Cytochrome P450-Dependent Metabolism in HepaRG Cells Cultured in a Dynamic Three-Dimensional Bioreactor

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ABSTRACT:
Reliable and stable in vitro cellular systems maintaining specific liver functions important for drug metabolism and disposition are urgently needed in preclinical drug discovery and development research. The cell line HepaRG exhibits promising properties such as expression and function of drug-metabolizing enzymes and transporter proteins, which resemble those found in freshly isolated human hepatocytes. In this study, HepaRG cells were cultured up to 68 days in a three-dimensional multicompartment capillary membrane bioreactor, which enables high-density cell culture under dynamic conditions. The activity of drug-metabolizing cytochrome P450 (P450) enzymes was investigated by a cocktail of substrates for CYP1A1/2 (phenacetin), CYP2C9 (diclofenac), CYP2B6 (bupropion), and CYP3A4 (midazolam). The model P450 substrates, which were introduced to the bioreactor system mimicking in vivo bolus doses, showed stable metabolism over the entire experimental period of several weeks with the exception of bupropion hydroxylase, which increased over time. Ketoconazole treatment decreased the CYP3A4 activity by 69%, and rifampicin induced the CYP3A4- and CYP2B6-dependent activity 6-fold, which predicts well the magnitude of changes observed in vivo. Moreover, polarity of transporter expression and formation of tissue-like structures including bile canaliculi were demonstrated by immune histochemistry. The long-lasting bioreactor system using HepaRG cells thus provides a promising and stable liver-like in vitro model for continuous investigations of the hepatic kinetics of drugs and of drug-drug interactions, which well predict the situation in vivo in humans.

Introduction

Drug-metabolizing enzymes and drug transporters in the liver play a critical role in the clearance and in drug-drug interactions of many medicinal products. Freshly isolated human hepatocytes represent a good model of the liver because they are able to perform the full range of known in vivo drug biotransformation pathways and retain many of the uptake and efflux functions of the liver cells (De Bartolo et al., 2006).

However, the use of primary human hepatocytes has several drawbacks such as scarce and unpredictable availability, huge variation in cell functions, especially cytochrome P450 (P450) activities, as well as a variable response to P450 inducers (Luo et al., 2002; Madan et al., 2003; Abadie-Viollon et al., 2010). The large variation in cell functions can be explained in part by interdonor variability. However, the variable loss of liver-specific functions during cell preparation and in standard in vitro culture conditions over time may cause additional variation of hepatocyte performance not related to the normal interindividual differences found in a population (Rodríguez-Antona et al., 2002).

In vivo, the liver cells are located in a perfused organ where they are arranged in three-dimensional (3D) structures forming cell-cell contacts, which are important for the intracellular function and also for maintaining their specific polarity. It has been shown that porcine or human liver cells retain in vivo-like properties and are arranged in tissue-like structures including formation of biliary canaliculi networks and neo-sinusoids when cultured in a perfused 3D bioreactor (Zeilinger et al., 2004; Schmelzer et al., 2009). More importantly, liver-specific functions such as urea and albumin synthesis, glucose metabolism, and P450 activities were all maintained for at least 14 days.

ABBREVIATIONS: P450, cytochrome P450; 2D, two-dimensional; 3D, three-dimensional; AST, aspartate aminotransferase; CK19, cytokeratin 19; DMSO, dimethyl sulfoxide; IHC, immunohistochemical staining; LDH, lactate dehydrogenase; MDR, multidrug resistance; P-gp, P-glycoprotein.
days in this type of bioreactor (Zeilinger et al., 2002). The multicomartment bioreactor technology initially described by Gerlach et al. (1994) was developed as a large-scale 800-ml clinical bioartificial liver support system. The technology is based on the use of interwoven hollow fiber capillary membranes that provide independent, decentralized medium and gas supply to the cells located between the capillaries (Fig. 1). In this study, an analytical scale bioreactor with a cell compartment volume of 2 ml was used for drug metabolism studies.

HepaRG cells are an attractive alternative to primary human hepatocytes because they exhibit important functions for drug metabolism and disposition such as P450, UDP glucuronosyltransferase, and transporter activities (Aninat et al., 2006; Le Vee et al., 2006; Kanebratt and Andersson, 2008a; Hart et al., 2010). Recently, HepaRG cells in two-dimensional (2D) cultures were evaluated as a valuable in vitro model for prediction of P450 induction in vivo in humans (Kanebratt and Andersson, 2008b).

In the present study, HepaRG cells cultured in the analytical scale 3D bioreactor system were used to study the function of four major drug-metabolizing enzymes, CYP1A1/2, CYP2C9, CYP2B6, and CYP3A4. In particular, the long-term stability of P450 enzyme expression and function was assessed by repeating P450 activity studies over several weeks. Furthermore, the ability to induce and inhibit P450 activities by the well characterized model inducer rifampicin and the inhibitor ketoconazole was sequentially investigated in the same bioreactor culture. The results were also compared with published in vivo results to evaluate the predictive value of the observed effects.

Materials and Methods

Bioreactor Technology. The bioreactor technology is based on three independent interwoven capillary systems integrated into a polyurethane housing (Fig. 1). Two of the capillary systems serve for countercurrent medium perfusion, and a third set of capillaries serves for direct, decentralized gas exchange via diffusion. The cells reside in the cell compartment between the capillaries, which form a 3D scaffold. A port enables substance injection, mimicking bolus administration, and sampling from the recirculating medium. Fresh medium is continuously supplied from the medium bottle, and excess medium flows out into the outflow bottle (Fig. 1C). A detailed description of the bioreactor structure is given elsewhere (Schmelzer et al., 2009). In this study, a downscaled bioreactor with a cell compartment volume of 2 ml was used (Fig. 1B).

Proliferation and Differentiation of HepaRG Cells. The cells were first proliferated in 2D flasks before bioreactor inoculation. The enriched HepaRG medium (Biopredic International, Rennes, France) composed of Williams’ medium E with GlutaMAX-I (Invitrogen, Darmstadt, Germany), supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml bovine insulin, and 50 μM hydrocortisone hemisuccinate, was changed five times in 2 weeks during 2D culture. The cells were trypsinated, and 83 ± 40 million cells were inoculated into the cell compartment of the bioreactor (n = 3). The cells had a viability above 90% as determined by trypan blue staining. The proliferation phase was continued in the bioreactor for 1 to 2 weeks using enriched HepaRG medium (Biopredic International). The differentiation of the cells was induced by changing the recirculating medium to high-DMSO HepaRG medium (Biopredic International) composed of enriched medium with the addition of 2% DMSO. The cells were allowed to differentiate for 2 weeks, then the high-DMSO medium was washed out and changed back to enriched HepaRG medium (Fig. 2). Because the cells were both expanded and differentiated in the bioreactor, an initial long lead time was needed before the first experiments could be performed. However, we are currently designing a bioreactor model in which fully differentiated cryopreserved HepaRG cells are inoculated into the bioreactor, which will significantly shorten the lead time.

Immunostaining. Samples from cell culture material were taken from different locations within the bioreactor capillary network upon culture termination. After fixation in 5% paraformaldehyde, the material was dehydrated, embedded in paraffin, and cut into approximately 5-μm sections. The immunohistochemical staining (IHC) was performed on the staining module Discovery XT (Ventana Medical Systems Inc., Tucson, AZ). Antibodies in use were MDR1 [P-glycoprotein (P-gp), clone F4; Sigma-Aldrich, St. Louis, MO], multidrug resistance-associated protein 2 (MRP2; clone M2 III-6; Abcam, Cambridge, UK), and CYP3A4 (Cypex, Scotland, UK). All solutions for deparaffinization, pretreatment, detection, counterstaining, and rinsing steps were supplied by Ventana Medical Systems Inc. Heat (20 min for MDR1 in a citrate buffer, pH 6, or 40 min for MRP2 in a Tris/borate/EDTA buffer, pH 8, at 98°C) or enzyme (protease, 8 min at 37°C, for CYP3A4) was used as antigen-retrieval pretreatment. Both primary and secondary antibodies were diluted in Discovery Ab Diluent. The IHC was visualized with diaminobenzidine chromogen, and the counterstaining was performed with hematoxylin.

For immunofluorescence staining of cytokeratin 19 (CK19), sections were deparaffinized with xylene and rehydrated with a series of decreasing concentrations of alcohol. Antigens were retrieved by boiling sections for 25 min in citrate buffer (0.01 citric acid monohydrate, pH 6.0; Merck, Darmstadt, Germany) followed by incubation for 20 min in 5% Triton/phosphate-buffered saline. Sections were blocked with 5% skimmed milk followed by incubation with monoclonal mouse anti-CK19 IgG1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 30 min. Cy2-conjugated polyclonal goat anti-mouse IgG (Dianova, Hamburg, Germany) was used as the secondary antibody. For nonspecific staining of the nuclei, sections were incubated with 4',6'-diamidino-2-phenylindole (Molecular Probes, Carlsbad, CA). Subsequently, the sections were mounted with Aqua Polymount solution (Polysciences Inc., Warrington, PA).

Determination of Chemical Cytometry Parameters. Biochemical parameters related to the cell integrity and to the synthesis capability of HepaRG cells were measured daily in samples from the culture perfusate and/or the medium outflow. Concentrations of lactate were determined by means of an automated cell culture analyzer (Bioprofile analyzer 100plus; IUL Instruments GmbH, Königswinter, Germany). Activities of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured with an automated clinical chemistry analyzer (Modular P800; Roche Diagnostics, Mannheim, Germany) using the corresponding assay kits from Roche/Cobas). Human albumin was
perfusion, but without feeding medium into the perfusion circuit (closed-system perfusion). Samples were taken 3 days after DMSO removal. P450 activities were assessed by adding marker substrates to the bioreactor medium and measuring metabolite formation in the perfusate over 6 h. The recirculating medium was washed out after each P450 activity experiment. The effects of ketoconazole and rifampicin on P450-dependent activities were sequentially investigated. The last P450 activity experiment (P450 activity 6) was performed 2 to 17 days after assessing the effect of rifampicin treatment on the P450 activities (P450 activity 5).

P450 Activity Measurements. P450 activity was assessed by adding a P450 cocktail through the sampling port close to the bubble trap (Fig. 1C). The medium in the bubble trap was mixed a few times before starting the recirculation of the medium (t = 0 h), allowing the substrate to reach the cells in the bioreactor. The P450 cocktail consisted of phenacetin (CYP1A1/2), midazolam (CYP3A4), diclofenac (CYP2C9), and bupropion (CYP2B6). The metabolites were measured in the medium over a period of 6 h. The parent drugs phenacetin and diclofenac (both from Sigma-Aldrich, Deisenhofen, Germany) were prepared as 20 mM stock solutions in DMSO and diluted in culture medium to achieve final concentrations of 26 and 9 μM in the bioreactor circuit. Midazolam was provided as an aqueous solution (Dormicum; Roche Pharma, Grenzach-Wyhlen, Germany) at 13.8 mM (5 mg/ml) and diluted to a final concentration of 3 μM. Bupropion (Toronto Research Chemicals Inc., Toronto, ON, Canada) was prepared as a 50 mM aqueous stock solution and diluted to a final concentration of 100 μM.

Analysis of the P450 metabolism was performed at continuous medium perfusion, but without feeding medium into the perfusion circuit (closed-system perfusion). Samples (200 μl) were taken at 0, 0.25, 0.5, 1, 2, 4, and 6 h. After each experiment, the bioreactor circuit was rinsed with 75 ml of culture medium (single-pass perfusion) and was then reset to the standard operation mode with continuous medium feed (open-system perfusion). Samples were frozen until further processing. The activities were measured as an increase in metabolite concentration in the medium. No attempts were made to relate the activities to the total amount of protein or number of cells, because this is difficult to assess in the bioreactor. However, the effects on P450 activities by ketoconazole and rifampicin are presented as relative changes in the bioreactors containing differentiated cells; thus, the absolute amount of proteins or cells is not important for interpretation of the results.

Analysis of Marker Substrates and Metabolites. 1'-Hydroxymidazolam, paracetamol, 4'-hydroxydiclofenac, and hydroxybupropion were analyzed using liquid chromatography/mass spectrometry. The liquid chromatography system consisted of an H TS PAL injector (CTC Analytics, Zwingen, Switzerland) combined with an HP 1100 LC binary pump and column oven (Agilent Technologies Deutschland, Waldbronn, Germany). The separation was performed on a reversed-phase Hypurity C18 analytical column (50 × 2.1 mm, 5 μm; Thermo Fisher Scientific, Waltham, MA) with a Hypurity C18 precolumn at 40°C and a flow rate of 750 μl/min. The mobile phases consisted of 0.1% (v/v) formic acid in 5% acetonitrile (A) and 0.1% (v/v) formic acid in 95% acetonitrile (B). The organic modifier content B was increased linearly from 2 to 40% over 2 min and from 40 to 98% over 1.4 min. Then, it was maintained at 98% for 0.5 min and decreased to 2% in 0.1 min. Detection was performed with a triple quadrupole mass spectrometer, API4000, equipped with an electrospray interface (Applied Biosystems/MDS Sciex, Concord, ON, Canada). The mass spectrometry parameters were optimized using each analyte. The compound-dependent parameters were as follows: the collision energy was set at 45, 20, 20, and −15 V for 1'-hydroxymidazolam, paracetamol, hydroxybupropion, and 4'-hydroxydiclofenac, respectively. The collision-activated dissociated gas was 10 for 1'-hydroxymidazolam, paracetamol, and hydroxybupropion and 7 for 4'-hydroxydiclofenac. The transitions chosen were 256.1 > 238 for hydroxybupropion, 309.9 > 265.8 for 4'-hydroxydiclofenac, 342.1 > 203.0 for 1'-hydroxymidazolam, and 152.1 > 109.9 for paracetamol. A dwell time of 100 ms was used, and the limit of quantification was 5 nM. Instrument control, data acquisition, and data evaluation were performed using Analyst 1.4 software (Applied Biosystems/MDS Sciex).
Calculations. Linear regression was used to describe data for 4′-hydroxy-diclofenac, 1′-hydroxymidazolam, paracetamol, and hydroxybupropion (see Figs. 6–8) using GraphPad Prisma 4.0.3 (GraphPad Software, Inc., La Jolla, CA). The data for 1′-hydroxymidazolam after rifampicin treatment were described by nonlinear regression using a polynomial second-order equation (see Fig. 8).

Formation rates of the metabolites were calculated over the 6-h experiment for paracetamol, 1′-hydroxymidazolam, 4′-hydroxydiclofenac, and hydroxybupropion using the slope of the amount (nanomoles) versus time (hours) graph. Only the first 2 h of the experiment were used to calculate the formation rate of 1′-hydroxymidazolam after rifampicin treatment (GraphPad Prism 4.0.3). The change in enzyme activities by rifampicin treatment was calculated by dividing formation rate values after rifampicin treatment by the corresponding values before rifampicin treatment.

Statistical Analysis. The possible significance of the effect of rifampicin or ketoconazole on the formation rate values of 1′-hydroxymidazolam or hydroxybupropion was calculated using the two-tailed Student’s t test. The same test was used to calculate the significance of differences between the first and last P450 activity experiment.

Results

Immunostaining of CYP3A4, P-gp, MRP2, and CK19. Cell material was removed from the bioreactor cell compartment after the P450 activity measurements and used for staining of P-gp (ABCB1), MRP2 (ABCC2), CYP3A4, and CK19 by monoclonal antibodies.

Histological pictures showed the formation of tissue-like cell aggregates between the capillaries (Fig. 3). As indicated in Fig. 3, CYP3A4 and the apical efflux transporters P-gp and MRP2 were all present in the bioreactor tissue. CYP3A4 was found to be expressed in the hepatocyte-like cells, and both P-gp and MRP2 were located to one side of the hepatocyte-like cells forming bile canaliculi-like structures. The biliary marker CK19 was detected in strand-like structures within the cell aggregates as shown in Fig. 4.

Clinical Chemistry Parameters. Clinical chemistry analyses of the medium perfusate were performed daily during the culture period. The mean values (n = 3) of the daily medium values are shown for the proliferation, differentiation, and experimental phases in Fig. 5. Lactate production significantly (p < 0.01) decreased by 34% during the differentiation phase compared with the proliferation phase, whereas no significant change was observed in albumin production (Fig. 5A). The lactate and albumin production in the bioreactor inoculated with HepaRG...
cells was comparable to what has been observed in bioreactors inoculated with human hepatocytes (Zeilinger et al., 2011). The release of LDH and AST was low and stable over the whole culture period (Fig. 5B).

**Basal Levels and the Effect of Ketoconazole and Rifampicin on P450 Activities.** The CYP2C9-, CYP1A1/2-, or CYP3A4-mediated activities, calculated as the formation rates of the metabolites over the 6-h experiment, showed no difference between the first (Fig. 2, P450 activity 1) and the last (Fig. 2, P450 activity 6) P450 experiment (Fig. 6, A–C). However, the rate of CYP2B6-dependent hydroxybupropion formation increased 3.6-fold between the first and last experiment (Fig. 6D).

Ketoconazole and rifampicin were introduced into the bioreactor as depicted in Fig. 2. The effects of ketoconazole and rifampicin on P450 activities were compared with the activities measured directly before introducing the interacting compounds. Ketoconazole was introduced into the system, and the direct effects on the P450-mediated activities were investigated. The 1'-hydroxymidazolam formation rate decreased by 69% \((p < 0.05)\) (Table 1; Fig. 7) upon ketoconazole treatment, whereas the CYP2C9-, CYP2B6-, and CYP1A1/A2-dependent activities were not affected (data not shown).

The cell culture was exposed to rifampicin for 60 h and washed out for 12 h before investigating P450 activities to avoid direct interaction of rifampicin with enzyme activity measurements. The CYP3A4-dependent 1'-hydroxymidazolam formation increased 6-fold upon rifampicin treatment \((p \leq 0.05)\) (Table 2; Fig. 8A). Furthermore, CYP2B6-dependent bupropion hydroxylase activity was 6-fold higher after rifampicin treatment \((p < 0.01)\) (Table 2; Fig. 8B), whereas phenacetin O-dealkylase (CYP1A1/2) and diclofenac 4'-hydroxylase (CYP2C9) activities were unaffected by rifampicin (data not shown).

**Discussion**

HepaRG cells represent a highly differentiated cell line that exhibits several important human liver functions such as drug metabolism and drug transporter activities (Aninat et al., 2006; Le Vee et al., 2006). In the present study, HepaRG cells were cultured in a dynamic 3D bioreactor system to evaluate the suitability of this experimental set up as a human hepatic in vitro model for drug metabolism and disposition studies.

Confluent HepaRG cells are known to differentiate into both hepatocyte-like and biliary epithelial-like cells (Aninat et al., 2006; Guilhouzo et al., 2007). Both cell types were present in the bioreactor, and the proportion between the two cell types was presumed to remain constant in the differentiated state when experiments were performed. The relative effects on P450 activities caused by ketoconazole and rifampicin that were evaluated in this study could thus reliably be calculated and compared with effects reported in other in vitro and in vivo studies. The hepatocyte-like cells were polarized as revealed by immunostaining of the transporter proteins P-gp and MRP2, which were located on one side of the cell. The P-gp- and MRP2-positive sides of the cells were also facing each other, which resembles the situation in vivo where these transporters are located at the apical membranes of hepatocytes forming bile canaliculi-like structures. Strand-like formations of CK19-positive cells indicate the formation of biliary structures. Thus, the HepaRG cells in the bioreactor seem to develop structures that resemble liver tissue in vivo. Studies are ongoing to characterize in more detail the biliary structures and the function of transporters in the bioreactor.

The stable albumin production observed over the whole culture period further implies a liver-like function of HepaRG cells when cultured in the bioreactor. Lactate production decreased significantly during differentiation, which can be explained by decreasing glucose metabolism due to a decrease in proliferation associated with ongoing differentiation. Furthermore, the albumin and lactate production were at the same level as observed for primary human hepatocyte cultured in the same bioreactor prototype (Zeilinger et al., 2011). The stable
The effect of ketoconazole on the 1'−hydroxymidazolam (CYP3A4) pharmacokinetics in HepaRG cells cultured in the bioreactor

The 1'−hydroxymidazolam pharmacokinetics in cells during ketoconazole exposure (P450 activity 3 in Fig. 2) are compared with the pharmacokinetics directly before (P450 activity 2 in Fig. 2) introducing ketoconazole into the system. The results are obtained from three different bioreactors. Published results on the effect of ketoconazole on midazolam pharmacokinetics in vivo are also shown.

<table>
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<tr>
<th>HepaRG bioreactor</th>
<th>Without Ketoconazole</th>
<th>With Ketoconazole</th>
<th>Change</th>
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<td>Formation rate1'−hydroxymidazolam (nmol/h)</td>
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<td>CLmidazolam (l/h)</td>
<td>26 ± 12</td>
<td>4.5 ± 1.8</td>
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*p < 0.05.

Data are mean ± S.D.; n = 3.

* Data are from Tham et al. (2006).

and low release of LDH and AST from cells indicates that no significant cell damage occurred during bioreactor cultivation. CYP1A1/2-, CYP2C9-, and CYP3A4-mediated activities were stable for several weeks in the bioreactor culture of differentiated cells, which is important for the performance of long-term studies on drug metabolism. CYP2B6 even showed an increased activity over time in the bioreactor. The reason for this observation should be studied further.

It is known that nonspecific binding of substances to material in different types of bioreactor systems (including conventional 2D systems) can be a problem (Toepke and Beebe, 2006; Chao et al., 2009). The nonspecific binding properties of the substances used in the present study were not investigated. However, the initial metabolite formation was linear, thus the substrate concentrations are not likely to be rate limiting for enzyme activities. In addition, the magnitude of induction by rifampicin and inhibition by ketoconazole seen in this study were comparable with HepaRG and hepatocytes cultured in two dimensions and with in vivo studies, as discussed below, indicating sufficient access to the substrates used.

Inhibition of P450 enzymes is the most common cause of drug–drug interactions in vivo. In this study, ketoconazole was used, which is a commonly used and well characterized strong CYP3A inhibitor in humans both in vitro and in vivo (Turpeinen et al., 2009). Midazolam is a sensitive CYP3A probe for CYP3A activity in vivo. In humans, the clearance of midazolam has been reported to be reduced by approximately 85% by ketoconazole (Tsunoda et al., 1999; Tham et al., 2006; Yong et al., 2008; Krishna et al., 2009), which is comparable with the 69% decrease in the formation rate of 1'−hydroxymidazolam seen in the bioreactor in this study using HepaRG cells. A comparable degree of inhibition (83−100%) of the 1'−hydroxymidazolam formation rate by 10 μM ketoconazole has also been reported in 2D-cultured primary human hepatocytes from 18 donors (Klieber et al., 2008). CYP2C9-dependent inhibition by ketoconazole was not recorded in this experiment. However, it has been shown that the potency by which CYP2C9 is inhibited by ketoconazole is approximately 10-fold lower than for CYP3A4 (Kumar et al., 2006). Thus, the ketoconazole concentration may be too low to significantly inhibit CYP2C9-dependent diclofenac hydroxylation in the bioreactor.

Induction of CYP3A in vivo is often measured as an increase in midazolam clearance. When midazolam was administered intravenously, the average induction of midazolam clearance by rifampicin was reported to be 2-fold (Floyd et al., 2003; Gorski et al., 2004; Kharasch et al., 2004; Yu et al., 2004). In the study by Gorski et al. (2004), the range of induction of midazolam clearance by rifampicin was reported to be between 1.4- and 7.4-fold. In this study, the rifampicin induction of CYP3A4 activity was 6 ± 4-fold, when measured as the formation rate of 1'−hydroxymidazolam. The fold induction in HepaRG cells cultured in the bioreactor is in the same range as that seen in vivo and also close to the 8 ± 3-fold induction seen for CYP3A4 activity by rifampicin in 2D-plated HepaRG cells (Kanebratt and Andersson, 2008b). In primary human hepatocytes, the effect of rifampicin on CYP3A4 can vary considerably. Abadie-Violon et al. (2010) reported a range of induction by rifampicin between 2- and 19-fold in hepatocytes from 21 donors. Thus, the range of CYP3A induction responses in primary human hepatocytes from different subjects by rifampicin is larger than the variation reported in vivo measured as midazolam clearance. The larger variation in induction response observed in human hepatocytes may be caused by the erratic loss of liver-specific functions in culture of human hepatocytes resulting in low basal levels of CYP3A4 enzymes before exposing the cells to potential inducers (Luo et al., 2002). However, the induction response of CYP3A activity by rifampicin measured in microsomes prepared from sandwich-cultured human hepatocytes from six different donors was close to the range seen in vivo (Luo et al., 2002; Gorski et al., 2004).

The 6 ± 2-fold increase (n = 3) in hydroxypyrimidolophen formation rates by rifampicin pretreatment in the bioreactor is consistent with the 3- to 5-fold increase (n = 10) in hydroxypyrimidolophen formation rates observed in humans in vivo (Kharasch et al., 2008) as well as with the 3- to 3.4-fold increased (n = 16) clearance of bupropion in vivo (Lobo et al., 2006). This result is also comparable with the 6 ± 2-fold CYP2B6 activity increase in 2D-cultured HepaRG cells (Kanebratt and Andersson, 2008b). In cultured primary human hepatocytes, however, a large variation in induction of bupropion hydroxylation activity by rifampicin has been reported. Abadie-Violon et al. (2010) described a 0- to 21-fold induction by rifampicin using cells from 22 donors. Thus, the magnitude of induction in vivo seems to be better reflected by HepaRG cells compared with primary human hepatocytes.

CYP2C9 was not significantly induced by rifampicin in this study. CYP2C9 is known to show a weaker induction response in vivo to rifampicin treatment than CYP3A (Kanebratt and Andersson, 2008b). In primary human hepatocytes, the effect of rifampicin on CYP3A4 can vary considerably. A considerably weaker induction response of CYP2C9 compared with CYP3A4 by rifampicin also has been reported in studies in cryopreserved human hepatocytes (Fahmi et al., 2010).

In conclusion, the bioreactor using HepaRG cells showed stable functions over several weeks, which allows the performance of long-term sequential studies using the same cell culture system. HepaRG cells aggregated in the bioreactor and formed tissue-like structures including bile canaliculi. Polarity of transporter protein expression similar to those seen in vivo was observed, which facilitates the formation of a bile-like environment.
The effect of rifampicin on 1'-hydroxymidazolam (CYP3A4) and hydroxybupropion (CYP2B6) pharmacokinetics in HepaRG cells cultured in the bioreactor

The 1'-hydroxymidazolam and hydroxybupropion pharmacokinetics in cells treated with rifampicin (P450 activity 5 in Fig. 2) are compared with the pharmacokinetics directly before (P450 activity 4 in Fig. 2) introducing rifampicin into the system. The results are obtained from three different bioreactors. Published results on the effect of rifampicin in vivo on midazolam and hydroxybupropion pharmacokinetics are also shown.

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**AUTHORSHIP CONTRIBUTIONS**

**Conceived and designed the experiments:** Andersson, Darnell, Schreiter, Zeilinger, Gerlach, and Berg.

**Performed the experiments:** Schreiter, Darnell, Urbaniak, Dillner, and Söderdahl.

**Contributed new reagents or analytic tools:** Gerlach.

**Wrote or contributed to the writing of the manuscript:** Andersson, Darnell, Schreiter, Dillner, and Urbaniak.

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**TABLE 2**

<table>
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<th>After Rifampicin Treatment</th>
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<td>Formation rate, 1'-hydroxymidazolam (nmol/h)</td>
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<td>Cl&lt;sub&gt;midazolam&lt;/sub&gt; (l/h · 70 kg)</td>
<td>33 ± 10</td>
<td>62 ± 36</td>
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<td><strong>CYP2B6</strong></td>
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<td>Formation rate, hydroxybupropion (nmol/h)</td>
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<td>0.38 ± 0.085**</td>
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<td>In vivo</td>
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<td>Formation Cl&lt;sub&gt;hydroxybupropion&lt;/sub&gt; (ml/min)</td>
<td>62 ± 47</td>
<td>249 ± 189</td>
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* p ≤ 0.05; ** p < 0.01.

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**FIG. 8.** The midazolam 1'-hydroxylase (CYP3A4) activity (A) and bupropion hydroxylase (CYP2B6) activity (B) in bioreactor cultured HepaRG cells before (solid line) and directly after (dotted line) rifampicin treatment, which are P450 activities 4 and 5, respectively, illustrated in Fig. 2. Results are given as means ± S.D. (n = 3 bioreactors).
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