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Induction of Cytochrome P450 (CYP)1A1, CYP1A2, and CYP3A4 but Not of CYP2C9, CYP2C19, Multidrug Resistance (MDR-1) and Multidrug Resistance Associated Protein (MRP-1) by Prototypical Inducers in Human Hepatocytes

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Human hepatocytes cultured serum-free for up to 6 weeks were used to study expression and induction of enzymes and membrane transport proteins involved in drug metabolism. Phase I drug metabolizing enzymes cytochrome P450 (CYP)1A1, CYP1A2, CYP2C9, CYP2C19, CYP2E1, and CYP3A4 were detected by Western blot analyses and, when appropriate, by enzymatic assays for ethoxyresorufin-*O*-deethylase(EROD)-activity and testosterone-6β-hydroxylase(T6H)-activity. Expression of the membrane transporter multi-drug resistance protein (P-glycoprotein, MDR-1), multidrug resistance-associated protein (MRP-1), and lung-resistance protein (LRP) was maintained during the culture as detected by RT-PCR and Western blot analyses. Model inducers like rifampicin, phenobarbital, or 3-methylcholanthrene and β-naphtoflavone were able to induce CYP1A or CYP3A4 as well as EROD or T6H activities for up to 30 days. CYP2C9, CYP2C19 and CYP2E1 expression was maintained but not inducible for 48 days. Also, rifampicin and phenobarbital were unable to increase MDR-1 and MRP-1 protein levels significantly.

Key Words: **human hepatocytes; antibiotics; barbiturates; aromatic hydrocarbons; Cytochrome P450; P-glycoprotein; multidrug-resistance-associated protein.**

Primary cultures of hepatocytes are an important tool to study liver-specific processes and functions. So far, metabolic studies have primarily been performed in rat hepatocyte cultures. However, interspecies differences have been reported especially with regards to cytochrome P450 induction (1, 2). Therefore, the cultivation of primary human hepatocytes seems to represent a more appropriate experimental system for the evaluation of liver specific processes, especially drug metabolism in man (3, 4). Several different attempts for cultivation of human hepatocytes have been followed using different substrates (5), media and hormonal conditions usually in the initial or ongoing presence of serum (6, 7). Most investigators analyzed the induction of cytochrome P450 enzymes, especially the CYP 1A and 3A isoforms (7–11). Most studies were done in short-term cultures, in which the cells were maintained for maximally 8 days, only a few data are available using hepatocytes cultured in the absence of serum for several weeks (8).

While cytochrome P450 proteins are responsible for phase I metabolism of xenobiotics, the membrane transport proteins MDR-1, MRP-1 and LRP take part in drug resistance due to active export of xenobiotics and their metabolites.

Recently, we have established culture conditions that allow serum-free cultivation of human hepatocytes in the presence of Hepatocyte Growth Factor (HGF) and Epidermal Growth Factor (EGF) (12). The cells maintain hepatocyte morphology as well as the expression of serum proteins and transcription factors for several weeks (13). Here we show that this culture system also serves as an appropriate model system to analyze regulation of proteins involved in drug metabolism.

It has been shown that in human hepatocyte cultures cytochrome P450 levels decline during the first 2–4 days in culture to about 15–30% compared to fresh isolated hepatocytes (14). Furthermore, cytochrome P450 protein levels and enzymatic activities require an

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adjustment period of about 4 days in culture before full responsiveness to added hormonal stimuli is regained (14). Therefore, we started our experiments on day 4 of the cultures. Basal expression of cytochrome P450 proteins CYP2C9, CYP2C19, CYP2E1 and CYP3A4 was maintained from day 4 on for 4–6 weeks. Model inducers like rifampicin (RIF) or phenobarbital (PB) were able to induce CYP3A protein and T6H activities, but had little effect on CYP2C9, CYP2C19, MDR-1 and MRP-1 protein levels. 3-methylcholanthrene (3MC) and β-naphtoflavone (β-NF) induced specifically CYP1A2 and corresponding EROD activity, β-NF also suppressed MDR-1 protein.

MATERIALS AND METHODS

The Δ5 variant of human HGF was a gift from Snow Brand Milk Products (ref. 15, Tochigi, Japan), natural mouse EGF was purchased from Collaborative Research (Bedford, MA). Vitrogen (bovine collagen type 1) was from Collagen Corp. (Fremont, CA). Gentamycin, Penicillin/Streptomycin, DMEM with and without Glucose (DMEM #11965 and #11966), and MEM (#41600) was purchased from GIBCO/BRL (Gaithersburgh, MD). Rabbit anti-human CYP2C9, rabbit anti-human CYP2C19, goat anti-rat CYP2E1 and goat anti-rat CYP1A antibody was purchased from Genetest Corp. (Woburn, MA). Rabbit anti-human CYP3A polyclonal antiserum was kindly provided by Dr. S. Wrighton (Lilly Research Laboratories). Monoclonal mouse anti-human p-glycoprotein (MDR-1) 6/1C antibody was obtained from Kamiya Biomedical company (Seattle, WA), monoclonal rat anti-human MRP-1 antibody MRPr-1 was from ICN Pharmaceuticals (Aurora, OH). Secondary antibodies were obtained from Sigma (St. Louis, MO). Culture dishes were from Corning Costar (Cambridge, MA). RNAzol B was from Tel-Test (Friendswood, TX), X-omat film was purchased from Kodak (Rochester, NY). Oligonucleotides for RT-PCR were from Genaxis (Spechbach, Germany), dNTPs were obtained from HYBAID-AGS (Heidelberg, Germany), RQ1 DNAse, M-MLV Reverse Transcriptase, Random Primer and Taq DNA Polymerase were purchased from Promega (Madison, WI).

Isolation and culture of human hepatocytes. Human hepatocytes were isolated from excess liver tissue from reduced human liver transplant procedures or from donor livers that had not been used for transplantation (13). Hepatocytes were isolated by perfusion with collagenase-P (Boehringer Mannheim, Indianapolis, IN) through the existing vasculature as described previously (16). Cells were plated onto collagen coated dishes and cultured in Human Hepatocyte Maintenance Medium. Briefly, the initial plating was done in MEM containing 500 ng/ml insulin and 50 μg/ml gentamycin for 3–12 h. The medium was then changed to a mixture of DMEM (no glucose): DMEM (plus glucose):MEM (25:25:50), with the following additions 1 g/l albumin, 5.5 mM galactose, 0.5 mM glutamine, 0.3 mM ornithine, 0.13 mM proline, 5 mM Hepes, 2.5 ng/ml Na-selenite, 2.5 μg/ml transferrin, 0.272 mg/l $ZnCl_2$, 0.1 mg/l $CuSO_4 \times 5\ H_2O$, 0.375 mg/l $ZnSO_4 \times 7\ H_2O$, 0.0125 mg/l $MnSO_4$, 2.75 μg/ml insulin, 25 μg/ml gentamycin, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 nM dexamethasone, 40 ng/ml HGF, and 20 ng/ml EGF. To simplify, we will refer to this medium composition as Human Hepatocyte Maintenance Medium (HHMM) with HGF and EGF.

Protein isolation and Western Blot analyses of whole cell lysates. Native protein extracts were isolated and subjected to Western blot analyses as described (17). Lysates were centrifuged at 10,000 rpm in a table top centrifuge for 20 min. Cytochrome P450 proteins, MDR-1, and MRP-1, but not LRP were detected in the supernatant of the 20 min × 10,000 rpm centrifugation. LRP and MRP-1, but not MDR-1 were detected in the pellets. For the detection of membrane transport proteins the transfer onto Immobilon membranes was modified as follows: The transfer was performed at 4°C for 18 h in a buffer consisting of 25 mM Tris, pH 8.3, 192 mM glycine, 0.15% (w/v) SDS, 10 mM borate, and 5% (v/v) methanol. For protein determination either the Bicinchonic acid Protein Assay (Sigma, St. Louis, MO) was used or protein was determined according to Lowry *et al.* (18). SDS polyacrylamide gel electrophoresis was performed according to Laemmli (19).

Immunochemical analysis of CYP4501A and 3A4 enzymes was conducted as described previously (20), using whole cell extracts. CYP3A4 was detected by a rabbit anti-human CYP3A antibody which detects both, CYP3A4 and CYP3A5 proteins (20–22). CYP1A1 and CYP1A2 were detected with a goat anti-rat CYP1A antibody detecting both proteins. Alkaline phosphatase-conjugated anti-rabbit or anti-goat antibodies and NBT/BCIP developing reagents were used to visualize blots. Baculovirus expressed CYP3A4, CYP1A1 and CYP1A2 were used as controls.

RNA isolation and RT-PCR analysis. RNA was isolated as described (17). DNase I digestion and reverse transcription reactions were performed according to the manufacturer's protocol. The following primer and reaction conditions were used for RT-PCR: MDR-1 mRNA was detected using primers 5′CCTATCATTGCAATAGCAGG3′ and 5′GTTCAAACTTCTGCTCCTGA3′, which amplify a 167 bp fragment (annealing temp. 55°C, 28 cycles, ref. 23), MRP mRNA was amplified with 5′CTGAGAAGGAGGCGCCCTG3′ and 5′GTGTCCGGATGGTGGACTG3′ primers that generate a 613 bp fragment (annealing temp. 56°C, 30 cycles, ref. 24). For detection of LRP 5′CCCCCATACCACTATATCCATGTG3′ and 5′TGGAAAAGCCAGTCATCTCCTG3′ primer were used to amplify a 405 bp fragment (annealing temp 55°C, 28 cycles, ref. 25). As internal control a 196 bp β-actin fragment was amplified in 25 cycles with 5′ACGGCTCCGGCATGTGCAAG3′ and 5′TGACGATGCCGTGCTGCATG3′ oligonucleotides (annealing temp 55° C, ref. 26) and a 120 bp β_2-microglobulin fragment was amplified in 25 cycles using 5′ACCCCCACTGAAAAAGATGA3′ and 5′ATCTTCAAACCTCCATGATG3′ oligonucleotides (annealing temp 55°C, ref. 23). The standard conditions for our RT-PCR reactions are as follows: PCR was performed starting with an initial denaturation at 94°C for 2 min, followed by 25–30 cycles of: 30 s denaturation at 94°C, 30 s annealing at 55°C or 56°C, and 45 s elongation at 72°C. In the last cycle, a 15 min elongation reaction at 72°C was carried out.

Cytochrome P450 enzyme activities. 7-ethoxyresorufin-*O*-deethylase activity catalyzed by CYP1A was determined directly in intact cultured hepatocytes. 48 h before assaying the enzymatic activity the cells were exposed to the indicated inducers 3-methylcholanthrene (3MC, 2 μg/ml) or β-naphthoflavone (β-NF, 50 μM). On days indicated in the corresponding figure or table culture media were changed and replaced with Williams E medium, containing 20 μM 7-ethoxyresorufin. Resorufin was measured as described previously with some modifications (27, 28), using the fluorescent spectrometer LS-50B from Perkin Elmer with a plate reader. 100 μl of culture medium were loaded on 96-well plates and fluorescence was detected at 535-nm excitation and 581-nm emission. Resorufin was quantitated by comparing the fluorescence to a standard curve of resorufin prepared in Williams E medium.

Testosterone-6β-hydroxylase activity catalyzed by CYP3A was determined directly in intact cultured hepatocytes, as described previously (29). After 48 h exposure to inducers rifampicin (RIF, 10 μM) or phenobarbital (PB, 2 mM), culture media were changed and replaced with Williams E medium containing 100 μM testosterone on days indicated in the corresponding figure or table. Formation of 6β-hydroxytestosterone was measured by HPLC (29) and quantitated by comparing the absorbance to a standard curve of 6β-hydroxytestosterone prepared in Williams E medium.

Transmission electron microscopy was performed as described previously (13).

TABLE 1

Testosterone-6-Hydroxylase Activity in Long-Term Serum-Free Cultures of Human Hepatocytes Induced by Phenobarbital or Rifampicin

Donor	Days in culture	Testosterone-6-hydroxylase [pmol/min/mg protein]		
		Untreated	Phenobarbital	Rifampicin
642	22	100 ± 16	190 ± 6	710 ± 23
	30	30 ± 43	170 ± 14	790 ± 41
660	22	n.d.	70 ± 8	80 ± 7
	30	n.d.	40 ± 4	70 ± 2

Note. Hepatocytes were cultured serum-free in HHMM with HGF [40 ng/ml] and EGF [20 ng/ml]. 48 hours before harvesting, rifampicin (final: 10 μM) or phenobarbital (final: 2 mM) was added as indicated. After 24 hours, medium was changed and inducers were added a second time. T6H activity was determined in intact hepatocytes. n.d. = not detectable.

RESULTS

Expression and induction of CYP3A4. CYP3A4 is the most abundant P450 isoform in liver, and therefore responsible for the majority of biotransformation processes. We evaluated the ability of the hepatocytes to retain the expression and inducibility of CYP3A4 even after prolonged culture in the absence of serum. Phenobarbital and rifampicin are known to be potent inducers for CYP3A4 in human hepatocytes. The induction was monitored by Western blot analyses and testosterone-6β-hydroxylase activities. We determined basal T6H activity in two cultures on days 22 and 30. Basal and inducible activities varied between these donors, probably reflecting individual differences. In one experiment (donor 642) the basal T6H activity in untreated cells was 3 fold lower on day 30 as compared to day 22. However, treatment with RIF resulted in similar increases in T6H activity on days 22 and 30. PB, although less effective than RIF, produced comparable increases in T6H activity (Table 1). In a second case (donor 660) T6H activity in untreated hepatocytes was not detectable. However, both RIF and PB were able to induce enzyme activity on days 22 and 30. The absolute increase in T6H activity observed in donor 660 was not as high as in donor 642 (Table 1). Western blot analysis detected CYP3A4 protein in untreated cells, diminishing after 4 weeks in both cultures (Fig. 1). Induction with RIF led to an about threefold increase in CYP3A4 protein at any given time of culture, independent of the donor (Fig. 1). Therefore, the increase in CYP3A4 protein seems to be responsible for the increase in T6H enzyme activity.

Expression and induction of CYP1A1 and CYP1A2. CYP1A1 and CYP1A2 can be induced by 3MC and β-NF. We were interested to determine if hepatocytes retained the ability to respond to these polycyclic aromatic hydro-

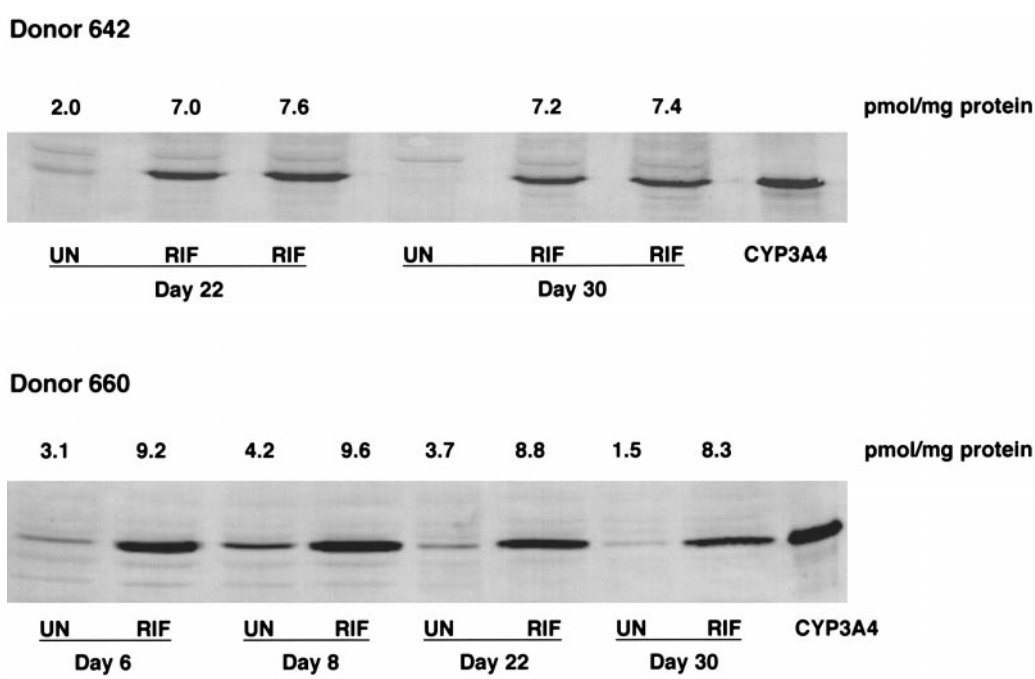

FIG. 1. Induction of CYP3A4 protein in cultured human hepatocytes by rifampicin. Hepatocytes from donors 642 and 660 were treated with 10 μM rifampicin (RIF) for 48 hours before harvesting on days indicated. 15 μg of whole cell lysates were separated by SDS–PAGE, transferred onto Immobilon-membrane and probed with rabbit anti human CYP 3A antibody. As a control 0.2 pmol of CYP3A4 standards was used. UN = untreated hepatocytes. Blots were subjected to densitometric analysis, the calculated amount of CYP3A4 [pmol/mg protein] is displayed on top of the lanes.

TABLE 2

Induction of Ethoxyresorufin-*O*-Deethylase Activity in Long-Term, Serum-Free Human Hepatocyte Cultures by 3-Methylcholanthrene or β-Naphtoflavone

Donor	Days in culture	Ethoxyresorufin-*O*-deethylase (pmol/min/mg protein)		Donor	Days in culture	Ethoxyresorufin-*O*-deethylase (pmol/min/mg protein)	
		Untreated	3-Methylcholanthrene			Untreated	β-Naphtoflavone
622	6	n.d.	0.71 ± 0.15	660	10	n.d.	18.4 ± 0.05
	12	n.d.	0.17 ± 0.04		22	n.d.	2.42 ± 0.36
	19	n.d.	0.31 ± 0.02		30	n.d.	0.37 ± 0.30
642	22	n.d.	2.43 ± 0.37	701	8	n.d.	6.49 ± 1.43
	30	n.d.	0.37 ± 0.39		16	n.d.	3.05 ± 0.12
					22	n.d.	0.69 ± 0.01

Note. Hepatocytes were cultured serum-free in HHMM with HGF [40 ng/ml] and EGF [20 ng/ml]. 48 hours before harvesting 3-methylcholanthrene (final: 2 μg/ml) or β-naphtoflavone (final: 50 μM) was added as indicated. After 24 hours medium was changed and inducers were added again. EROD activity was detected in intact hepatocytes at 535-nm excitation and 581-nm emission using a fluorescent plate reader. Protein amounts were determined using the Bicinchoninic Acid Protein Assay. n.d. = not detectable.

carbons during continous culture without serum. The enzymatic activity can be estimated by ethoxyresorufin-*O*-deethylation, a reaction that is mainly catalysed by CYP1A1. We used 3MC (2 μg/ml) and β-NF (50 μM) to induce EROD activity and CYP1A protein. In non-induced hepatocytes the enzyme activity was below the limit of detection. Both chemicals induced CYP1A mediated EROD activity (Table 2). Treatment with β-NF led to a stronger increase in activity, probably because it induced both CYP1A1 and CYP1A2 proteins whereas 3MC induced only CYP1A2 (Fig. 2). Although the induced protein amounts were similar on day 22 and day 30, inducible EROD activity decreased with time independent of the inducer and the donor (Table 2).

Expression of CYP2C9, CYP2C19 and CYP2E1. These proteins were expressed for up to 48 days in culture (Fig. 3). Since there is little known about the

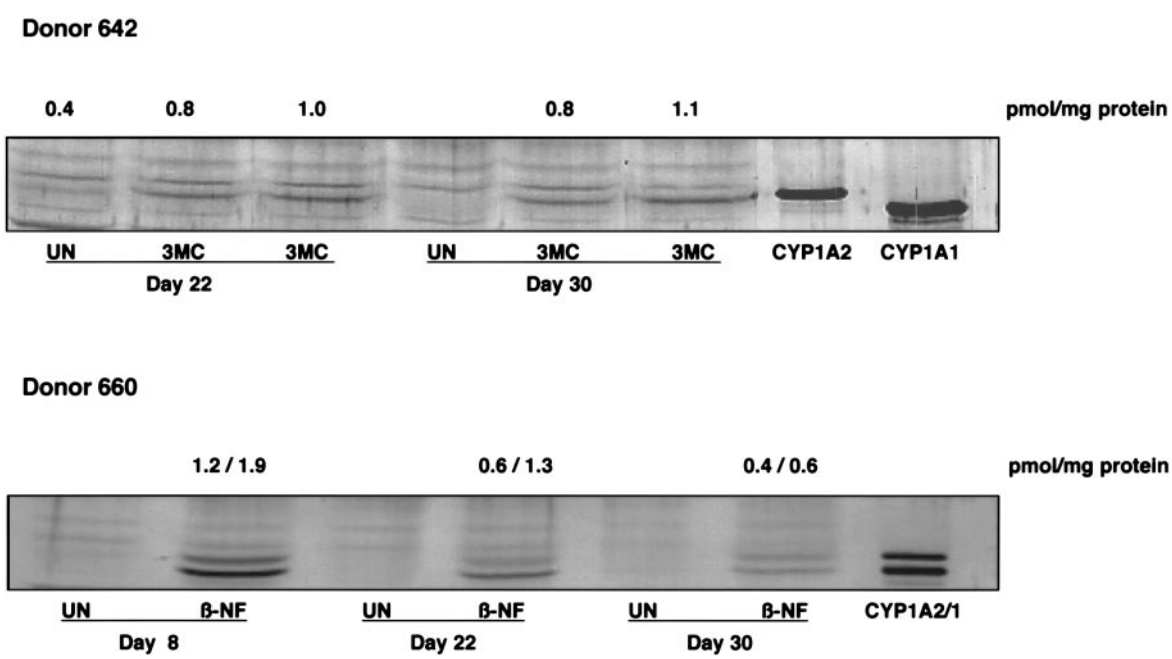

FIG. 2. Induction of CYP1A1 and CYP1A2 protein by β-naphtoflavone [β-NF] and 3-methylcholanthrene [3MC]. Hepatocytes from donors 642 and 660 were treated with 2 μg/ml 3MC or 50 μM β-NF for 48 h before harvesting on indicated days. 20 μg of whole cell lysates were separated by SDS–PAGE, transferred onto a Immobilon-membrane and probed with specific antiserum against CYP1A. As a control 0.1 pmol of CYP1A1 and CYP1A2 standard were used in (A) and a mixture of protein standards CYP1A1 and CYP1A2 at 0.05 pmol each in (B). UN = untreated hepatocytes. Results of duplicate treatment is shown in (A). Blots were subjected to densitometric analysis, the calculated amount of CYP1A2 (A and B/first number, [pmol/mg/protein]) and CYP1A1 (B/second number, [pmol/mg protein]) is displayed on top of the lanes.

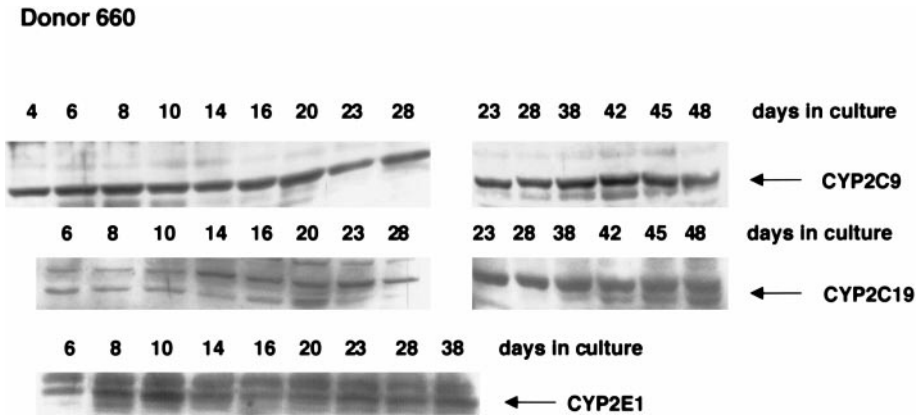

FIG. 3. Expression of CYP2C9, CYP2C19 and CYP2E1 in human hepatocyte cultures. Hepatocytes from donor 660 were cultured in HHMM with HGF and EGF. On days indicated cells were harvested. 50 μg of whole cell extracts were separated by SDS–PAGE, transferred onto Immobilon-membrane and probed with specific anti-sera as indicated.

inducibility of CYP2C family members in human hepatocytes, we investigated if antibiotics, barbiturates and aromatic hydrocarbons were able to modify CYP2C9 and CYP2C19 expression. Although application of RIF and PB increased CYP3A4 protein, none of the inducers at the given concentrations had any effect on CYP2C9 and CYP2C19 expression. 3MC and β-NF did not modulate the expression of these proteins in our cultures (data not shown).

Cell morphology. In addition to cytochrome P450s membrane transport proteins play an important role in detoxifying processes. Since we were interested to examine expression of membrane transport proteins we first analyzed the cell structure by transmission electron microscopy. At all time points, bile canaliculi were readily apparent. Furthermore, other membrane structures like desmosmes and tight junctions were established and maintained throughout the culture (Fig. 4).

Expression of multi-drug resistance proteins. Messenger RNA of MDR-1, MRP, and LRP was present for up to 36 days as determined by RT-PCR. A minor increase of MRP mRNA was observed at later time points, while LRP and MDR-1 mRNA levels remained relatively constant during culture (Fig. 5). Expression and inducibility MDR-1, MRP-1, and LRP proteins was characterized by interindividual differences. LRP protein was not detectable in fresh isolated hepatocytes. Protein levels increased during the culture, reaching maximal levels at around day 6–10, then decreased again (Fig. 6). Basal expression of MDR-1 and MRP-1

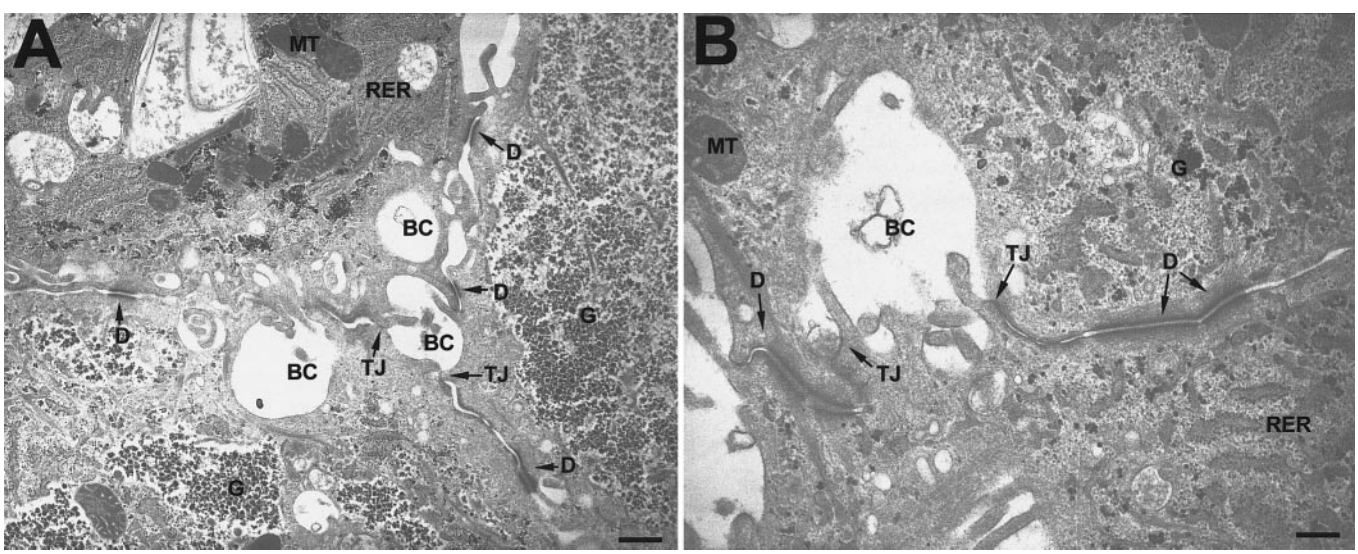

FIG. 4. Morphology of human hepatocytes in long-term serum-free cultures. Transmission electron microscopy of human hepatocytes (donor 622). (A) Day 11 in culture; Bar, 500 nm. (B) Day 24 in culture; Bar, 200 nm. BC, bile canaliculus; D, desmosome; TJ, tight junction; G, glycogen; MT, mitochondria; RER, rough endoplasmatic reticulum.

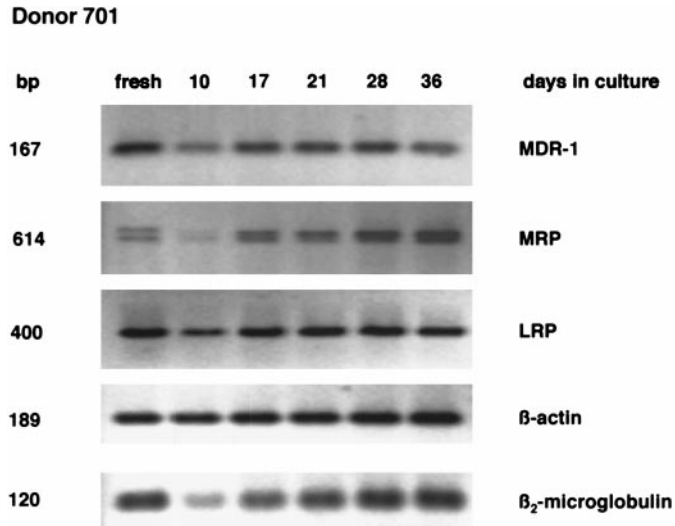

FIG. 5. Expression of MDR-1, MRP and LRP mRNA in human hepatocytes. Human hepatocytes (donor 701) were cultured in HHMM with HGF and EGF. On days indicated, cells were harvested, total RNA was extracted and subjected to RT-PCR using primers specific for MDR-1, MRP, LRP, β-actin and β_2-microglobulin.

was detectable by Western blot analyses of whole cell lysates. Expression of both proteins was maximal at day 4, decreasing with time (Fig. 6). CYP1A and CYP3A/2B model inducers failed to modulate MDR-1 and MRP-1 expression, especially during the first few days of culture. However, as the basal expression of both transporters decreased with aging cultures, they seemed to become more sensitive to application of antibiotics, barbiturates, and aromatic hydrocarbons (Fig. 7). In one case (donor 660) application of RIF and PB led to a slight induction of MDR-1 at day 10 of culture, while β-NF decreased MDR-1 levels. In another case (donor 622) and RIF and 3MCfailed to induce MDR-1 and MRP-1 expression, while PB increased MRP-1 but not MDR-1 at day 19 (Fig. 7).

DISCUSSION

In human hepatocytes detoxifying pathways remain to be characterized. Nevertheless, primary cultures of human hepatocytes are already used as a tool to evaluate the potential of novel drugs to induce cytochrome P450 activities (30). In this study, we have investigated the effects of model inducer on cytochrome expression and enzymatic activity. Furthermore, the expression and inducibility of transmembrane transport proteins involved in drug efflux (MDR-1 and MRP-1) and in nuclear-cytoplasmatic trafficking (LRP) was analysed. The study presented here demonstrates that primary human hepatocytes can be cultured serum-free for 48 days. The cells are characterized by a well preserved morphology and function including the formation of bile canaliculi, storage of glycogen, and induction of CYP P450 proteins for at least 30 days. Cellular organelles like mitochondria were preserved, cell-cell contacts in form of desmosomes and tight junctions were established and maintained throughout the culture period (Figs. 1, 2, and 4).

Expression of Drug Metabolizing Proteins

CYP3A4 is the predominant constitutive P450 isoform in human liver and is responsible for the metabolism of many therapeutic drugs. It is hormonally regulated, glucocorticoids and growth hormone increase 3A4 mRNA, protein and the associated T6H activity, while tri-iodothyronine decreases mRNA and protein levels as well as its enzymatic activity (14). CYP 3A4 metabolizes a number of therapeutic agents including cyclosporin, erythromycin, lidocain, midazolam, nifedipine, and taxol (21, 31–36). In short-term human hepatocyte cultures it is induced by numerous xenobiotics. In our culture system basal 3A4 protein expression was maintained for at least 22 days and was inducible 3- to 4-fold to a new steady state level at any time point in our culture system (Fig. 1). The corresponding testosterone-6β-hydroxylase activity was induced

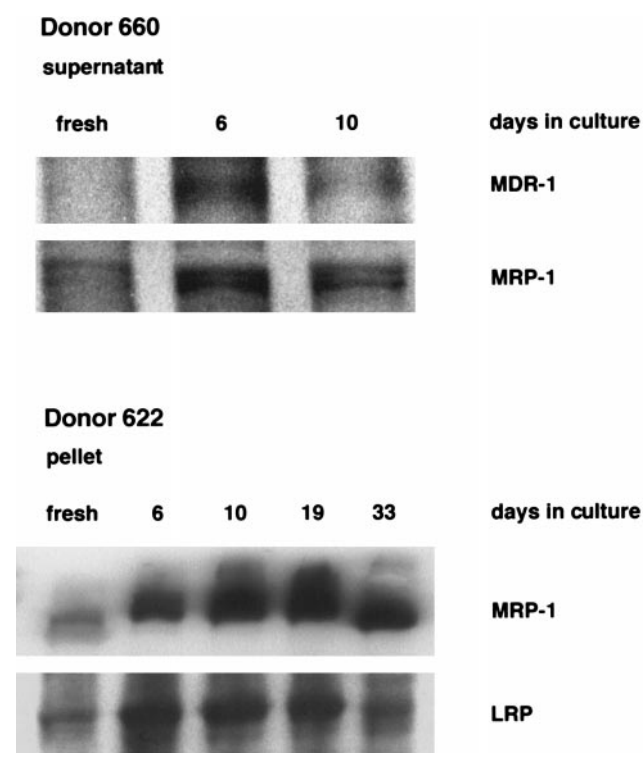

FIG. 6. Expression of MDR-1, MRP-1 and LRP in human hepatocytes. Hepatocytes from donors 622 and 660 were cultured serum-free in HHMM with HGF and EGF. On days indicated cells were harvested and whole cell extracts were prepared as described in Materials and Methods. Donor 660: supernatants of the 20 min $\times$ 10,000 rpm centrifugation were used to detect MDR-1 and MRP-1. Donor 622: Pellets of the 20 min $\times$ 10,000 rpm centrifugation were used to detect MRP-1 and LRP.

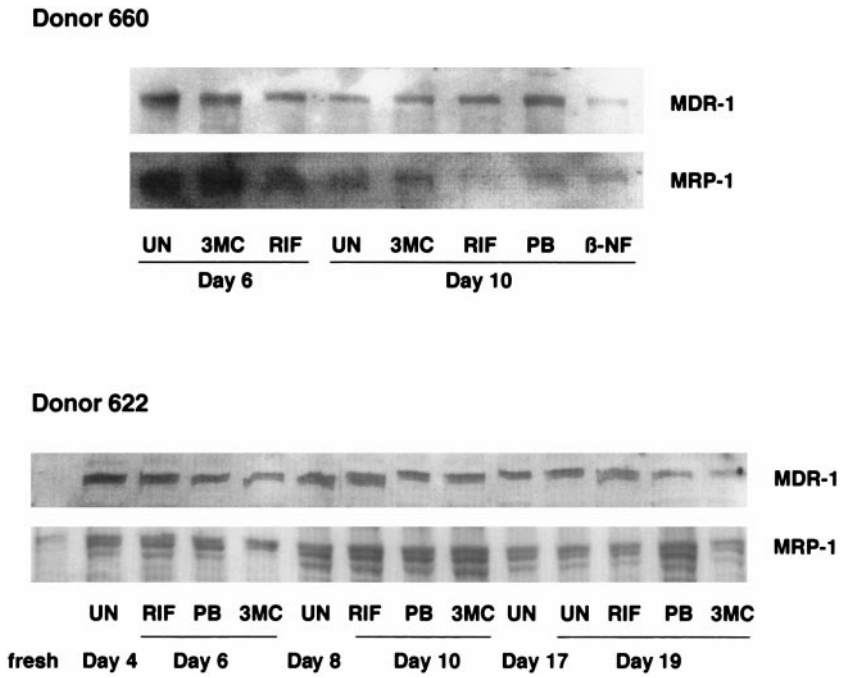

FIG. 7. Rifampicin, phenobarbital, 3-methylcholanthrene and β-naphtoflavone have little effect on MDR-1 and MRP-1 expression in human hepatocyte cultures. Hepatocytes form donors 622 and 660 were treated with 10 μM RIF, 2 mM PB, 2 μg/ml 3MC or 50 μM β-NF for 48 h before harvesting on indicated days. 50 μg of whole cell extracts were separated by SDS–PAGE, transferred onto Immobilon-membrane and probed with specific anti-sera as indicated.

about sevenfold by RIF and thereby correlated with the increase in protein in one case (donor 642). In a second experiment (donor 660) the protein amounts induced by RIF reached about the levels as in case 642, however, the inducible T6H activity was approximately tenfold lower than in donor 642 (Table 1). The observation that protein amounts can be induced to higher levels than enzymatic activities was also reported by other groups in hepatocyte cultures for CYP3A4 protein and T6H activity (14) and after partial hepatectomy (37).

Basal CYP1A1 and CYP1A2 expression was lost during prolonged culture. However, protein inducibility was retained for up to 30 days. Addition of 3MC led to induction of 1A2, and incubation in the presence of βNF to induction of 1A1 and 1A2. As seen for CYP3A4 the actual amount of protein induced by 3MC varied only slightly between earlier and later time points in the cultures (Fig. 2). The inducible enzymatic activity did not correlate with steady state protein levels and dropped during the culture in donor 642 (Table 2). However, there was a correlation between the decrease in β-NF induction of CYP1A1 and CYP1A2 proteins and the decrease in EROD activity in donor 660. A possible explanation for the differences observed in CYP1A and CYP3A protein levels and the corresponding enzyme activities is that cofactors, like the heme component, might be less stable and become degraded. Further studies will be necessary to elucidate these differences.

Proteins of the CYP2C subfamily are characterized by a general lack of inducibiliy by xenobiotics (38). In an uncontrolled study a small increase in CYP2C9 activity by RIF was reported in patients with cirrhosis and cholestasis (39). In our cultures these proteins were expressed, but RIF, PB, 3MC, and β-NF were unable to increase CYP2C9 and CYP2C19 protein levels significantly (data not shown), which is in line with the observed weak inducibility of these proteins by others (for review: 38).

Expression of Membrane Transport Proteins

MDR-1, MRP-1 and LRP are associated with multidrug resistance. LRP plays a role in rapid relocation of drugs from the nucleus to the cytoplasm (40, 41). In human liver MDR-1 expression is restricted to bile canaliculi, while MRP is present in canalicular and lateral membrane domains of hepatocytes (42–44). Our cultures express cytokeratin 19, which serves as a marker for bile ducts, furthermore, electron microscopy revealed that bile canaliculi are established and maintained throughout the culture (ref. 13, Fig. 4). In rat hepatocyte cultures 3MC is able to induce expression of CYP1A and MDR-1 mRNA and protein (45, 46). However, a previous report on MDR-1 regulation in human hepatocytes in response to treatment with car-

cinogens has shown that inter-individual differences exist with regards to MDR-1 mRNA levels. Aromatic hydrocarbons like 3MC were able to induce MDR mRNA expression in 60% of human donor cells, remaining unchanged in 40% of the donors. In this particular study only data on mRNA expression were obtained (47). In our study 3MC application did not change MDR-1 protein levels. This implies, that MDR-1 expression might be regulated differently in rats and humans, and, in addition, that in humans posttranscriptional regulatory mechanisms may play a role.

LRP, MDR-1, and MRP-1 were expressed in our cultures at relatively low levels, as detected by RT-PCR and Western blot analyses (Figs. 5 and 6). This is in line with a previous report, in which MRP expression was detected in canalicular and lateral membrane domains of hepatocytes (43) but somewhat in contrast to a more recent study where MRP-1 and LRP expression could be detected in various organs, but not in liver (44). The reasons for these discrepancies are not obvious. Our results suggest, that inter-individual or age related differences might exist in the expression of these proteins: hepatocytes of adult donors seem to have much lower levels of MDR-1 and MRP-1. However, the number of donors we have analyzed so far is very small. More experiments will be necessary to evaluate these preliminary observations. All prototypical inducers used in this study induced specific members of cytochrome P450 families but failed to significantly induce membrane transport proteins involved in export of drugs and xenobiotics (Figs. 1, 2, and 7). We summarize that the described culture system for human hepatocytes represents a suitable and stable system to evaluate the expression of cytochrome P450s, membrane transport proteins and drug metabolism. Since it utilizes a completely serum-free medium it should allow inducer screening in drug metabolism under chemically defined conditions. Furthermore, the establishing of long-term culture conditions should allow repeated treatment/wash-out cycles to screen for drug activity especially if well established noninvasive assays as for testosterone-6β-hydroxylase (CYP3A4), ethoxyresorufin-deethylase (CYP1A) or chlorzoxazone-6-OH-hydroxylase (CYP2E1) can be used to determine enzymatic activities.

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REFERENCES

1. Kocarek, T. A., Schuetz, E. G., Strom, S. C., Fisher, R. A., and Guzelian, P. S. (1995) *Drug Metab. Dispos.* **23,** 415–421.
2. Silva, J. M., Morin, P. E., Day, S. H., Kennedy, B. P. Payette, P., Rushmore, T., Yergey, Y. A., and Nicoll-Griffith, D. A. (1998) *Drug Metab. Dispos.* **26,** 490–496.
3. Moshage, H., and Yap, S. H. (1992) *J. Hepatol.* **15,** 404–413.
4. Li, A. P., Maurel, P., Gómez-Lechón, M. J., Cheng, L. C., and Jurima-Romet, M. (1997) *Chemico. Biol. Interact.* **107,** 5–16.
5. Kane, R. E., Li, A. E., and Kaminski, D. R. (1995) *Drug Metab. Dispos.* **23,** 303–307.
6. Kono, Y., Yang, S., and Roberts, E. A. (1997) *In Vitro Cell. Dev. Biol.* **33,** 467–472.
7. Donato, M. T., Gómez-Lechón, M. J., Jover, R., Nakamura, T., and Castell, J. V. (1998) *J. Pharmacol. Exp. Therapeutics* **284,** 760–767.
8. Ferrini, J. B., Pichard, L., Domergue, J., and Maurel, P. (1997) *Chemico.-Biol. Interactions* **107,** 31–45.
9. Langouët, S., Coles, B., Morel, F., Beaune P., Guengerich, F. P., Ketterer, B., and Guillouzo, A. (1995) *Cancer Res.* **55,** 5574–5579.
10. Chang, T. K. H., Yu, L., Maurel, P., and Waxman D. J. (1997) *Cancer Res.* **57,** 1946–1954.
11. Li, A. P., Reith, M. K., Rasmussen, A., Gorski, J. C., Hall, S. D., Xu, L., Kaminski, D. L., and Cheng, L. K. (1997) *Chemico-Biol. Interact.* **107,** 17–30.
12. Runge, D. M., Runge, D., Dorko, K., Pisarov, L. A., Leckel, K., Kostrubsky, V. E., Thomas, D., Strom, S. C., and Michalopoulos, G. K. (1999) *J. Hepatol.* **30,** 365–274.
13. Runge, D., Runge, D. M., Jäger, D., Lubecki, K. A., Beer Stolz, D., Karathanasis, S., Kietzmann, T., Strom, S. C., Jungermann K., Fleig, W. E., and Michalopoulos, G. K. (2000) *Biochem. Biophys. Res. Comm.* **269,** 46–53.
14. Liddle, C., Goodwin, B. J., George, J., Tapner, M., and Farrell, G. C. (1998) *J. Clin. Endocrin. Metab.* **83,** 2411–2416.
15. Shima, N., Tsuda, E., Goto, M., Yano, K., Hayasaka, H., Ueda, M., and Higashio K. (1994). *Biochem. Biophys. Res. Comm.* **200,** 808–815.
16. Strom, S. C., Dorko, K., Thompson, M. T., Pisarov, L. A., and Nüssler, A. K. (1998) *in* Îlots de Langerhans et Hépatocytes. Vers une Utilisation Therapeutique. (Franco, D., Boudjema, K., and Varet, B., Eds.), pp. 195–205, Les editions Inserm, Paris.
17. Runge, D., Runge, D. M., Bowen, W. C., Locker, J., and Michalopoulos, G. K. (1997) *Biol. Chem.* **378,** 873–881.
18. Lowry, O. N., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
19. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
20. Kostrubsky, V. E., Strom, S. C., Wood, S. G., Wrighton, S. A., Sinclair, P. R., and Sinclair J. F. (1995) *Arch. Biochem. Biophys.* **322,** 516–520.
21. Kostrubsky, V. E, Lewis, L. D., Strom, S. C., Wood, S. G., Schuetz, E. G., Schuetz, J. D., Sinclair, P. R., Wrighton, S. A., and Sinclair, J. F. (1998) *Arch. Biochem. Biophys.* **355,** 131–136.
22. Wrighton, S. A., Brian, W. R., Sari, A. A., Iwasaki, M., Guengerich, P. F., Raucy, J. L., Molowa, D. T., and Vandenbranden, M. (1990) *Mol. Pharmacol.* **38,** 207–213.
23. Noonan, K. E., Beck C., Holzmayer, T. A., Chin, J. E., Wunder, J. S., Andrulis, I. L., Gazdar, A. F., Willman, C. L., Griffith, B., von Hoff, D. D., and Roninson, I. B. (1990). *Proc. Natl. Acad. Sci USA* **87,** 7160–7164.
24. Barrand, M. A., Heppell-Parton, A. C., Wright, K. A., Rabbitts, P. H., and Twentyman, P. R. (1994) *J. Natl. Cancer Inst.* **86,** 110–117.
25. Stein, U., Walther W., Laurencot, C. M., Scheffer, G. L., Scheper R. J., and Shoemaker, R. H. (1997) *J. Natl. Cancer Inst.* **11,** 807–813.

26. Jantsch, B. (1997) Dissertation, University of Leipzig.
27. Donato, M. T., Gómez-Lechón, M. J., and Castell, J. V. (1993) *Anal. Biochem.* **213,** 29–33.
28. Lubinski, J., Flint, O. P., and Durham, S. K. (1994) *In vitro Toxicol.* **7,** 13–23.
29. Kostrubsky, V. E., Ramachandran, V., Venkataramanan, R., Dorko, K., Esplen, J. E., Zhang, S., Sinclair, J. F., Wrighton, S. A., and Strom, S. C. (1999) *Drug Metab. Dispos.* **27,** 887–894.
30. Pichard-Garcia, L., Hyland, R., Baulieu, J., Fabre, J. M., Milton, A., and Maurel, P. (2000) *Drug Metab. Dispos.* **28,** 51–57.
31. Kronbach, T., Fischer, V., and Meyer, U. A. (1988) *Clin. Pharmacol. Therapeutics* **43,** 630–635.
32. Kronbach, T., Mathys, D., Umeno, M., Gonzalez, F. J., and Meyer, U. A. (1989) *Mol. Pharmacol.* **36,** 89–96.
33. Watkins, P. B., Wrighton, S. A., Maurel, P., Schuetz, E. G., Mendez-Picon, G., Parker, G. A., and Guzelian, P. S. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 6310–6314.
34. Brian, W. R., Sari, M. A., Iwasaki, M., Shimada, T., Kaminsky, L. S., and Guengerich, F. P. (1990) *Biochemistry* **29,** 11280–11292.
35. Bargetzi, M. J., Aoyama, T., Gonzalez, F. J., and Meyer, U. A. (1989) *Clin. Pharm. Therapeutics* **46,** 521–752.
36. Guengerich, F. P., Brian, W. R., Iwasaki, M., Sari, M. A., Bäärnhielm, C., and Berntsson, P. (1991) *J. Med. Chem.* **34,** 1838–1844.
37. Trautwein, C., Rakemann, T., Obermayer-Straub, P., Niehof, M., and Manns, M. P. (1997) *J. Hepatol.* **261,** 48–54.
38. Ronis, M. J. J., and Ingelman-Sundberg, M. (1999) ISSX Short Course Manual, Budapest.
39. Zilly, W., Breimer, D. D., and Richter, E. (1977) *Eur. J Clin. Pharmacol.* **11,** 287–293.
40. Silverman, J. A., and Schrenk, D. (1997) *FASEB J.* **11,** 308–313.
41. Schuurhuis, G. J., Broxterman, H. J., de Lange, J. H., Pinedo, H. M., van Heijningen, T. H., Kuiper, C. M., Scheffer, G. L., Scheper, R. J., van Kalken, C. K., and Baak, J. P., (1991) *Br. J. Cancer* **64,** 857–861.
42. Cordon-Cardo, C., O'Brien, J. P., Boccia J., Casals, D., Bertino, J. R., and Melamed, M. R. (1990) *J. Histochem. Cytochem.* **38,** 1277–1287.
43. Mayer, R., Kartenbeck, J., Büchler, M., Jedlitschky, G., Leier, I., and Keppler, D. (1995) *J. Cell Biol.* **131,** 137–150.
44. Sugarawa, I., Akiyama, S., Scheper, R. J., and Itoyama, S. (1997) *Cancer Lett.* **112,** 23–31.
45. Gant, T. W., Silverman, J. A., Bisgaard, H. C., Burt, R. K., Marino, P. A., and Thorgeirsson, S. S. (1991) *Mol. Carcinogen.* **4,** 499–509.
46. Chieli, E., Sontoni-Rugio, E., Cervelli, F., Sabatini, A., Petrini, M., Romiti, N., Paolicchi, A., and Tongiani, R. (1994) *Carcinogenesis* **15,** 335–341.
47. Schuetz, E. G., Schuetz, J. D., Thompson, M. T., Fisher, R. A., Madariage, J. R, and Strom, S. C. (1995) *Mol. Carcinogen.* **12,** 61–65.