EXPRESSiON AND INDUCTION POTENTIAL OF CYTOCHROMES P450 IN HUMAN CRYOPRESERVED HEPATOCYTES

Dirk Roymans, Pieter Annaert, Jos Van Houdt, Adri Weygers, Jan Noukens, Carlo Sensenhauser, José Silva, Cis Van Looveren, Jan Hendrickx, Geert Mannens, and Willem Meuldermans


ABSTRACT:

Fresh human hepatocytes are still considered as the “gold standard” to screen in vitro for cytochrome P450 (P450) induction. However, sparse availability of good quality human liver tissue for research purposes and 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 P450 induction. Cryopreserved cells from four different donors were plated and cultured for 48 h, followed by incubation in the presence of typical P450 inducers. During the experiments, quality of the cultured cells was monitored both physiologically and morphologically. Concomitantly, the activity of CYP1A2, 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 P450s 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 (P450s) are a superfamily of heme-thiolate-containing mixed function monoxygenases 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 P450s are induced severalfold 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. Therefore, drugs and new chemical entities (NCEs) are often screened for their ability to induce P450s or other drug-metabolizing enzymes with the aim of predicting or explaining pharmacokinetic tolerance or drug-drug interactions.

In the past, many P450 induction studies, performed in various animal species, have proven to be beneficial. However, species differences in the induction of P450s 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 P450 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., 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 P450s (Kostrubsky et al., 1999; LeCluyse et al., 2000; Silva and Nicoll-Griffith, 2002). When in vitro P450 induction experiments are conducted at therapeutically relevant concentrations of the NCEs, in most cases, the in vitro data correlate well with clinical observations (Dilger et al., 1999; LeCluyse 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 is 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 immediate use.

In 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,b; Alexandre et al., 2003).
hepatocyte plating medium was removed and replaced with incubation medium from Ultrafine Chemicals (Manchester, UK). Dexamethasone was obtained from Sigma-Aldrich (St. Louis, MO).

7-ethoxyresorufin, resorufin, 4-hydroxymethyl tolbutamide, tolbutamide, and testosterone were obtained from Sigma-Aldrich (St. Louis, MO).

Hepatocytes from four separate human donors were obtained from cryopreserved hepatocyte bank maintained at In Vitro Technologies (Baltimore, MD). 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 obtained from Invitrogen (Merelbeke, Belgium). Trypan blue, DMSO, omeprazole, rifampicin, phenobarbital, 7-ethoxyresorufin, resorufin, 4-hydroxymethyl tolbutamide, tolbutamide, and testosterone were obtained from Sigma-Aldrich (St. Louis, MO). Clofibric acid and chlorozoxazone were obtained from Ultrafine Chemicals (Manchester, UK). Dexamethasone was obtained from Cytokine (New Brunswick, NJ). Clofibric acid and chlorozoxazone were obtained from Serva (100x), Zandhoven, Belgium) and Ortho-McNeil (Raritan, NJ) respectively. 6β-Hydroxytestosterone was obtained from Steraloids (Newport, RI). β-Glucuronidase and β-glucuronidase/arylsulfatase were obtained from Roche Diagnostics (Vilvoorde, Belgium).

Materials and Methods

Materials. Hepatocytes from four separate human donors were obtained from the cryopreserved hepatocyte bank maintained at In Vitro Technologies (Baltimore, MD). 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 obtained from Invitrogen (Merelbeke, Belgium). Trypan blue, DMSO, omeprazole, rifampicin, phenobarbital, 7-ethoxyresorufin, resorufin, 4-hydroxymethyl tolbutamide, tolbutamide, and testosterone were obtained from Sigma-Aldrich (St. Louis, MO). Clofibric acid and chlorozoxazone were obtained from Ultrafine Chemicals (Manchester, UK). Dexamethasone was obtained from Cytokine (New Brunswick, NJ). Clofibric acid and chlorozoxazone were obtained from Serva (100x), Zandhoven, Belgium) and Ortho-McNeil (Raritan, NJ) respectively. 6β-Hydroxytestosterone was obtained from Steraloids (Newport, RI). β-Glucuronidase and β-glucuronidase/arylsulfatase were obtained from Roche Diagnostics (Vilvoorde, Belgium).

Polyclonal antibodies against CYP1A2 (catalog no. 458124), CYP2B6 (catalog no. 458226), CYP2C9 (catalog no. 458209), CYP2E1 (catalog no. 458219), and CYP3A4 (catalog no. 458234) were obtained from BD Gentest (Woburn, MA). Horseradish peroxidase-conjugated donkey-anti-goat (catalog no. 705-035-147) and donkey-anti-rabbit (catalog no. 712-035-153) were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). The β-actin rabbit-anti-human antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

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 confinement 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, three separate wells per treatment condition were used.

Human Albumin Enzyme-Linked Immunosorbent Assay. Human albumin secretion was measured with an ELISA quantitation kit from Bethyl Laboratories (Montgomery, TX) according to the manufacturer’s instructions. Briefly, cells were cultured as described above and human albumin was 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 three times 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 postcoat solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0). Wells were washed again three times with wash solution and incubated for another 60 min with 100 μl of standard, sample, or blank. Subsequently, wells were washed five times with wash solution and incubated for 60 min at RT with a goat-anti-human albumin-HP blot conjugate antibody (1:100,000) diluted in conjugate diluent (100 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0). Wells were washed again five times with wash solution, and colorimetric reactions were initiated in the wells using 3.3′,5.5′-tetramethylbenzidine as a substrate. Finally, reactions were stopped after 5 min by administration of 2 M H2SO4 and absorbance was measured at 450 nm using a Safire spectrophotometer (Tecan, Grödig, Austria). To allow the albumin secretion to be expressed as pg/cell, 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 (Madison, Wisconsin, 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 was stabilized for 10 min at RT. Luminescence was recorded on an Victor2 1420 Multilabel counter (PerkinElmer Life and Analytical Sciences, Boston, MA). 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 Hanks’ 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 and incubated at 37°C for 2 h in the presence of 4 × 10^{-5} U/ml β-glucuronidase, and the reaction was stopped by addition of methanol (1:1). Samples were centrifuged at 1700g for 30 min at RT, and liberated resorufin in the supernatant was fluorimetrically analyzed (excitation, 550 nm; emission, 585 nm) in a Safire spectrophotometer. CYP2B6 activity was determined by N-demethylation of S-mephénytoin. Briefly, cells were incubated for 2 h at 37°C in the presence of 200 μM S-mephénytoin in HBSS, and the reaction was stopped by probe sonication with a Vibracell sonicator (Sonics & Materials Inc., Newton, CT) at 40 W for 10 s. 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 propranolol as internal standard, samples were centrifuged and the supernatant was 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 was injected onto the column for liquid chromatography–tandem mass spectrometry 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 × 50 mm) column (Phenomenex, Torrance, CA). 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 and 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 (Applied Biosystems/MDS Sciex, Foster City, CA). The mass spectrometer was equipped with an electrospray ionization source and operated in the negative ion mode or 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 analyzed with liquid chromatography–tandem mass spectrometry by determining the 4-methylhydroxylation and the metabolite of the substrate. For nirvanol, the precursor-to-product ion reaction monitored was m/z 205→134.

RNA was prepared using the RNeasy method (Qiagen, Valencia, CA) according to the manufacturer’s instructions and included an on-column DNase I digestion to minimize genomic DNA contamination. RNA quantification was determined fluorimetrically using RiboGreen RNA quantitation reagent (Molecular Probes, Eugene, OR). 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 of total RNA from each sample was added to a reaction mixture containing 100 ng of random hexamer primers, 0.5 mM deoxynucleoside-5'-triphosphates, RT buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 5 mM MgCl2, 10 mM dithiothreitol, 40 U of ribonuclease inhibitor, and incubated at 25°C for 2 min. Subsequently, 50 μl of 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 μl of Escherichia 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 P450s (Table 2) were designed with Primer Express software (Applied Biosystems, Foster City, CA) 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 10000Rxn 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). Assays were performed using an Applied Biosystems ABI Prism 7900HT sequence detection system. An initial heating step to 95°C for 10 min was followed by 50 cycles of 95°C for 15 s, 60°C for 1 min. 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 (three wells/treatment).

Protein Isolation. After 48 h of incubation with compounds, the cell culture medium was completely aspirated and the cells were washed two times with ice-cold phosphate-buffered saline. The cells were placed on ice, 100 μl of ice-cold homogenization 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 s. The cell homogenate was snap-frozen in liquid nitrogen. The samples were stored at −70°C until use.

SDS-PAGE. The protein concentration of the samples was determined with the Coomassie Plus assay from Pierce Chemical (Rockford, IL)., 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 Abs5 values against a BSA standard curve, ranging from 0 to 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% reducing agent (Invitrogen). The samples were boiled for 5 min and loaded onto a 4 to 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 an

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Forward Primer (5‘→3’)</th>
<th>Reverse Primer (5‘→3’)</th>
<th>Probe (5‘FAM→3’TAMRA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>NM_000761</td>
<td>CTCCTCCCTCTTGCCCTCCA</td>
<td>TCTAGAGCGACTTTGTTG</td>
<td>CATCCCATCACACACAAACAGAG</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>NM_000767</td>
<td>GTGGTCATGTTTGGTGGAATAGA</td>
<td>TTTCTCCCGCGCCGAGAAAG</td>
<td>CCAACTCGGCGGCTTTGAGC</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>NM_000773</td>
<td>CATGAGTTAACCCTGTTGACA</td>
<td>GGTCTGCTGCTGTCCCTCCA</td>
<td>TCTGCTGGCTACACCTGCC</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>AF182273</td>
<td>CCTGGCTCTTCTCAGGACTA</td>
<td>TAAGCTCGGAGGAAGTTAATGG</td>
<td>TCCACACCACCGGAGTTACC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>NM_001101</td>
<td>TGAAGCGGCTACACGTTT</td>
<td>TCTCGTATTGCTCACGACATT</td>
<td>ACCACCACCGGCGGAGGG</td>
</tr>
</tbody>
</table>
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 polyvinylidene difluoride membrane in NuPAGE transfer buffer (25 mM Bis-Tris, 25 mM Bicine, 1 mM EDTA, 1 mM chlorobutanol, 10% methanol, and 0.001% antioxidant) (Invitrogen). Membranes were blocked for 1 h at RT in 5% nonfat milk in TBST (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20). Blots were incubated with a goat-anti-rat CYP1A1/1A2 (1:10,000), a rabbit-anti-human CYP2B6 (1:5000), a rabbit-anti-human CYP2C9 (1:5000), a goat-anti-rat CYP2E1 (1:10,000), a rabbit-anti-human CYP3A4 (1:5000), or a rabbit-anti-human β-actin (1:2000) antibody in 0.5% nonfat milk in TBST for 1 h at RT. After four final washes of the membranes for 5 min in TBST, 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 F. Hoffman-La Roche (Basel, Switzerland). β-Actin expression was used to normalize relative P450 protein expression. Protein expression was determined for each separate well (three wells/treatment).

**Statistical Analysis.** Differences were evaluated using 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 by measurement of both 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 × 10⁴ 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 four 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).

**P450 Activity and Expression Profiling.** Cells originating from four 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 clofibric 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 up-regulated in cells from the different lots (Fig. 3E). Cells from lots NLR, BDF, 059, and 082 up-regulated 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 whether measurement of the P450 mRNA expression can be predictive for the assessment of P450 activity induction, the correlation between P450 mRNA expression and activity was scored. In all of the experimental conditions where CYP1A2 activity was significantly increased, a significant increase in CYP1A2 mRNA expression also 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 four 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. 4, A and B). Incubation of the cells with rifampicin significantly induced CYP2B6 activity 3.5-, 3.0-, 2.0-, and 2.7-fold in cells from lots 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. 4, C and D). Upon rifampicin treatment, the mRNA expression increased 3.4-, 4.2-, 4.5-, and 2.1-fold in cells from lots 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
FIG. 3. Measurement of CYP1A2 activity, protein, and mRNA expression in cryopreserved human hepatocytes. A, CYP1A2 activity was determined by measuring 7-ethoxyresorufin O-deethylase activity in four 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 mean ± S.E.M. from three separate wells per treatment. B, overall CYP1A2 activity was 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 presented as mean ± S.E.M. (n = 4 lots). C, measurement of relative CYP1A2 mRNA expression in four different lots of cryopreserved human hepatocytes incubated with different prototypical inducers for 24 h. For each lot, results are presented as mean ± S.E.M. from three separate wells per treatment. mRNA expression in untreated cells was set as 100%. D, mean CYP1A2 mRNA expression calculated from four different lots of cryopreserved human hepatocytes. Relative mRNA expression (%) is presented as mean ± S.E.M. and calculated relative to the control condition (100%). Overall mRNA expression was calculated from the expression obtained in each of the four different lots of cryopreserved human hepatocytes. *, p < 0.05 compared with the control condition. 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. F, correlation between CYP1A2 activity and mRNA expression. Y/Y, statistically significant induction in both CYP1A2 activity and mRNA expression; N/N, no significant induction measured in CYP1A2 activity and mRNA expression; Y/N, significant induction measured in CYP1A2 activity but not in mRNA expression; N/Y, no change observed in CYP1A2 activity but a significant induction of CYP1A2 mRNA expression.
FIG. 4. Measurement of CYP2B6 activity, protein, and mRNA expression in cryopreserved human hepatocytes. A, CYP2B6 activity was determined by measuring the 7-ethoxyresorufin O-deethylase activity in four 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 mean ± S.E.M. from three separate wells per treatment. B, overall CYP2B6 activity was 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 presented as mean ± S.E.M. (n = 4 lots). C, measurement of relative CYP2B6 mRNA expression in four different lots of cryopreserved human hepatocytes incubated with different prototypical inducers for 24 h. For each lot, results are presented as mean ± S.E.M. from three separate wells per treatment. mRNA expression in untreated cells was set as 100%. D, mean CYP2B6 mRNA expression calculated from four different lots of cryopreserved human hepatocytes. Relative mRNA expression (%) is presented as mean ± S.E.M. and calculated relative to the control condition (100%). Overall mRNA expression is calculated from the expression obtained in each of the four different lots of cryopreserved human hepatocytes. *p < 0.05 compared with the control condition. E, CYP2B6 protein expression in the different lots of cryopreserved human hepatocytes, determined under the different incubation conditions. Blots represent the expression in pooled samples obtained from three separate wells. F, correlation between CYP2B6 activity and mRNA expression. Y/Y, statistically significant induction in both CYP2B6 activity and mRNA expression; N/N, no significant induction measured in CYP2B6 activity and mRNA expression; Y/N, significant induction measured in CYP2B6 activity but not in mRNA expression; N/Y, no change observed in CYP2B6 activity but significant induction of CYP2B6 mRNA expression.
phenobarbital-stimulated cells, confirming the observations in CYP2B6 activity assays and mRNA expression experiments (Fig. 4E). In 20 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 with the control cells. Activity was up-regulated 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-hydroxyethyl 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 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 chlorozoxazine varied approximately 4.5-fold among the four 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 lots 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 with 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 post-transcriptional 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 up-regulation. 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 up-regulation. In general, analysis of CYP3A4 protein expression confirmed the CYP3A4 activity measurements (Fig. 7E). Hepatocytes from all four lots demonstrated up-regulated 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 with 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 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 P450 induction experiments (Li et al., 1999). Besides a significantly lower P450 enzyme activity after induction in these cells compared with freshly isolated human hepatocytes, their conclusion was also based on the limited availability of P450 induction data in cryopreserved human hepatocytes. Indeed, the number 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 behavior 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 P450s was selected to be included in the study based on their importance in the metabolism of drugs. At present, three categories can be classified: P450s of major importance, those of emerging importance, and those of low importance (Bjornsson et al., 2003).
FIG. 5. Measurement of CYP2C9 activity, protein, and mRNA expression in cryopreserved human hepatocytes. A, CYP2C9 activity was determined by measuring the 7-ethoxyresorufin O-deethylase activity in four 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 mean ± S.E.M. from three separate wells per treatment. B, overall CYP2C9 activity was 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 presented as mean ± S.E.M. (n = 4 lots). C, measurement of relative CYP2C9 mRNA expression in four different lots of cryopreserved human hepatocytes incubated with different prototypical inducers for 24 h. For each lot, results are presented as mean ± S.E.M. from three separate wells per treatment. mRNA expression in untreated cells was set as 100%. D, mean CYP2C9 mRNA expression calculated from four different lots of cryopreserved human hepatocytes. Relative mRNA expression (%) is presented as mean ± S.E.M. and calculated relative to the control condition (100%). Overall mRNA expression is calculated from the expression obtained in each of the four different lots of cryopreserved human hepatocytes. * p < 0.05 compared with the control condition. 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. F, correlation between CYP2C9 activity and mRNA expression. Y/Y, statistically significant induction in both CYP2C9 activity and mRNA expression; N/N, no significant induction measured in CYP2C9 activity and mRNA expression; Y/N, significant induction measured in CYP2C9 activity but not in mRNA expression; N/Y, no change observed in CYP2C9 activity but significant induction of CYP2C9 mRNA expression.
FIG. 6. Measurement of CYP2E1 activity, protein, and mRNA expression in cryopreserved human hepatocytes. A, CYP2E1 activity was determined by measuring 7-ethoxyresorufin O-deethylase activity in four different lots of cryopreserved human hepatocytes incubated with 0.1% DMSO (con), 25 μM omeprazole (ome), 25 μM rifampicin (rif), 10 μM dexamethasone (dex), 10 μM phenobarbital (phe), 100 μM clofibric acid (clo), or 40 mM ethanol (eth) for 48 h. For each lot, results are presented as mean ± S.E.M. from three separate wells per treatment. B, overall CYP2E1 activity was 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 presented as mean ± S.E.M. (n = 4 lots). C, measurement of relative CYP2E1 mRNA expression in four different lots of cryopreserved human hepatocytes incubated with different prototypical inducers for 24 h. For each lot, results are presented as mean ± S.E.M. from three separate wells per treatment. mRNA expression in untreated cells was set as 100%. D, mean CYP2E1 mRNA expression calculated from four different lots of cryopreserved human hepatocytes. Relative mRNA expression (%) is presented as mean ± S.E.M. and calculated relative to the control condition (100%). Overall mRNA expression was calculated from the expression obtained in each of the four different lots of cryopreserved human hepatocytes. *, p < 0.05 compared with the control condition. 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. F, correlation between CYP2E1 activity and mRNA expression. Y/Y, statistically significant induction in both CYP2E1 activity and mRNA expression; N/N, no significant induction measured in CYP2E1 activity and mRNA expression; Y/N, significant induction measured in CYP2E1 activity but not in mRNA expression; N/Y, no change observed in CYP2E1 activity but significant induction of CYP2E1 mRNA expression.
FIG. 7. Measurement of CYP3A4 activity, protein, and mRNA expression in cryopreserved human hepatocytes. A, CYP3A4 activity was determined by measuring 7-ethoxyresoru- 
fin O-deethylase activity in four 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 mean ± S.E.M. from three 
separate wells per treatment. B, overall CYP3A4 activity was 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 presented as mean ± S.E.M. (n = 4 lots). C, measurement of relative CYP3A4 mRNA expression in four different lots of cryopreserved 
human hepatocytes incubated with different prototypical inducers for 24 h. For each lot, results are presented as mean ± S.E.M. from three separate wells per treatment. mRNA 
expression in untreated cells was set as 100%. D, mean CYP3A4 mRNA expression calculated from four different lots of cryopreserved human hepatocytes. Relative mRNA 
expression (%) is presented as mean ± S.E.M. and calculated relative to the control condition (100%). Overall mRNA expression was calculated from the expression obtained in 
each of the four different lots of cryopreserved human hepatocytes. * p < 0.05 compared with the control condition. 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. 
F, correlation between CYP3A4 activity and mRNA expression. Y/Y, statistically significant induction in both CYP3A4 activity and mRNA expression; N/N, no significant 
induction measured in CYP3A4 activity and mRNA expression; Y/N, significant induction measured in CYP3A4 activity but not in mRNA expression; N/Y, no change observed 
in CYP3A4 activity but significant induction of CYP3A4 mRNA expression.
CYP1A2, 2C9, and 3A4 were selected for their established role in drug metabolism. The panel of P450s 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 P450 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 to be 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 to and in the same order of magnitude of 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; LeCluyse, 2001; Madan et al., 2003). No significant induction of CYP1A2 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 et al., 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 et al., 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. Whereas 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 2 orders of magnitude lower than that 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 efficiently than CYP2E1 does. 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 ubiquitinylolation and proteasomal degradation (Koop and Tierney, 1990; Fuhr, 2000; Novak and Woodcroft, 2000). Our findings support these observations.

CYP3A44 is inducible by many compounds including rifampicin, dexamethasone, and phenobarbital (Donato et al., 1995, 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 CYP3A44 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 CYP3A44 observed in the cryopreserved cells is approximately 1 to 2 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 CYP3A44 was observed when the cryopreserved cells were treated with 10 μM dexamethasone. Besides the observation that in different batches of cryopreserved hepatocytes the CYP3A44 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, pregnane X receptor-independent pathway for the induction of CYP3A44 by glucocorticoids. A point mutation in a HNF-3/c/EBPα consensus binding sequence in the promoter of the CYP3A44 gene disrupted the ability of glucocorticoids to induce CYP3A44, whereas it did not affect the response of CYP3A44 to compounds like rifampicin.

The mRNA levels of P450 enzymes can be quantified accurately by real-time QRT-PCR (Worboys and Carlyle, 2000, and references therein). Therefore, we investigated whether the P450 induction potential of a NCE can be predicted by measurement of the P450 mRNA expression in human cryopreserved hepatocytes. It was observed that in cases where P450 induction is regulated mainly at the transcriptional level, determination of the different P450 mRNA levels correlated, in at least 82%, with the measurement of P450 activity. Moreover, in the 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 P450 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 P450 activity and induction potential is observed. This is not disadvantageous since the population of the hepatocytes can be carefully selected by function of the experimental aim. However, isolation of human hepatocytes causes a decrease in P450 enzyme activity. Moreover, some basal P450 activities were lower in the cryopreserved cells compared with freshly isolated hepatocytes. Although this lowered basal P450 activity may increase the sensitivity of the model system, it may also complicate interpretation of the in vitro obtained P450 induction data in relation to the in vivo situation (discussed in detail by Madan et al., 2003).

Nevertheless, cryopreserved human hepatocytes have been shown to be a reliable model system to evaluate the potential of a NCE to induce CYP1A2, CYP2B6, CYP2C9, CYP2E1, or CYP3A4, or CYP3A4, provided the data are interpreted with the advantages and limitations of the system in mind. Together, these P450s are involved in the metabolism of more than 75% of all marketed drugs.

References


Address correspondence to: Dirk Roymans, Lead Discovery Operations, Tibotec bvba, General De Wittelaan 11B3, B-2800 Mechelen, Belgium. E-mail: droymans@tibbe.jnj.com