was also increased under the same incubation conditions, but no change in the CYP2E1 mRNA expression was observed under the different experimental conditions. The molecular mechanism by which CYP2E1 is regulated in humans has not been fully elucidated yet, but mechanisms include both increased translational efficiency and stabilization of the protein from degradation, which appears to occur primarily through ubiquitinylation and proteasomal degradation (Koop and Tierney, 1990; Fuhr, 2000; Novak and Woodcroft, 2000). Our findings support these observations.

CYP3A4 is inducible by many compounds including rifampicin, dexamethasone, and phenobarbital (Donato et al, 1995; Donato et al, 1998; Guillén et al, 1998; Silva et al, 1998; Jang and Maurel, 2000; Meunier et al, 2000; Madan et al, 2003; Roymans et al, 2004). It has also been reported that omeprazole is able to induce CYP3A4 in some but not all primary hepatocyte preparations (Curi-Pedrosa et al, 1994). In general, our data are in agreement with these reports. Although the mean basal activity of CYP3A4 observed in the cryopreserved cells is approximately one to two orders of magnitude lower than the mean activity measured in former studies on human hepatocytes (LeCluyse et al, 2000; LeCluyse, 2001; Madan et al, 2003), it is comparable to the activity determined in other studies using
primary human hepatocytes (Donato et al, 1998; Guillén et al, 1998). In addition, the induction response to compounds like rifampicin, phenobarbital or omeprazole was comparable to the response observed in these previous studies (Curi-Pedrosa et al, 1994; Donato et al, 1998; Guillén et al, 1998; Madan et al, 2003). However, no induction of CYP3A4 was observed when the cryopreserved cells were treated with 10 µM dexamethasone. Besides the observation that in different batches of cryopreserved hepatocytes the CYP3A4 induction response to dexamethasone may be different (Roymans et al, 2004), the observations may also be explained by a study from El-Sankary et al (2002) which demonstrated an alternative, PXR-independent pathway for the induction of CYP3A4 by glucocorticoids. A point mutation in a HNF-3/c/EBPα consensus binding sequence in the promotor of the CYP3A4 gene, disrupted the ability of glucocorticoids to induce CYP3A4, while it did not affect the response of CYP3A4 to compounds like rifampicin.

The mRNA levels of CYP enzymes can be quantified accurately by real-time QRT-PCR [Worboys and Carlile (2000) and references therein]. Therefore we investigated whether the CYP induction potential of a NCE can be predicted by measurement of the CYP mRNA expression in human cryopreserved hepatocytes. It was observed that in cases were CYP induction is regulated mainly at the transcriptional level, determination of the different CYP mRNA levels correlated in at least 82 % with the measurement of CYP activity. Moreover, in case of CYP2E1, induced mainly by xenobiotics via protein stabilization (Fuhr, 2000; Novak and Woodcroft, 2000), no induction of its mRNA expression was observed in cases where CYP2E1 activity was significantly increased. Together these results demonstrate that in cases where CYP induction is mainly regulated by a transcriptional mechanism, determination of the mRNA expression levels has a predictive value.

From the results it is clear that the use of cryopreserved human hepatocytes has many advantages but unfortunately also limitations. Use of the cells allows a better planning of induction experiments and provides researchers with standardized hepatocyte populations. As in cultures of fresh human hepatocytes or in vivo, a high variability in basal CYP activity and induction potential is observed. This is not disadvantageous since the population of the hepatocytes can be carefully selected in function of the experimental aim. However, isolation of human hepatocytes causes a decrease in CYP enzyme activity. Moreover, some basal
CYP activities were lower in the cryopreserved cells compared to freshly isolated hepatocytes. While this lowered basal CYP activity may increase the sensitivity of the model system, it may also complicate interpretation of the in vitro obtained CYP induction data in relation to the in vivo situation [discussed in detail by Madan et al (2003)]. Nevertheless, cryopreserved human hepatocytes have shown to be a reliable model system to evaluate the potential of a NCE to induce CYP1A2, CYP2B6, CYP2C9, CYP2E1 or CYP3A4, provided the data are interpreted with the advantages and limitations of the system in mind. Together, these CYPs are involved in the metabolism of more than 75 % of all marketed drugs.
References


Footnotes

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Figure legends

Fig 1: Determination of albumin secretion and cell viability.
Panel A: The rate of albumin secretion was measured in an untreated pool of human cryopreserved hepatocytes originating from 4 different donors (NLR, BDF, 059, 082) as a function of the culturing time. The albumin secretion rate is expressed as pg/cell.hour and is presented as the mean ± SEM calculated from three separate wells. Panel B: The ATP content was determined in an untreated pool of human cryopreserved hepatocytes originating from 4 different donors (NLR, BDF, 059, 082) as a function of time. The ATP content is expressed as fmol/cell and is presented as the mean ± SEM calculated from three separate wells. Panel C: Relative cell viability was determined in cultures of the different lots of cryopreserved human hepatocytes after a 48 h treatment with different prototypical inducers by measurement of the intracellular ATP concentration. Cells were incubated with 0.1 % DMSO (con), 25 µM omeprazole (ome), 25 µM rifampicin (rif), 10 µM dexamethasone (dex), 100 µM phenobarbital (phe), 100 µM clofibric acid (clo), or 40 mM ethanol (eth), the ATP content measured and calculated relatively to the ATP content in control cells (100 %). Bars represent the mean + SEM and are obtained from three separate wells. Panel D: Calculation of the mean relative ATP concentration from the four different lots of cryopreserved human hepatocytes under the different incubation conditions. Results were presented as mean + SEM and calculated from three separate wells.

Fig 2: Morphology of the cryopreserved human hepatocytes.
Light microscopic images (400 X) of cryopreserved human hepatocyte cultures obtained from lots NLR, BDF, 059, and 082 at 96 h after plating. Bar = 50µm.

Fig 3: Measurement of the CYP1A2 activity, protein and mRNA expression in cryopreserved human hepatocytes.
Panel A: CYP1A2 activity was determined by measuring the 7-ethoxyresorufin O-deethylase activity in 4 different lots of cryopreserved human hepatocytes incubated with 0.1 % DMSO (con), 25 µM omeprazole (ome), 25 µM rifampicin (rif), 10 µM dexamethasone (dex), 100 µM phenobarbital (phe), 100 µM clofibric acid (clo), or 40 mM ethanol (eth) for 48 h. For each lot, results are presented as the mean + SEM from three separate wells per treatment. Panel B:
Overall CYP1A2 activity is calculated from the activity obtained in each of the four different lots of cryopreserved human hepatocytes. Activity is expressed as pmol/min.mg protein and is represented as the mean + SEM (n = 4 lots). Panel C: Measurement of the relative CYP1A2 mRNA expression in 4 different lots of cryopreserved human hepatocytes incubated with different prototypical inducers for 24 h. For each lot, results are presented as the mean + SEM from three separate wells per treatment. The mRNA expression in untreated cells was set as 100 %. Panel D: Mean CYP1A2 mRNA expression calculated from 4 different lots of cryopreserved human hepatocytes. The relative mRNA expression (%) is presented as the mean + SEM and calculated relative to the control condition (100 %). The overall mRNA expression is calculated from the expression obtained in each of the four different lots of cryopreserved human hepatocytes. * p < 0.05 compared to the control condition. Panel E: CYP1A2 protein expression in the different lots of cryopreserved human hepatocytes was determined under the different incubation conditions. Blots represent the expression in pooled samples obtained from three separate wells. Panel F: Correlation between CYP1A2 activity and mRNA expression. Y/Y, represents statistically significant induction in both CYP1A2 activity and mRNA expression. N/N, represents no significant induction measured in CYP1A2 activity and mRNA expression. Y/N, represents a significant induction measured in CYP1A2 activity but not in mRNA expression. N/Y, represents no change observed in CYP1A2 activity but a significant induction of CYP1A2 mRNA expression.

Fig 4: Measurement of the CYP2B6 activity, protein and mRNA expression in cryopreserved human hepatocytes.

Panel A: CYP2B6 activity was determined by measuring the 7-ethoxyresorufin O-deethylase activity in 4 different lots of cryopreserved human hepatocytes incubated with 0.1 % DMSO (con), 25 µM omeprazole (ome), 25 µM rifampicin (rif), 10 µM dexamethasone (dex), 100 µM phenobarbital (phe), 100 µM clofibric acid (clo), or 40 mM ethanol (eth) for 48 h. For each lot, results are presented as the mean + SEM from three separate wells per treatment. Panel B: Overall CYP2B6 activity is calculated from the activity obtained in each of the four different lots of cryopreserved human hepatocytes. Activity is expressed as pmol/min.mg protein and is represented as the mean + SEM (n = 4 lots). Panel C: Measurement of the relative CYP2B6 mRNA expression in 4 different lots of cryopreserved human hepatocytes incubated with
different prototypical inducers for 24 h. For each lot, results are presented as the mean + SEM from three separate wells per treatment. The mRNA expression in untreated cells was set as 100 %. Panel D: Mean CYP2B6 mRNA expression calculated from 4 different lots of cryopreserved human hepatocytes. The relative mRNA expression (%) is presented as the mean + SEM and calculated relative to the control condition (100 %). The overall mRNA expression is calculated from the expression obtained in each of the four different lots of cryopreserved human hepatocytes. * p < 0.05 compared to the control condition. Panel E: CYP2B6 protein expression in the different lots of cryopreserved human hepatocytes was determined under the different incubation conditions. Blots represent the expression in pooled samples obtained from three separate wells. Panel F: Correlation between CYP2B6 activity and mRNA expression. Y/Y, represents statistically significant induction in both CYP2B6 activity and mRNA expression. N/N, represents no significant induction measured in CYP2B6 activity and mRNA expression. Y/N, represents a significant induction measured in CYP2B6 activity but not in mRNA expression. N/Y, represents no change observed in CYP2B6 activity but a significant induction of CYP2B6 mRNA expression.

Fig 5: Measurement of the CYP2C9 activity, protein and mRNA expression in cryopreserved human hepatocytes.

Panel A: CYP2C9 activity was determined by measuring the 7-ethoxyresorufin O-deethylase activity in 4 different lots of cryopreserved human hepatocytes incubated with 0.1 % DMSO (con), 25 µM omeprazole (ome), 25 µM rifampicin (rif), 10 µM dexamethasone (dex), 100 µM phenobarbital (phe), 100 µM clofibric acid (clo), or 40 mM ethanol (eth) for 48 h. For each lot, results are presented as the mean + SEM from three separate wells per treatment. Panel B: Overall CYP2C9 activity is calculated from the activity obtained in each of the four different lots of cryopreserved human hepatocytes. Activity is expressed as pmol/min.mg protein and is represented as the mean + SEM (n = 4 lots). Panel C: Measurement of the relative CYP2C9 mRNA expression in 4 different lots of cryopreserved human hepatocytes incubated with different prototypical inducers for 24 h. For each lot, results are presented as the mean + SEM from three separate wells per treatment. The mRNA expression in untreated cells was set as 100 %. Panel D: Mean CYP2C9 mRNA expression calculated from 4 different lots of cryopreserved human hepatocytes. The relative mRNA expression (%) is presented as the
mean + SEM and calculated relative to the control condition (100 %). The overall mRNA expression is calculated from the expression obtained in each of the four different lots of cryopreserved human hepatocytes. * p < 0.05 compared to the control condition. Panel E: CYP2C9 protein expression in the different lots of cryopreserved human hepatocytes was determined under the different incubation conditions. Blots represent the expression in pooled samples obtained from three separate wells. Panel F: Correlation between CYP2C9 activity and mRNA expression. Y/Y, represents statistically significant induction in both CYP2C9 activity and mRNA expression. N/N, represents no significant induction measured in CYP2C9 activity and mRNA expression. Y/N, represents a significant induction measured in CYP2C9 activity but not in mRNA expression. N/Y, represents no change observed in CYP2C9 activity but a significant induction of CYP2C9 mRNA expression.

**Fig 6: Measurement of the CYP2E1 activity, protein and mRNA expression in cryopreserved human hepatocytes.**

Panel A: CYP2E1 activity was determined by measuring the 7-ethoxyresorufin O-deethylase activity in 4 different lots of cryopreserved human hepatocytes incubated with 0.1 % DMSO (con), 25 µM omeprazole (ome), 25 µM rifampicin (rif), 10 µM dexamethasone (dex), 100 µM phenobarbital (phe), 100 µM clofibric acid (clo), or 40 mM ethanol (eth) for 48 h. For each lot, results are presented as the mean + SEM from three separate wells per treatment. Panel B: Overall CYP2E1 activity is calculated from the activity obtained in each of the four different lots of cryopreserved human hepatocytes. Activity is expressed as pmol/min.mg protein and is represented as the mean + SEM (n = 4 lots). Panel C: Measurement of the relative CYP2E1 mRNA expression in 4 different lots of cryopreserved human hepatocytes incubated with different prototypical inducers for 24 h. For each lot, results are presented as the mean + SEM from three separate wells per treatment. The mRNA expression in untreated cells was set as 100 %. Panel D: Mean CYP2E1 mRNA expression calculated from 4 different lots of cryopreserved human hepatocytes. The relative mRNA expression (%) is presented as the mean + SEM and calculated relative to the control condition (100 %). The overall mRNA expression is calculated from the expression obtained in each of the four different lots of cryopreserved human hepatocytes. * p < 0.05 compared to the control condition. Panel E: CYP2E1 protein expression in the different lots of cryopreserved human hepatocytes was
determined under the different incubation conditions. Blots represent the expression in pooled samples obtained from three separate wells. Panel F: Correlation between CYP2E1 activity and mRNA expression. Y/Y, represents statistically significant induction in both CYP2E1 activity and mRNA expression. N/N, represents no significant induction measured in CYP2E1 activity and mRNA expression. Y/N, represents a significant induction measured in CYP2E1 activity but not in mRNA expression. N/Y, represents no change observed in CYP2E1 activity but a significant induction of CYP2E1 mRNA expression.

**Fig 7: Measurement of the CYP3A4 activity, protein and mRNA expression in cryopreserved human hepatocytes.**

Panel A: CYP3A4 activity was determined by measuring the 7-ethoxyresorufin O-deethylase activity in 4 different lots of cryopreserved human hepatocytes incubated with 0.1 % DMSO (con), 25 µM omeprazole (ome), 25 µM rifampicin (rif), 10 µM dexamethasone (dex), 100 µM phenobarbital (phe), 100 µM clofibric acid (clo), or 40 mM ethanol (eth) for 48 h. For each lot, results are presented as the mean + SEM from three separate wells per treatment. Panel B: Overall CYP3A4 activity is calculated from the activity obtained in each of the four different lots of cryopreserved human hepatocytes. Activity is expressed as pmol/min.mg protein and is represented as the mean + SEM (n = 4 lots). Panel C: Measurement of the relative CYP3A4 mRNA expression in 4 different lots of cryopreserved human hepatocytes incubated with different prototypical inducers for 24 h. For each lot, results are presented as the mean + SEM from three separate wells per treatment. The mRNA expression in untreated cells was set as 100 %. Panel D: Mean CYP3A4 mRNA expression calculated from 4 different lots of cryopreserved human hepatocytes. The relative mRNA expression (%) is presented as the mean + SEM and calculated relative to the control condition (100 %). The overall mRNA expression is calculated from the expression obtained in each of the four different lots of cryopreserved human hepatocytes. * p < 0.05 compared to the control condition. Panel E: CYP3A4 protein expression in the different lots of cryopreserved human hepatocytes was determined under the different incubation conditions. Blots represent the expression in pooled samples obtained from three separate wells. Panel F: Correlation between CYP3A4 activity and mRNA expression. Y/Y, represents statistically significant induction in both CYP3A4 activity and mRNA expression. N/N, represents no significant induction measured in CYP3A4 activity and mRNA expression.
activity and mRNA expression. Y/N, represents a significant induction measured in CYP3A4 activity but not in mRNA expression. N/Y, represents no change observed in CYP3A4 activity but a significant induction of CYP3A4 mRNA expression.
Table 1: Donor demographics.

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<th>Substances</th>
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M, male; F, female; C, Caucasian; H, Hispanic; ICH, intracranial hemorrhage; Y, yes; N, no.
Table 2: TaqMan primers and probes.

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Fig 1
Fig 3
Fig 6

(A) CYP2E1 activity

(B) CYP2E1 mRNA expression

(C) CYP2E1 mRNA expression

(D) CYP2E1 mRNA expression

(E) Western blot analysis of CYP2E1

(F) Distribution of genotypes

* indicates significant difference from control.
Fig 7