Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 427–437
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Determination of cytochrome P450 1A2 and cytochrome P450 3A4 induction in cryopreserved human hepatocytes

Dirk Roymans[a,*], Cis Van Looveren[a], Angelique Leone[b], J. Brandon Parker[b], Michael McMillian[b], Mark D. Johnson[b], Aruna Koganti[c], Ron Gilissen[d], Paul Silber[c], Geert Mannens[a], Willem Meuldermans[a]

[a]*Preclinical Pharmacokinetics, Johnson & Johnson Pharmaceutical Research & Development, Turnhoutseweg 30, B-2340 Beerse, Belgium*
[b]*Drug Discovery Support, Johnson & Johnson Pharmaceutical Research & Development, 1003 US Route 202, Raritan, NJ, USA*
[c]*In Vitro Technologies, Inc., 1450 South Rolling Road, Baltimore, MD 21227, USA*
[d]*ADME/TOX, Johnson & Johnson Pharmaceutical Research & Development, Turnhoutseweg 30, B-2340 Beerse, Belgium*

Received 4 July 2003; accepted 17 September 2003

Abstract

Freshly prepared human hepatocytes are considered as the 'gold standard' for *in vitro* testing of drug candidates. However, several disadvantages are associated with the use of this model system. The availability of hepatocytes is often low and consequently the planning of the experiments rendered difficult. In addition, the quality of the available cells is in some cases poor. As an alternative, cryopreserved human hepatocytes were validated as a model to study cytochrome P450 1A2 (CYP1A2) and cytochrome P450 3A4 (CYP3A4) induction. In a single blinded experiment, hepatocytes from three separate lots were incubated with three concentrations of different compounds, and compared to non-treated cells and cells incubated with omeprazole or rifampicin. CYP1A2 and CYP3A4 induction was determined by measuring 7-ethoxyresorufin-*O*-deethylation activity and 6β-hydroxytestosterone formation, respectively. CYP1A2 and CYP3A4 mRNA and protein expression were analyzed by TaqMan QRT–PCR and immunodetection. Cells responded well to the prototypical inducers with a mean 38.8- and 6.2-fold induction of CYP1A2 and CYP3A4 activity, respectively. Similar as with fresh human hepatocytes, high batch-to-batch variation of CYP1A2 and CYP3A4 induction was observed. Except for 1 and 10 μM rosiglitazone, the glitazones did not significantly affect CYP1A2. A similar result was observed for CYP3A4 activity although CYP3A4 mRNA and protein expression were dose-dependently upregulated. In conclusion, cryopreserved human hepatocytes may be a good alternative to fresh hepatocytes to study CYP1A and 3A induction.
© 2003 Elsevier Inc. All rights reserved.

Keywords: Cytochrome P450; CYP1A2; CYP3A4; Induction; Human hepatocytes; Gene and protein expression

1. Introduction

Cytochromes P450 (CYP) form a superfamily of mixed-function monooxygenases involved in the biotransformation of a variety of chemically diverse substances including endogenous compounds and xenobiotics like food compounds, pollutants, and drugs. Some of these CYP-genes are induced several fold by specific drugs, causing variation in enzymatic activity. As a consequence, variability in the pharmacokinetics of drugs may appear, as well as drug–drug interactions, or even therapeutic failure. Although clinical induction may still be debatable [1], profound therapeutic consequences have been observed when it occurs. For instance, woman taking oral contraceptives lose protection and suffer from menstrual bleeding irregularities caused by induction of CYP3A [2]. In addition, when the CYP3A inducers carbamazepine or phenytoin are co-administered with cyclosporine, serious clinical complications, such as organ rejection, are observed due to lowered serum concentrations of the immunosuppressant cyclosporine [3–5]. Likewise, the interaction between warfarin and phenobarbital, causing disturbed anti-coagulation effects in patients, is a notorious example in this respect [6].

* Corresponding author. Tel.: +32-14-60-77-99; fax: +32-14-60-37-68.
E-mail address: droymans@prdbe.jnj.com (D. Roymans).

Abbreviations: CYP, cytochrome P450; DMSO, dimethylsulfoxide; EROD, 7-ethoxyresorufin-*O*-deethylation; NCE, new chemical entity; TaqMan QRT–PCR, TaqMan quantitative reverse-transcriptase polymerase chain reaction; SEM, standard error of the mean; TBS-T, Tris buffered saline-Tween 20.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2003.09.022

Table 1
Donor demographics of the different hepatocyte lots

Lot	Age	Sex	Race	Cause of death	Tobacco	Alcohol	Substances	CMV	HepB/HIV	Medical history
130	2	F	C	Anoxia	N	N	N	+	–	None
QKR	35	M	C	Seizure	Y	Y	Y	–	–	Seizure disorder
XPK	39	M	H	Head trauma	Y	Y	Y	–	–	Seizure

Age is presented in years. F, female; M, male; C, Caucasian; H, Hispanic; N, no; Y, yes.

For the pharmaceutical industry, clinical CYP-induction may also have severe financial consequences. The major part of the expenses to develop a new chemical entity (NCE) into a marketed drug are made in the clinical phase. Consequently, failure of the NCE in late stages of development or withdrawal from the market due to clinical CYP-induction causes a tremendous financial loss. Knowledge of possible CYP-induction potential of drug candidates in drug discovery or the early preclinical phase of development would greatly enhance the ability to develop drugs that are free of CYP-inducing properties, and may thus help in preventing the industry from investing in the wrong compounds.

In the past, many CYP-induction studies, performed in various animal species, have proven to be beneficial. However, in late discovery or early drug development, these studies are difficult to carry out because of the large number of animals needed, and compounds to be tested [7]. Moreover, species differences in the induction of CYPs make the extrapolation from animals to humans 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 [7].

At present, several *in vitro* models are available to study CYP-induction. Among them, fresh human hepatocytes are considered as the 'gold standard' for *in vitro* testing of drug candidates [8]. An extensive number of studies have been reported demonstrating the soundness of primary cultures of human hepatocytes for toxicological, metabolic and pharmacological experiments [9–13]. 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 liver tissue, the supply of these cells is also unpredictable, and limited by legal and ethical issues [14]. Moreover, the large amount of hepatocytes isolated from liver (approximately 5×10^6 cells/g liver) may not be necessarily required for an immediate use. To allow a better planning of the experiments and a more efficient use of the available human hepatocytes, cryopreservation techniques have been developed allowing a high percentage of viable and plateable hepatocytes after thawing [15–17]. However, in 1999, an international expert panel recommended that for *in vitro* CYP-induction studies it is advisable not to use cryopreserved human hepatocytes, because the enzymatic activity of CYPs such as CYP1A2 and CYP3A4 decrease by approximately 50% per day, stabilizing at 10–20% of the original activity [18].

The present study was undertaken to investigate the usefulness of cryopreserved human hepatocytes as an *in vitro* model to study the induction of CYP1A2 and CYP3A4. Both CYPs were selected in this study because they are highly inducible by xenobiotics, and together they are involved in the metabolism of approximately 70% of all marketed drugs.

2. Materials and methods

2.1. Materials

For this study, hepatocytes from three separate human lots were obtained from the cryopreserved hepatocyte bank maintained at In Vitro Technologies. Hepatocytes from In Vitro Technologies lots 130, QKR, and XPK (Table 1) were used and were all obtained from livers that were donated for research and which could not be used for transplantation. Complete hepatocyte plating medium, sandwich medium, incubation medium, and collagen precoated 48-well plates were all obtained from In Vitro Technologies. Trypan blue, methanol, dimethylsulfoxide (DMSO), salicylamide, omeprazole, rifampicin, ethoxyresorufin, resorufin, and Krebs-Henseleit buffer were obtained from Sigma Chemical Company. 6β-Hydroxytestosterone was obtained from Steraloids, Inc.

2.2. Hepatocyte isolation, cryopreservation and thawing

Hepatocytes were isolated using a two-step collagenase digestion procedure [19]. After isolation, viability of the hepatocyte suspensions was measured using Trypan blue exclusion; only hepatocytes with ≥70% viability were used and purified by a Percoll gradient. Subsequently, the hepatocytes were cryopreserved in a medium containing 10% DMSO and 90% fetal bovine serum [20,21] using a controlled rate freezer. Frozen hepatocytes were stored in liquid nitrogen. Thawing was achieved by shaking the vials of cryopreserved hepatocytes in a 40° water bath. The vials were placed immediately on ice as soon as all the ice crystals disappeared, diluted with plating medium at 4° and washed by centrifugation at 50 *g* for 5 min. Viability was measured using Trypan blue exclusion. The viability of the cell suspensions was 83.7, 84.8, and 84.1% for lots 130, QKR and XPK, respectively.

2.3. Establishment of hepatocyte cultures

Isolated hepatocytes were transferred to collagen precoated 48-well plates, each well containing a cell density of 1.75×10^5 viable cells in 0.25 mL of plating medium. Plating medium was removed and replaced with sandwich medium the day after plating. After cultures had been established, the confluence of the hepatocytes was visually assessed using phase contrast microscopy. At the time of dosing, hepatocytes from lot 130 and lot QKR were ~90% confluent, whereas hepatocytes from lot XPK were 70–75% confluent. The cells were then incubated for an additional day (a total of 48 hr) to establish the cultures. All incubations were conducted at $37 \pm 1^\circ$, 95% air/5% CO_2, and saturating humidity. The sample size was $N = 3$ replicates for experimental groups and $N = 6$ replicates for control groups.

2.4. Hepatocyte incubation and dosing

Following the initial 2-day equilibration phase, the sandwich medium was removed and the hepatocytes were dosed with incubation medium including 1, 10, or 50 μM lansoprazole or 1, 10, or 100 μM dexamethasone, pioglitazone, rosiglitazone or troglitazone for 24 ± 1.5 hr. Vehicle controls (hepatocytes dosed with incubation medium without any test article) and positive controls (hepatocytes dosed with incubation medium containing 50 μM omeprazole or 25 μM rifampicin) were also included to demonstrate the responsiveness of the hepatocytes to known CYP1A2 and CYP3A4 inducers. The incubation medium was aspirated and replaced with fresh incubation medium containing the same concentrations of test or control articles, and then incubated for an additional 24 ± 1.5 hr. The total treatment period with each test or control article was 48 ± 3 hr. To determine the effect of test and control compounds on CYP1A2 by analysis of enzymatic activity, after treatment, the incubation medium was replaced with 300 μL of Krebs-Henseleit buffer containing 2 μM ethoxyresorufin and 3 mM salicylamide. The hepatocytes were then incubated for 1 hr. Reactions were terminated with the addition of 300 μL of 2% DMSO in methanol, and the samples were transferred to cryovials and stored at -70° until analysis. To determine the effect of test and control compounds on CYP3A4 by analysis of enzymatic activity, after treatment, the incubation medium was replaced with 300 μL of Krebs-Henseleit buffer containing 125 μM testosterone. The hepatocytes were then incubated for 1 hr. Reactions were terminated with the addition of 300 μL of cold methanol, and the samples were transferred to cryovials and stored at 4° until analysis.

2.5. TaqMan QRT–PCR

After 24 hr of incubation with compounds, total cellular RNA was prepared using the RNeasy method from Qiagen 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) and quality was ascertained on an Agilent 2100 Bioanalyzer. An aliquot of each RNA sample was adjusted to a final concentration of 2 ng/μL and used for subsequent TaqMan quantitative reverse-transcriptase polymerase chain (TaqMan QRT–PCR) assays. All RNA samples were stored at -80° until further use. CYP3A4 and CYP1A2 mRNA levels were determined using standard TaqMan QRT–PCR methods. Sequence specific primers and TaqMan probe for CYP3A4 were synthesized (Applied Biosystems) using nucleotide sequences reported previously [22] and are as follows: CYP3A4 forward 5′-TCAGCCTGGTGCTCCTCTATC-TAT-3′, CYP3A4 reverse 5′-AAGCCCTTATGGTAGGA-CAAAATATTT-3′, CYP3A4 TaqMan probe 5′-FAM-TCCAGGGCCCACACCTCTGCCT-TAMRA-3′. CYP1A2 specific primers and probe were purchased as a pre-developed gene expression assay and used according to manufacturer's instructions (Applied Biosystems). TaqMan one-step RT–PCR assays were performed with 10 ng of each RNA sample in a final reaction volume of 50 μL prepared from TaqMan one-step RT–PCR Master Mix Reagents Kit (Applied Biosystems). CYP3A4 assays contained 0.9 μM each of forward and reverse primer and 0.25 μM TaqMan probe. Assays were performed using an Applied Biosystems' ABI Prism 7900HT sequence detection system. An initial RT step occurred for 30 min at 48° and was subsequently followed by heating to 95° for 10 min followed by 40 cycles of 95° for 15 s, 60° 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.

2.6. Protein isolation

After 48 hr of incubation with compounds, the cell-culture medium was completely aspirated and the cells were washed twice 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 Vibra cell sonicator (Sonics and Materials Inc.) at 40 W for 20 s. The cell homogenate was snap frozen in liquid nitrogen. The samples were stored at -70° until use.

2.7. SDS–PAGE gelelectrophoresis

The protein concentration of the samples was determined with the Coomassie Plus assay from Pierce, 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 Spectramax plus 384 spectrophotometer (Molecular Devices). The protein concentration of the different samples was determined by comparing the A_{595} values against a bovine serum albumine 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× LDS sample loading buffer and 10× reducing agent (Invitrogen). The samples were boiled for 5 min and loaded onto a 4–12% Bis–Tris Gel from Invitrogen and electrophorized in MOPS SDS running buffer [50 mM MOPS, 50 mM Tris, 3.5 mM SDS and 1 mM EDTA] in a Xcell SureLockTM Electrophoresis Cell from Invitrogen according to the manufacturer's instructions.

2.8. Western blot analysis

After electrophoresis, proteins were electroblotted overnight at 36 V onto a PVDF 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 hr at room temperature in 5% non-fat milk in Tris buffered saline-Tween 20 (TBS-T) [25 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.1% Tween 20]. Blots were incubated with a goat-anti-rat CYP1A1/1A2 (1:1000), a rabbit-anti-human CYP3A4 (1:500), or a rabbit-anti-human β-actin (1:2000) antibody in 0.5% non-fat milk in TBS-T for 1 hr at room temperature. After washing the membranes for 3 × 5 min in TBS-T, they were incubated with a secondary HRP-conjugated donkey-anti-goat (1:60,000) or a HRP-conjugated donkey-anti-rabbit (1:100,000) antibody in 0.5% non-fat milk in TBS-T for 1 hr at room temperature. After four final washes of the membranes for 5 min in TBS-T, protein expression was detected with a SuperSignal R West Pico ECL kit from Pierce. Relative quantitation of the target signals was performed with a Lumi-Imager from Roche. β-Actin expression was used to normalize relative CYP protein expression.

2.9. Cytochrome P450 activity assays

The activity of CYP1A2 in hepatocytes was quantified by adding ethoxyresorufin to the samples and measuring the formation of its metabolite, resorufin, based on the procedure described by Burke *et al*. [23]. Briefly, the samples were analyzed for fluorescence at 530 nm excitation and 590 nm emission wavelengths. The activity of CYP3A4 in hepatocytes was quantified by adding testosterone to the samples and measuring the formation of its metabolite, 6β-hydroxytestosterone. The procedures used were modified from Easterbrook *et al*. [24]. Briefly, the samples were centrifuged to remove the cells and the supernatant was analyzed without extraction using high pressure liquid chromatography. Testosterone and the 6β-hydroxytestosterone metabolite were separated on a C18 column and detected at a wavelength of 247 nm. Enzyme activity for each CYP450 isoform was reported as specific activity (pmol/min × mg protein) and expressed as mean ± SEM.

3. Results and discussion

3.1. Induction of CYP1A2 in cryopreserved human hepatocytes

The extent of CYP1A2 induction in cryopreserved human hepatocytes was performed by measurement of the CYP1A2 mRNA and protein expression in comparison with the determination of CYP1A2 enzymatic activity.

Forty-eight hours after seeding, hepatocytes from three different lots were incubated for an additional 48 hr in the presence of 1, 10, or 50 μM lansoprazole, or 1, 10, or 100 μM dexamethasone, pioglitazone, rosiglitazone or troglitazone, respectively. CYP1A2 enzymatic activity in these cells was compared to the activity in control cells (0.1% DMSO) or cells incubated in the presence of a typical CYP1A2 (omeprazole) or CYP3A4 (rifampicin) inducer. A high batch-to-batch variation was observed in CYP1A2 inducibility (Fig. 1A) similar to findings using freshly prepared human hepatocytes. Cells incubated in the presence of 25 μM omeprazole demonstrated a mean 38.8-fold induction of CYP1A2 activity compared to control cells (Fig. 2A). CYP1A2 activity varied between 16.4 ± 1.8 pmol/min mg and 4.4 ± 0.3 pmol/min mg protein in the three different batches of hepatocytes. In agreement with a report from Curi-Pedrosa *et al*. [25] we also observed that incubation of cryopreserved hepatocytes with 50 μM lansoprazole, a benzimidazole derivative proton pump inhibitor equipotent to omeprazole, resulted in a mean 31.5-fold induction of CYP1A2 activity (Fig. 2A). Incubation of the cells with different concentrations of glitazones resulted, albeit not significantly, in a dose-dependent upregulation of CYP1A2 activity (Figs. 1 and 2A). Troglitazone and pioglitazone appeared to be equipotent as CYP1A2 inducer. CYP1A2 activity increased from 0.23 ± 0.06 pmol/min mg at 1 μM to 0.8 ± 0.2 pmol/min mg protein at 100 μM pioglitazone or troglitazone, giving rise to a mean maximal 3.4-fold induction of CYP1A2 activity. These findings of troglitazone and pioglitazone are in agreement with results from Sahi *et al*. [26]. They also did not observe any significant effect of troglitazone on the expression of CYP1A2 in primary cultures of human and rat hepatocytes. However, rosiglitazone significantly induced CYP1A2 activity leading to a mean maximal 36.7-fold induction at 10 μM.

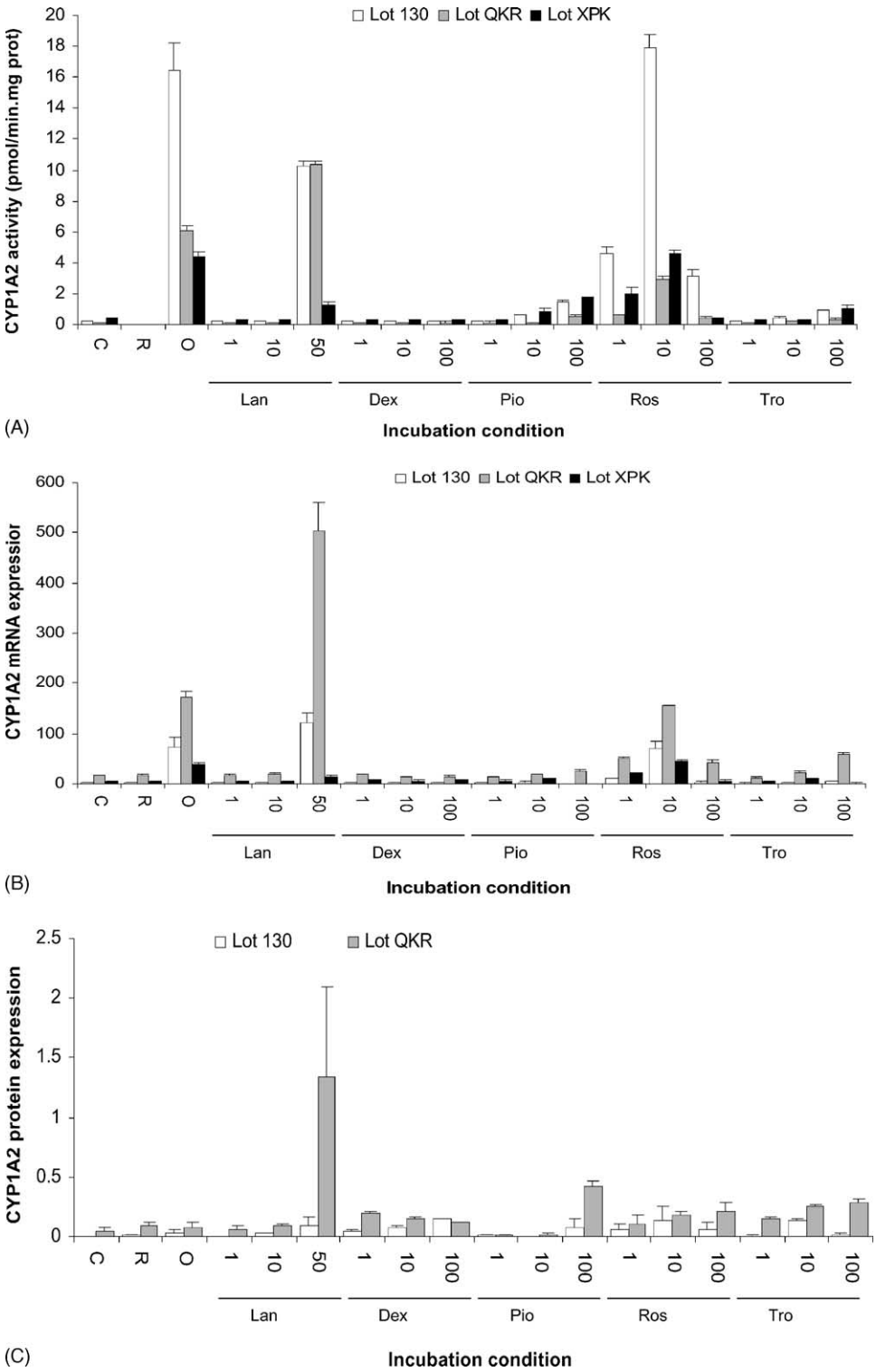


Fig. 1. CYP1A2 induction in cryopreserved human hepatocytes. Cryopreserved human hepatocytes of three different lots were incubated for 48 hr in the presence of three concentrations of different compounds as described in Section 3. Control cells (0.1% DMSO) are represented as C, cells stimulated with 25 μM rifampicin as R, and hepatocytes incubated in the presence of 50 μM omeprazole as O. CYP1A2 enzymatic activity was determined by the fluorescent measurement of the *O*-deethylation of 7-ethoxyresorufin and represented in the graph as pmol/min mg protein (A). The CYP1A2 mRNA (B) and protein (C) expression were determined by TaqMan real-time RT–PCR and immunoblotting, respectively. Both the mRNA and protein expression are represented as the relative expression compared to the control cells (0.1% DMSO) and are plotted in arbitrary units. The error bars represent the SEM and are calculated from three independent incubations.

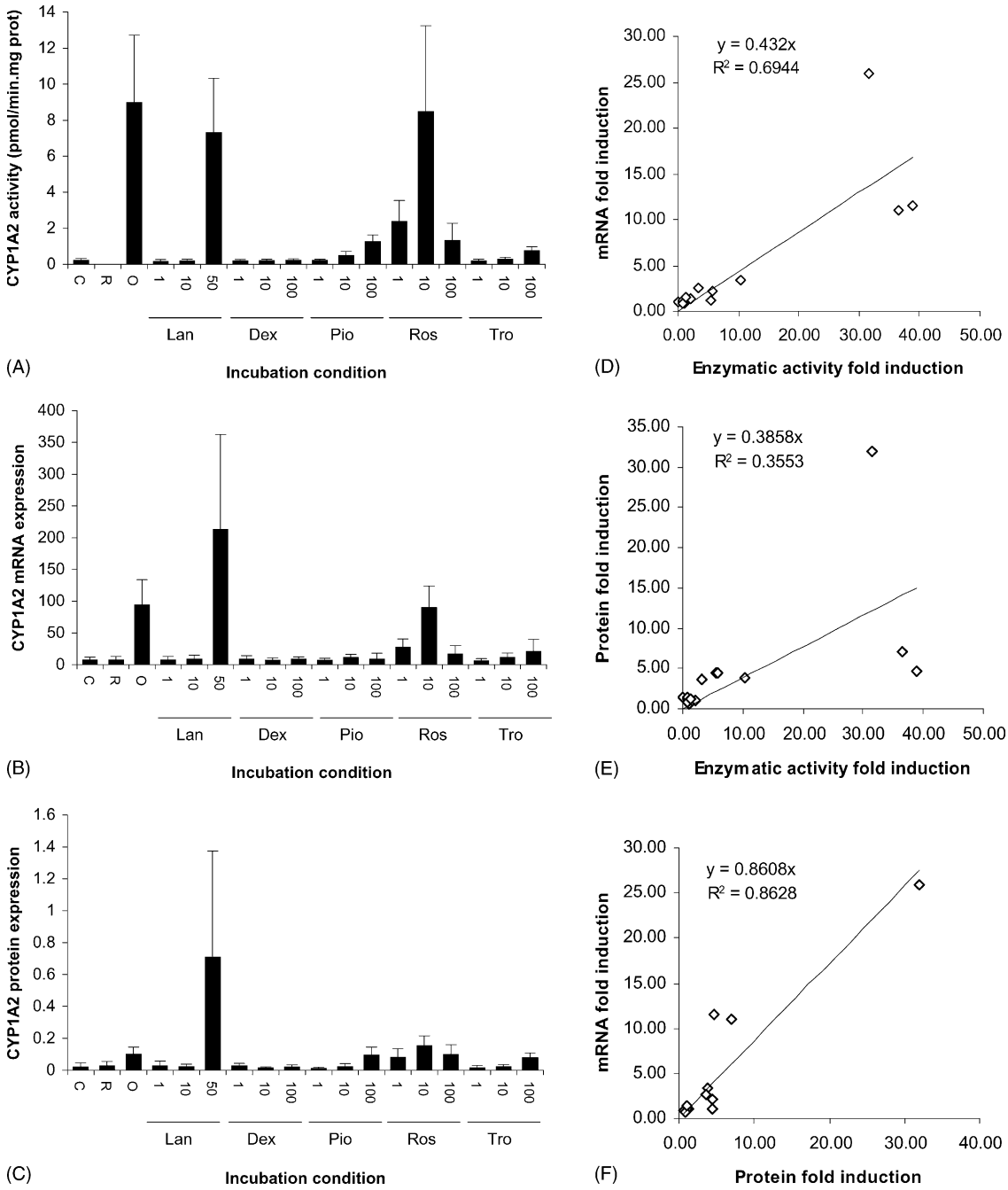


Fig. 2. Mean CYP1A2 induction and correlation of the fold induction values between the three end-points determined. Panels A–C: The mean enzymatic activity of CYP1A2 and of the CYP1A2 mRNA and protein expression. Data are given as the mean value of three different lots. The enzymatic activities are presented as pmol/min mg protein (A), while the mRNA (B) and protein (C) expression is represented as the relative expression compared to the control cells (0.1% DMSO) and plotted in arbitrary units. Control cells are represented as C, cells stimulated with 25 μM rifampicin as R, and hepatocytes incubated in the presence of 50 μM omeprazole as O. The error bars represent the SEM and are calculated from three independent incubations of each of the three lots. Panels D–F: Calculation of the correlation coefficients of the fold inductions measured with different techniques. Panel D represents the comparison in fold induction between CYP1A2 mRNA and the enzymatic activity. A comparison of the fold inductions of the CYP1A2 protein expression vs. the enzymatic activity is represented in panel E. Panel F represents the comparison in fold induction between CYP1A2 mRNA and the protein expression.

Incubation of the cells with 100 μM rosiglitazone caused a profound decrease again of the CYP1A2 activity which was probably due to cytotoxic effects.

In general, quantitation of the CYP1A2 mRNA and protein expression after 24 and 48 hr of induction of hepatocytes, respectively, correlated well with CYP1A2 enzymatic activity (Fig. 2D and E). Again, the same large batch-to-batch variation in CYP1A2 mRNA and protein expression was observed (Fig. 1B and C). Incubation of the cells with 50 μM of the typical CYP1A2 inducers omeprazole and lansoprazole caused a mean 11.5- and 26.0-fold upregulation of CYP1A2 mRNA and a 4.6- and 32.0-fold induction of CYP1A2 protein expression, respectively (Fig. 2B and C). In agreement with the deter-

mination of the CYP1A2 enzymatic activity, the glitazones ranked in the same order of potency to induce CYP1A2 mRNA and protein. A dose-dependent upregulation of CYP1A2 mRNA and protein was observed and a mean maximal 11.0- and 7.0-fold induction of mRNA and protein was measured, respectively (Fig. 2B and C). Protein expression of lot XPK could not be analyzed due to a very low amount of protein available in this sample set.

At present, CYP enzymatic activity is still considered as the reference parameter to study CYP-induction. However, some of these assays are time-consuming, and in cases were mechanistic information is needed on an inducer it is interesting to know what happens with the mRNA and/or protein expression of a CYP. Therefore, it was assessed if CYP mRNA or protein expression analysis is predictive for investigating CYP-induction by measurement of the enzymatic activity. Our results demonstrated a correlation coefficient of approximately 0.69 and 0.36 between the fold induction of the activity and the fold induction of mRNA and protein, respectively. In addition, a 0.86 correlation coefficient was observed between mRNA fold induction and protein fold induction.

The mean basal *O*-deethylation activity of CYP1A2 was calculated to be 0.23 pmol/min mg protein in cryopreserved human hepatocytes. Compared to data in the literature [27,28], this is between 2.0- and 13.0-fold lower than in fresh human hepatocytes. Despite this disadvantageous lower basal expression, our data demonstrate that CYP1A2 induction can be measured precisely.

3.2. Induction of CYP3A4 in cryopreserved human hepatocytes

In a second set of samples CYP3A4 enzymatic activity was measured after incubation of cryopreserved human hepatocytes as described above. Concomitantly, CYP3A4 mRNA and protein expression was determined. As expected, a large variation of CYP3A4 activity was observed in cells from the different lots (Fig. 3A). The 6β-hydroxylation of testosterone varied between 45.6 ± 0.8 pmol/min mg and 757.6 ± 18.1 pmol/min mg protein with a mean activity of 430.1 ± 207.5 pmol/min mg protein in cells stimulated with 25 μM rifampicin. In comparison, the mean CYP3A4 activity in control cells was 68.9 ± 23.8 pmol/min mg protein, which means an approximate mean 6.2-fold induction of CYP3A4 activity in cells incubated in the presence of rifampicin (Fig. 4A). When we compare this to the 6β-hydroxylation activity of CYP3A4 in cell lysates of fresh human hepatocytes, the measured basal activity is in the same order of magnitude [27,28]. Compared to measurements in microsome fractions of fresh hepatocytes, of which is known that they exhibit approximately 5.0-fold higher 6β-hydroxylation activity than would be detected in their respective cell lysates [17], the 6β-hydroxylation activity in our cells is between 3.0- and 16.0-fold [29,30]. In addition, in cells stimulated with 25 μM rifampicin a mean 10.5- and 4.5-fold upregulation of CYP3A4 mRNA and protein was observed, respectively (Fig. 4B and C). Incubation of the cells with 1, 10, and 100 μM dexamethasone resulted in a dose-dependent upregulation of CYP3A4 mRNA and protein expression, and 6β-hydroxylation of testosterone. The mean CYP3A4 activity was increased from 76.2 ± 19.0 pmol/min mg protein in the presence of 1 μM dexamethasone to 214.6 ± 88.3 pmol/min mg protein when 100 μM of the glucocorticoid was administered to the culture medium. Cells from lot XPK were the least responsive with a maximum 1.2-fold increase in CYP3A4 activity when the cells were stimulated with 100 μM dexamethasone. These results are in accordance with studies from Lindley *et al.* [29] and Luo *et al.* [30] in fresh hepatocytes who observed a dose-dependent upregulation of the CYP3A4 activity ranging from approximately 1.5-fold (2 μM dex) to 6.9-fold (250 μM dex). The mechanism of dexamethasone induction of CYP3A4 is not completely clear yet. However, a recent report from Pascussi *et al.* [31] demonstrates a biphasic effect of dexamethasone treatment on CYP3A4 induction in human hepatocytes. Administration of submicromolar concentrations of dexamethasone to the culture medium of the cells results in a low amplitude (3- to 4-fold) increase in CYP3A4 mRNA, while addition of supramicromolar concentrations results in a high amplitude (15- to 30-fold) mRNA increase. These results were not observed in our study with cryopreserved human hepatocytes. Increase of CYP3A4 mRNA was dose-dependent with a maximal 5.3-fold induction in the presence of 100 μM dexamethasone in the cell-culture medium (Fig. 3B). This discrepancy may be explained by subtle changes in the mechanisms involved in the regulation of CYP3A4 gene activity upon cryopreservation of human hepatocytes. Alternatively, due to the low number of lots in the study together with a large batch-to-batch variation in CYP3A4 expression and activity, we might not have detected this. A comparable dose-dependent upregulation of CYP3A4 protein was observed when incubating cryopreserved hepatocytes with dexamethasone (Figs. 3C and 4C). Cells were also stimulated for 48 hr with the benzimidazole derivatives, omeprazole and lansoprazole. Incubation of the hepatocytes with 50 μM omeprazole did not result in an increase in CYP3A4 mRNA or protein expression, and activity (Figs. 3 and 4). Administration of 50 μM lansoprazole to the culture medium resulted in a moderate 1.4-, 3.2-, and 6.3-fold increase in CYP3A4 activity, protein expression and mRNA abundance, respectively. These data are in agreement with a study from Curi-Pedrosa *et al.* [25] demonstrating that omeprazole and lansoprazole are mixed CYP1A- and CYP3A-inducers, and support the hypothesis stated by these authors that the induction of CYP3A in response to these molecules could be polymorphic in humans. The observation that, contrary to lansoprazole, omeprazole did not induce CYP3A4 could be explained by a difference in potency of both

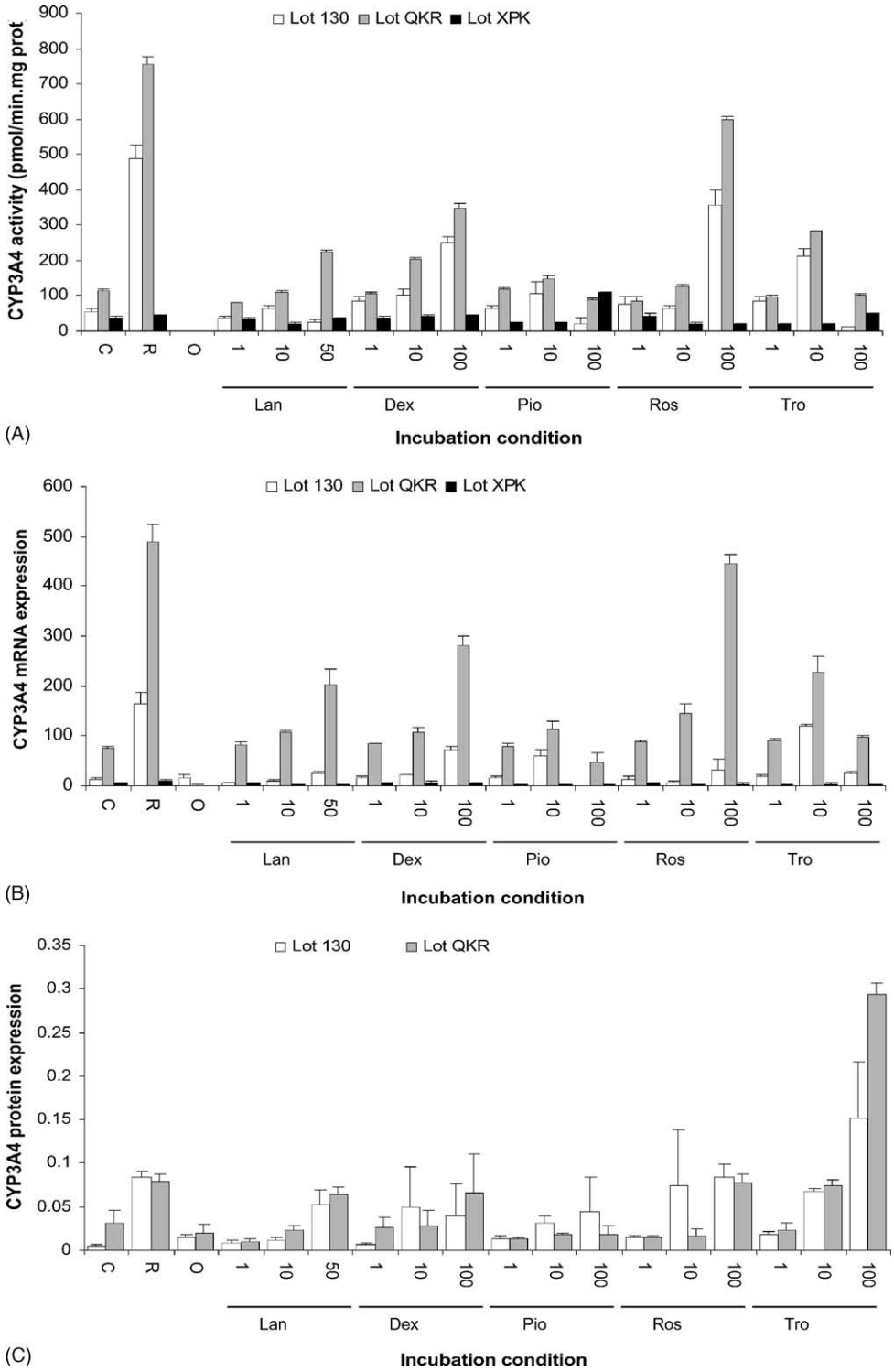
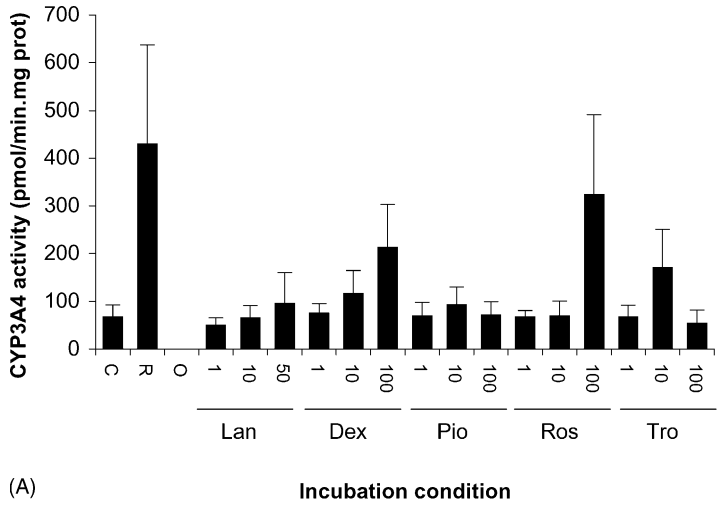
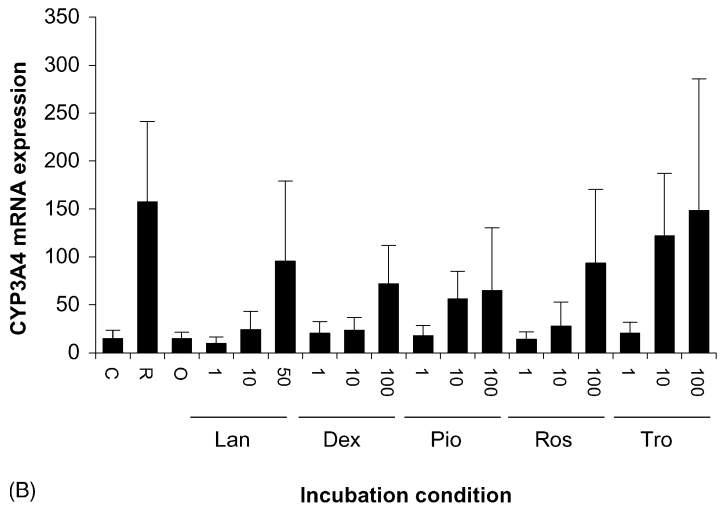


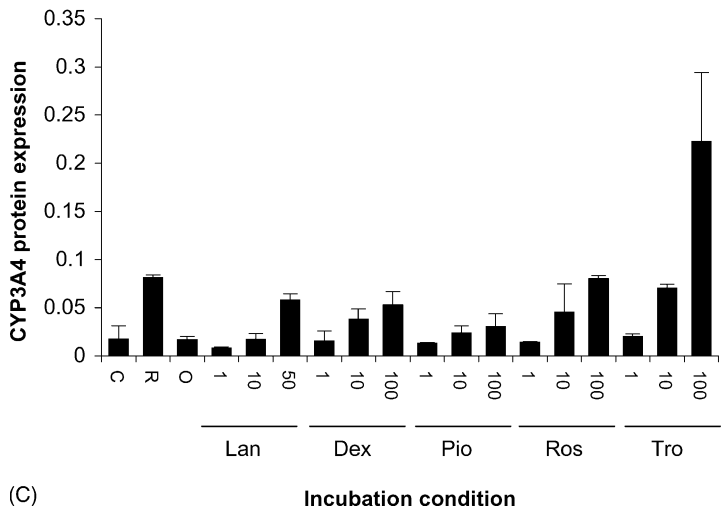
Fig. 3. CYP3A4 induction in cryopreserved human hepatocytes. Cryopreserved human hepatocytes of three different lots were incubated for 48 hr in the presence of three concentrations of different compounds as described in Section 3. Control cells (0.1% DMSO) are represented as C, cells stimulated with 25 μM rifampicin as R, and hepatocytes incubated in the presence of 50 μM omeprazole as O. CYP3A4 enzymatic activity was determined by the radio-HPLC measurement of the 6β-hydroxylation of testosterone and represented in the graph as pmol/min mg protein (A). The CYP3A4 mRNA (B) and protein (C) expression were determined by TaqMan real-time RT–PCR and immunoblotting, respectively. Both the mRNA and protein expression are represented as the relative expression compared to the control cells (0.1% DMSO) and are plotted in arbitrary units. The error bars represent the SEM and are calculated from three independent incubations.



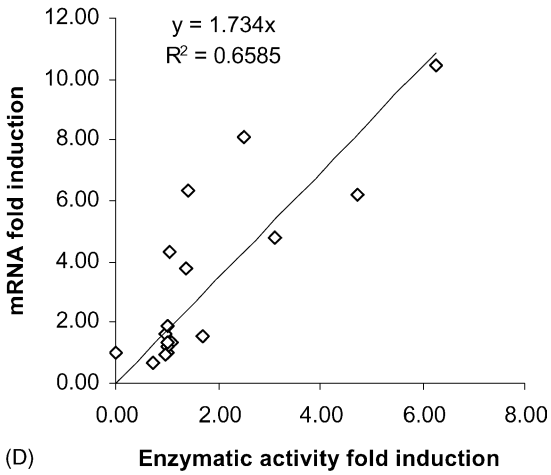
(A)



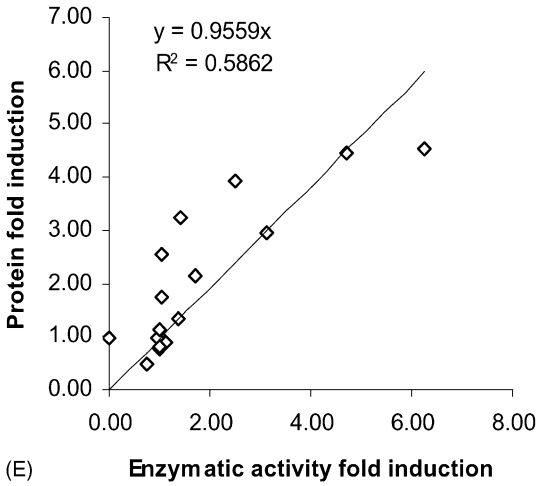
(B)



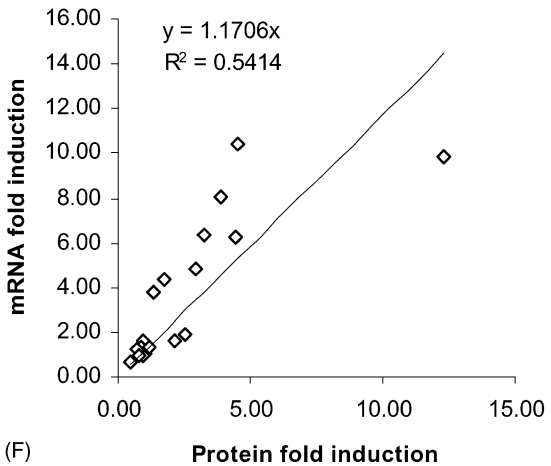
(C)



(D)



(E)



(F)

Fig. 4. Mean CYP3A4 induction and correlation of the fold induction values between three end-points determined. Panels A–C: The mean enzymatic activity of CYP3A4 and of the CYP3A4 mRNA and protein expression. Data are given as the mean value of three different lots. The enzymatic activities are presented as pmol/min mg protein (A), while the mRNA (B) and protein (C) expression is represented as the relative expression compared to the control cells (0.1% DMSO) and plotted in arbitrary units. Control cells are represented as C, cells stimulated with 25 μM rifampicin as R, and hepatocytes incubated in the presence of 50 μM omeprazole as O. The error bars represent the SEM and are calculated from three independent incubations of each of the three lots. Panels D–F: Calculation of the correlation coefficients of the fold inductions measured with different techniques. Panel D represents the comparison in fold induction between CYP3A4 mRNA and the enzymatic activity. A comparison of the fold inductions of the CYP3A4 protein expression vs. the enzymatic activity is represented in panel E. Panel F represents the comparison in fold induction between CYP3A4 mRNA and the protein expression.

compounds to activate a factor necessary in the induction of CYP3A4.

Cryopreserved human hepatocytes from three different lots were incubated for 48 hr with different concentrations of glitazones. A dose-dependent upregulation of both the mRNA and protein expression was observed. At maximum dose, a mean 4.3-, 6.2-, and 9.9-fold induction of CYP3A4 mRNA was determined when cells were stimulated with piolgitazone, rosiglitazone and troglitazone, respectively. Similarly, CYP3A4 protein expression increased 1.7-, 4.5-, and 12.3-fold and CYP3A4 activity responded in a comparable fashion. However, CYP3A4 activity at the highest dose of pioglitazone and troglitazone (100 μM) was low, and is probably due to cytotoxic effects. Interestingly, lot XPK demonstrated almost no response to glitazone stimulation. Based on these experiments, the ranking of the glitazones with respect to their induction potential of CYP3A4 corresponds to that found in fresh human hepatocytes.[1] It should be pointed out that these experiments were specifically designed to compare the basal and induced activity of CYP1A2 and CYP3A4 in cryopreserved human hepatocytes with those in cultures of fresh cells. In addition, calculation of the plasma concentration at steady state of lansoprazole in patients dosed daily with 30 mg lansoprazole results in 0.19–0.24 μM in adolescents and adult, respectively [32]. Patients treated daily with therapeutically relevant doses of troglitazone (400 mg), rosiglitazone (8 mg), and pioglitazone (45 mg) demonstrated plasma concentrations at steady state of 1.1, 0.3 and 1.68 μM, respectively [33–35]. It is clear that when the CYP-induction potential of a NCE is investigated in cultures of fresh or cryopreserved human hepatocytes, ideally, one should try to use clinically relevant concentrations. Finally, correlations in CYP3A4 fold induction between the different techniques were analyzed. By comparing the fold induction of the mRNA expression vs. enzymatic activity, protein expression vs. enzymatic activity, and mRNA expression vs. protein expression, a correlation coefficient of 0.66, 0.58 and 0.54 was calculated, respectively (Fig. 4D–F).

In conclusion, compared to previous reports, the basal enzymatic activity of CYP1A2 is 2.0- to 13.0-fold lower than in fresh human hepatocytes. In contrast, CYP3A4 basal activity in cryopreserved cells is comparable to that in fresh hepatocytes. Similar to freshly prepared hepatocytes, a large batch-to-batch variation in CYP1A2 and CYP3A4 expression and inducibility was observed. However, induction of both CYPs could be precisely measured. An important advantage of using cryopreserved cells is that induction studies can be carefully planned, and the population of cells be chosen in function of the type of experiment. In conclusion, our data demonstrate that cryopreserved human hepatocytes are a useful tool to study CYP1A2 and CYP3A4 induction.

[1] Habucky *et al*. (unpublished results).

References

[1] Smith DA. Induction and drug development. Eur J Pharm Sci 2000;11:185–9.

[2] Li AP. Primary hepatocyte cultures as an *in vitro* experimental model for the evaluation of pharmacokinetic drug–drug interactions. Adv Pharmacol 1997;43:103–30.

[3] Freeman DJ, Laupacis A, Keown PA, Stiller CR, Carruthers SG. Evaluation of cyclosporin–phenytoin interaction with observations on cyclosporin metabolites. Br J Clin Pharmacol 1984;18:887–93.

[4] DeSouza MJ, Pollock SH, Solomon HM. Cyclosporine–phenytoin interaction. Drug Metab Disp 1988;16:256–8.

[5] Cooney GF, Mochon M, Kaiser B, Dunn SP, Goldsmith B. Effects of carbamazepine on cyclosporine metabolism in pediatric renal transplant recipients. Pharmacotherapy 1995;15:353–6.

[6] Breckenridge A, Orme M. Clinical implications of enzyme induction. Ann NY Acad Sci 1971;179:421–31.

[7] Silva JM, Morin PE, Day SH, Kennedy BP, Payette P, Rushmore T, Yergey JA, Nicoll-Griffith D. Refinement of an *in vitro* cell model for cytochrome P450 induction. Drug Metab Disp 1998;26:490–6.

[8] LeCluyse EL. Human hepatocyte culture systems for the *in vitro* evaluation of cytochrome P450 expression and regulation. Eur J Pharm Sci 2001;13:343–68.

[9] Donato MT, Castell JV, Gomez-Lechon MJ. Effect of model inducers on cytochrome P450 activities of human hepatocytes in primary culture. Drug Metab Disp 1995;23:553–8.

[10] Ferrini JB, Pichard L, Domergue J, Maurel P. Long-term primary cultures of adult human hepatocytes. Chem Biol Interact 1997; 107:31–45.

[11] Guillouzo A, Morel F, Langouet S, Maheo K, Rissel M. Use of hepatocyte cultures for the study of hepatotoxic compounds. J Hepatol 1997;26:73–80.

[12] Kern A, Bader A, Pichlmayr R, Sewing KF. Drug metabolism in hepatocyte sandwich cultures of rats and humans. Biochem Pharmacol 1997;54:761–72.

[13] Maurel P. The use of adult human hepatocytes in primary culture and other *in vitro* systems to investigate drug metabolism in man. Adv Drug Deliv Rev 1996;22:105–32.

[14] Skett P, Tyson C, Guillouzo A, Maier P. Report on the international workshop on the use of human liver preparations to study drug metabolism in drug development. Biochem Pharmacol 1995;50: 280–5.

[15] Alexandre E, Viollon-Abadie C, David P, Gandillet A, Coassolo P, Heyd B, Mantion G, Wolf P, Bachellier P, Jaeck D, Richert L. Cryopreservation of adult human hepatocytes obtained from resected liver biopsies. Cryobiology 2002;44:103–13.

[16] Hengstler JG, Ringel M, Biefang K, Hammel S, Milbert U, Gerl M, Klebach M, Diener B, Platt KL, Böttger T, Steinberg P, Oesch F. Cultures with cryopreserved hepatocytes: applicability for studies of enzyme induction. Chem Biol Interact 2000;125:51–73.

[17] Hengstler JG, Utesch D, Steinberg P, Platt KL, Diener B, Ringel M, Swales N, Fischer T, Biefang K, Gerl M, Böttger T, Oesch F. Cryopreserved primary hepatocytes as a constantly available *in vitro* model for the evaluation of human and animal drug metabolism and enzyme induction. Drug Metab Rev 2000;32:81–118.

[18] Li AP, Gorycki PD, Hengstler JG, Kedderis GL, Koebe HG, Rahmani R, de Sousas G, Silva JM, Skett P. Present status of the application of cryopreserved hepatocytes in the evaluation of xenobiotics: consensus of an international expert panel. Chem Biol Interact 1999;121: 117–23.

[19] Li AP, Roque MA, Beck DJ, Kaminski DL. Isolation and culturing of hepatocytes from human liver. J Tissue Cult Methods 1992;14: 139–46.

[20] Loretz LJ, Li AP, Flye MW, Wilson AG. Optimization of cryopreservation procedures for rat and human hepatocytes. Xenobiotica 1989;19:489–98.

[21] Ruegg CE, Silber PM, Mughal RA, Ismail J, Lu C, Bode DC, Li AP. Cytochrome-P450 induction and conjugated metabolism in primary human hepatocytes after cryopreservation. In Vitro Toxicol 1997; 10:217–22.

[22] Gellner K, Eiselt R, Hustert E, Arnold H, Koch I, Haberl M, Deglmann CJ, Burk O, Buntefuss D, Escher S, Bishop C, Koebe HG, Brinkmann U, Klenk HP, Kleine K, Meyer UA, Wojnowski L. Genomic organization of the human CYP3A locus: identification of a new, inducible CYP3A gene. Pharmacogenomics 2001;11:111–21.

[23] Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T, Mayer RT. Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P450. Biochem Pharmacol 1985;34:3337–45.

[24] Easterbrook J, Fackett D, Li AP. A comparison of Aroclor 1254-induced and uninduced rat liver microsomes to human liver microsomes in phenytoin *O*-deethlyation, coumarin 7-hydroxylation, tolbutamide 4-hydroxylation, *S*-mephenytoin 4′-hydroxylation, chlorzoxazone 6-hydroxylation and testosterone 6β-hydroxylation. Chem Biol Interact 2001;134:243–9.

[25] Curi-Pedrosa R, Daujat M, Pichard L, Ourlin JC, Clair P, Gervot L, Lesca P, Domergue J, Joyeux H, Fourtanier G, Maurel P. Omeprazole and lansoprazole are mixed inducers of CYP1A and CYP3A in human hepatocytes in primary culture. J Pharmacol Exp Ther 1994;269: 384–92.

[26] Sahi J, Hamilton G, Sinz M, Barros S, Huang SM, Lesko LJ, LeCluyse EL. Effect of troglitazone on cytochrome P450 enzymes in primary cultures of human and rat hepatocytes. Xenobiotica 2000;30:273–84.

[27] Donato MT, Gómez-Lechón MJ, Jover R, Nakamura T, Castell JV. Human hepatocyte growth factor down-regulates the expression of cytochrome P450 isoenzymes in human hepatocytes in primary culture. J Pharmacol Exp Ther 1998;284:760–7.

[28] Guillén MI, Donato MT, Jover R, Castell JV, Fabra R, Trullenque R, Gómez-Lechón MJ. Oncostatin M down-regulates basal and induced cytochromes P450 in human hepatocytes. J Pharmacol Exp Ther 1998;285:127–34.

[29] Lindley C, Hamilton G, McCune JS, Faucette S, Shord SS, Hawke RL, Wang H, Gilbert D, Jolley S, Yan B, LeCluyse EL. The effect of cyclophosphamide with and without dexamethasone on cytochrome P450 3A4 and 2B6 in human hepatocytes. Drug Metab Disp 2002; 30:814–21.

[30] Luo G, Cunningham M, Kim S, Burn T, Lin J, Sinz M, Hamilton G, Rizzo C, Jolley S, Gilbert D, Downey A, Mudra D, Graham R, Carroll K, Xie J, Madan A, Parkinson A, Christ D, Selling B, LeCluyse E, Gan L-S. Cyp3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. Drug Metab Disp 2002;30:795–804.

[31] Pascussi J-M, Drocourt L, Gerbal-Chaloin S, Fabre J-M, Maurel P, Vilarem M-J. Dual effect of dexamethasone on *CYP3A4* gene expression in human hepatocytes. Eur J Biochem 2001;268:6346–57.

[32] Gremse DA. Lansoprazole: pharmacokinetics. Expert Opin Pharmacother 2001;2:1663–70.

[33] Prueksaritanont T, Vega JM, Zhao J, Gagliano K, Kuznetsova O, Musser B, Amin RD, Liu L, Roadcap BA, Dilzer S, Lasseter KC, Rogers JD. Interactions between simvastatin and troglitazone or pioglitazone in healthy subjects. J Clin Pharmacol 2001;41:573–81.

[34] Mamidi NVSR, Mullangi R, Katneni K, Ravikanth B, Babu AP, Rani UP, Naidu MUR, Srinivas NR, Rajagopalan R. Lack of effect of sucralfate on the absorption and pharmacokinetics of rosiglitazone. J Clin Pharmacol 2002;42:670–5.

[35] Budde K, Neumayer H-H, Fritsche L, Sulowicz W, Stompôr T, Eckland D. The pharmacokinetics of pioglitazone in patients with impaired renal function. Br J Clin Pharmacol 2003;55:368–74.