In Vitro Evaluation of Major In Vivo Drug Metabolic Pathways Using Primary Human Hepatocytes and HepaRG Cells in Suspension and a Dynamic Three-Dimensional Bioreactor System

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Received May 10, 2012; accepted July 6, 2012

ABSTRACT

Major human specific metabolites, not detected during in vivo and in vitro preclinical studies, may cause unexpected drug interactions and toxicity in human and delays in clinical programs. Thus, reliable preclinical tools for the detection of major human metabolites are of high importance. The aim of this study was to compare major drug metabolic pathways in HepaRG cells, a human hepatoma cell line, to fresh human hepatocytes, cryopreserved human hepatocytes, and human in vivo data. Furthermore, the maintenance of cytochrome P450 (P450) and UDP-glucuronosyltransferase (UGT) activities in a dynamic three-dimensional (3D) bioreactor were evaluated over time by using HepaRG cells and human hepatocytes. 14C-diclofenac and a candidate from AstraZeneca’s drug development program, 14C-AZD6610, which are metabolized by P450 and UGT in vivo, were used as model substrates. The proportion of relevant biotransformation pathways of the investigated drug was clearly different in the various cell systems. The hydroxylation route was favored in primary human hepatocytes, whereas the glucuronidation route was favored in HepaRG cells. The human in vivo metabolite profile of AZD6610 was best represented by human hepatocytes, whereas all major diclofenac metabolites were detected in HepaRG cells. Moreover, the metabolite profiles in cryopreserved and fresh human hepatocytes were essentially the same. The liver bioreactor using both fresh human hepatocytes and HepaRG cells retained biotransformation capacity over 1 week. Thus, the incubation time can be increased from a few hours in suspension to several days in 3D cultures, which opens up for detection of metabolites from slowly metabolized drugs.

Introduction

Metabolism in the liver is one of the important determinants of the overall disposition of drugs, and metabolites formed can have an impact on efficacy and safety in humans. Comparisons of in vitro metabolite profiles across species can provide an early signal if a new drug candidate could have a different major metabolic pathway in humans than in the animal species used for safety evaluations. Thus, liver in vitro systems from which reliable predictions of major human in vivo metabolic pathways can be made are highly desired. Freshly isolated human hepatocytes represent a good in vitro liver model because they are able to perform the full range of in vivo hepatic drug biotransformation pathways and retain many of the uptake and efflux functions of liver cells (De Bartolo et al., 2006). However, the use of fresh human hepatocytes has several drawbacks, such as scarce and unpredictable availability, interdonor variability, and significant variation in cell functions, especially cytochrome P450 (P450) activities (Luo et al., 2002; Ohtsuki et al., 2012; this study was in part supported by SCR&Tox, which is funded by the European Commission within its seventh framework Programme and Cosmetics Europe, the European Cosmetics Association, as part of the SEURAT (Toward the replacement of in vivo repeated dose system toxicity) Cluster [Contract HEALTH-F5–2010-266573].

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Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

http://dx.doi.org/10.1124/jpet.112.195834.

[The online version of this article (available at http://jpet.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: P450, cytochrome P450; 2D, two-dimensional; 3D, three-dimensional; ACN, acetonitrile; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; HLM, human liver microsomes; LC, liquid chromatography; PCR, polymerase chain reaction; RAM, radioactivity monitoring; UGT, UDP-glucuronosyltransferase.
Rogue et al., 2012; Schaefer et al., 2012). Today, pooled cryopreserved human hepatocytes from several donors are used routinely in drug discovery and development to overcome the inconsistencies associated with fresh human hepatocytes. However, both fresh hepatocytes and cryopreserved hepatocytes suffer a rapid loss of functions over time in culture, and formation of metabolites from slowly metabolized drugs may be undetectable in such systems (Rodríguez-Antona et al., 2002; Wang et al., 2010). The loss of liver-specific functions in freshly isolated cells may partly be explained by the rupture of the three-dimensional (3D) structure of the tissue.

To mimic the situation in the liver, fresh human hepatocytes can be cultured in bioreactors, which enables a 3D structure, cell-cell contact, and a constant medium flow and oxygen supply that are important for the intracellular functions and maintenance of the cell polarity (Tilles et al., 2001; Zeilinger et al., 2004; Schmelzer et al., 2009; Vinci et al., 2011). It has been shown previously that fresh human hepatocytes can maintain their liver-specific functions such as urea and albumin synthesis, glucose metabolism, and P450 activities for at least 14 days in 3D cultures (Zeilinger et al., 2002, 2011).

The human hepatoma cell line HepaRG is recognized as an acceptable alternative to human hepatocytes because the cells maintain P450 activities in both 2D and 3D culture for several weeks (Jossé et al., 2008; Anthériéu et al., 2010; Darnell et al., 2011). In addition, HepaRG cells have been documented to provide reliable prediction of P450 drug induction and drug clearance in vivo in humans (Kanebratt and Andersson, 2008a; Zanelli et al., 2012). However, UDP-glucuronosyltransferase (UGT) phase II enzyme activity and detailed studies of metabolite profiles of drugs in HepaRG cells need further investigation (Aninat et al., 2006; Jossé et al., 2008; Anthériéu et al., 2010).

In the present study, the major metabolic pathways of diclofenac and AZD6610, a candidate peroxisome proliferator-activated receptor α/γ agonist with an unusual and unknown enzymatic hydroxylation pathway, were evaluated in five different human hepatic in vitro systems. The compounds undergo both phase I and phase II metabolism in vivo in humans. P450 enzymes are often involved in phase I reactions, whereas UGTs catalyze glucuronidation, which is the most important phase II reaction. The compounds were incubated in suspensions of cryopreserved human hepatocytes, fresh human hepatocytes, and cryopreserved differentiated HepaRG cells, and the major metabolic pathways were compared with clinical in vivo results. In addition, the maintenance of P450 and UGT activities over time in HepaRG cells and fresh human hepatocytes cultured in 3D hollow fiber bioreactors were evaluated by using these two model substances.

### Materials and Methods

**Chemicals and Reagents.**[^1] 14C-diclofenac, 14C-AZD6610, and the authentic standard M6 were provided by AstraZeneca R&D Mölndal (Mölndal, Sweden). The specific radioactivity was 2.68 kBq/nmol for 14C-diclofenac and 2.06 kBq/nmol for 14C-AZD6610, and the compounds were used as received. Ultra-pure water was obtained from a water purification system (Elgastat Maxima, Elga, Chicago, IL), and acetonitrile (ACN) was of liquid chromatography (LC)/mass spectrometry (MS) grade (Rathburn Chemical Ltd, Walkerburn, Scotland). Formic acid was purchased from Riedel-de Haën AG (Seelze, Germany), and phosphate-buffered saline, L-glutamine, and Williams Medium E were obtained from Sigma-Aldrich (St. Louis, MO).

**Hepatocyte Isolation.** All tissues were obtained from Karolinska University Hospital (Huddinge, Sweden) by qualified medical staff, with donor-informed consent following ethical and institutional guidelines. Liver tissues from three patients (donors 1–3) undergoing liver resection caused by metastatic colon or liver cancer were used in this study. Donor information regarding age, sex, disease, and cell viability is summarized in Table 1. Residual tissue not needed for diagnostic purposes was transported to the laboratory from the surgeries in cold Eagle’s minimum essential medium (Lonza Walkerville, Inc., Walkersville, MD) within 30 min after removal. Tissue dissociation and hepatocyte isolation were performed by using a two-step collagenase perfusion procedure originally developed by Seglen (1976 and modified as described previously (Gramignoli et al., 2011). The isolated fresh human hepatocytes were transported from Karolinska University Hospital to AstraZeneca R&D Mölndal, in a cold package within the same day. Immediately upon arrival, the cells were centrifuged for 3 min at 100g, and the supernatant was removed. The cells were washed with 45 ml of Williams Medium E without phenol red supplemented with 25 mM HEPES and 2 mM L-glutamine, pH adjusted to 7.4 (modified Williams Medium E) and centrifuged for 3 min at 100g. The cells were counted by using a Bürker chamber (Danlab, Helsinki, Finland), and the trypan blue exclusion test was used to calculate cell viability.

**Thawing of Cryopreserved Human Hepatocytes and HepaRG Cells.** Cryopreservation of human hepatocytes was conducted at Celsis In Vitro Technologies (Brussels, Belgium) by using a controlled freezing protocol according to in-house procedures. Pooled cryopreserved human hepatocytes from three different batches (IRK, UMJ, and PHL), each containing hepatocytes from 10 donors, were used in the experiments. Immediately before experiments in suspension, cryopreserved human hepatocytes were rapidly thawed by gently shaking the vial in a 37°C water bath. The content was transferred to a 50-ml tube containing prewarming (37°C) modified Williams Medium E. The cryogenic tubes were rinsed twice, and the 50-ml tube was centrifuged at 60g for 5 min. After removal of the supernatant, the pellet was resuspended in 2 to 3 ml of buffer.

HepaRG cells were differentiated and cryopreserved at Biopredic International (Rennes, France), and three different batches (HPRI16046, HPRI16048, and HPRI16070) of cryopreserved HepaRG cells were used in the experiments. Immediately before experiments in suspension or inoculation of cells into the bioreactor, HepaRG cells were rapidly thawed by gently shaking the vial in a 37°C water bath. The content was transferred to a 50-ml tube containing prewarming (37°C) basal hepatic cell medium MIL600 supplemented with ADD670 according to the manufacturer’s instructions (Biopredic International). The cryogenic tubes were rinsed twice. The 50-ml tube was centrifuged at 360g for 2 min. After removal of the supernatant, the pellet was resuspended in 2 to 3 ml of basal hepatic cell medium MIL600 supplemented with serum-free ADD650 according to the manufacturer’s instructions (Biopredic International). These reagents are part of a commercially available assay that anyone can use to replicate the work.

Cryopreserved human hepatocytes and HepaRG cells were counted by using a Bürker chamber, and the trypan blue exclusion test was used to determine cell viability. The cell suspensions

### Table 1

<table>
<thead>
<tr>
<th>Donor ID</th>
<th>Age</th>
<th>Sex</th>
<th>Disease</th>
<th>Cell Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>81</td>
<td>Female</td>
<td>Hepatocellular carcinoma</td>
<td>76</td>
</tr>
<tr>
<td>Donor 2</td>
<td>83</td>
<td>Female</td>
<td>Metastasis (colon cancer)</td>
<td>75</td>
</tr>
<tr>
<td>Donor 3</td>
<td>41</td>
<td>Female</td>
<td>Metastasis (rectal cancer)</td>
<td>70</td>
</tr>
</tbody>
</table>

[^1]: In Vivo Drug Metabolic Pathways in Hepatic In Vitro Systems 135
were diluted with buffer to the required concentration and used immediately.

Identification of P450 and UGT Isoenzymes Responsible for the Metabolism of AZD6610. AZD6610 was incubated with human liver microsomes, human recombinant CYP2J2 expressed in Escherichia coli (Cypex Ltd, Dundee, UK), and human recombinant CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP4A11, CYP4F2, CYP4F12, and CYP4F3B (BD Gentest, Woburn, MA) expressed in baculovirus-infected cells to evaluate the possible involvement of these P450 enzymes in the metabolism of AZD6610. 14C-AZD6610 was incubated at a concentration of 10 μM (0.6 μCi/ml) with 0.05 μM of the recombinant P450 proteins and 0.5 mg/ml microsomal protein in 0.1 M phosphate buffer for 30 min. The incubation started with the addition of NADPH to a final concentration of 0.05 M with 0.05 mg/ml microsomal protein. Each recombinant P450 protein was incubated at 37°C for 2, 6, and 22 h and terminated with 100 μl of ice-cold ACN. The fresh human hepatocyte plates were incubated at 37°C for 2, 14, and 22 h and terminated with 100 μl of ice-cold ACN. The 0-h plate was terminated with 100 μl of ACN before adding the substrates. The plates were placed in the freezer (−20°C) for 20 min and then centrifuged for 20 min at 4000 g and 4°C. The supernatant was mixed with an equal volume of water, and the samples were stored at −80°C until analyzed by using LC/MS (QToF MS Xevo; Waters, Milford, MA).

AZD6610 was also incubated with 11 recombinant UGTs expressed in baculovirus-infected cells (UGT1A1, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, and UGT2B15) and human liver microsomes, to identify possible enzymes responsible for the glucuronidation of AZD6610. AZD6610 was incubated at a concentration of 100 μM with 0.2 mg/ml of each of the recombinant UGT proteins and 0.1 mg/ml human liver microsomal protein. Each recombinant UGT protein was incubated for 60 min with and without UDP glucuronic acid. The samples were diluted with ACN (1:2) to terminate the reaction, placed in the freezer (−20°C) for 20 min, and then centrifuged for 20 min at 4000 g and 4°C. Aliquots were injected directly onto the chromatographic column and analyzed by using a LC/MS/MS system (PE Scieix API 3000; SpectraLab Scientific Inc., Markham, Canada).

RNA Isolation and cDNA Synthesis. Total RNA was isolated from fresh human hepatocytes, cryopreserved human hepatocytes, and HepaRG cells by using TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). cDNA was prepared from 0.5 μg of total RNA by using the SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (PCR) with random hexamer primers according to the manufacturer’s protocol (Invitrogen).

Quantitative Real-Time PCR. CYP2C8 (Hs00258314_m1), CYP2C9 (Hs00426397_m1), CYP2J2 (Hs00951113_m1), CYP4A11 (Hs04194779_g1), CYP4F3B (Hs01587857_mH), UGT1A3 (Hs04194922_g1), UGT1A6 (Hs01592477_m1), UGT1A9 (Hs02516855_s1), and UGT2B7 (Hs00425592_m1) were analyzed by quantitative real-time PCR using TaqMan Gene Expression Assays with specific primers and probes (Applied Biosystems, Foster City, CA). The Hs01587857_mH CYP4F3 probe spans exons 2 and 3 and was chosen to detect the splicing variant CYP4F3B. The reaction mixtures (25 μl/well) contained 30 ng of cDNA, 1× TaqMan Universal Master Mix (Applied Biosystems), and 1.25 μl of Gene Expression Assays Mix and RNase free water. The cDNA quantification was performed by using an 7500 Sequence detector (Applied Biosystems), and the thermal cycle conditions comprised 2 min at 50°C, 10 min at 95°C, followed by 40 PCR cycles alternating 95°C for 15 s and 60°C for 1 min. Amplification curves were analyzed by using the 7500 sequence detection software version 1.3.1 (Applied Biosystems). The expression of all genes was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1). The relative gene expression (∆∆Ct) and the fold change of gene expression (2^ΔΔCt) were calculated by using the cryopreserved human hepatocytes as a calibrator. The significant differences in mRNA expression in HepaRG cells and fresh human hepatocytes compared with cryopreserved human hepatocytes were calculated by using the two-tailed Student’s t test.

Metabolism Studies in Cell Suspension. Specific incubations for metabolite profiling were performed in three different batches of pooled cryopreserved human hepatocytes, three different batches of cryopreserved differentiated HepaRG cells, and fresh human hepatocytes from donors 1, 2, and 3. Cryopreserved human hepatocytes and fresh human hepatocytes were diluted to a cell concentration of 4 × 10^7 cells/ml in modified Williams Medium E, whereas MIL600 cell medium (Biopredic International) supplemented with serum-free ADD650 was used to dilute the HepaRG cells to the same concentration. Fifty microliters of cell suspension was added to 96-well tissue culture plates with flat bottoms (TPP; Trasadingen, Switzerland) and preincubated for 10 min at 37°C. Fifty microliters of prewarmed (37°C) substrate solution was added to a final concentration of 10 μM 14C-diclofenac (1.0 μCi/ml) or 14C-AZD6610 (0.6 μCi/ml), with a final cell concentration of 2 × 10^5 cells/ml. The HepaRG cells and the cryopreserved human hepatocyte plates were incubated at 37°C for 2, 6, and 22 h and terminated with 100 μl of ice-cold ACN. The fresh human hepatocyte plates were incubated at 37°C for 2, 14, and 22 h and terminated with 100 μl of ice-cold ACN. The 0-h plate was terminated with 100 μl of ACN before adding the substrates. The plates were placed in the freezer (−20°C) for 20 min and then centrifuged for 20 min at 4000 g and 4°C. The supernatant was mixed with an equal volume of water. Cells from each batch/donor were run in three separate wells, and cells from different batches (n = 3/donors) were run on three different occasions. The triplicates from the same batch/donor were pooled, and the samples were stored at −80°C until analysis.

Bioreactor and Perfusion System. The bioreactor was made of two hollow fiber capillary layers integrated into a polyurethane housing. Each capillary layer consisted of hydrophobic oxygenation capillaries and hydrophilic medium perfusion capillaries (Fig. 1). The medium capillaries were arranged as two layers with counter-current perfusion to increase mass exchange between the inside of capillaries and the cells located within the intercapillary space. A port enabled substance injection, mimicking bolus administration, and sampling from the recirculating medium (Fig. 1). Fresh medium was continuously supplied from the medium bottle, and excess medium flowed into the outflow bottle (Fig. 1). A detailed description of the bioreactor structure is given elsewhere (Schmelzer et al., 2009).

In this study, a down-scaled bioreactor with a cell compartment volume of 0.5 ml was used to be compared with the 2-ml cell compartment bioreactor used in previous studies (Darnell et al., 2011; Zeilinger et al., 2011). The bioreactors were operated by means of an electronically controlled perfusion device (Zeilinger et al., 2011) allowing two bioreactors to be run in parallel. Temperature, medium feed, and medium recirculation rates were monitored and regulated via a connected computer. A valve-controlled gas mix unit was used for air/oxygen/CO2 supply. Bioreactors, tubing systems, and perfusion device were purchased from Stem Cell Systems (Berlin, Germany).

Bioreactor Cell Inoculation and Culture. Bioreactors were connected with preassembled sterile tubing systems and rinsed with phosphate-buffered saline and medium before cell inoculation. Two and a half milliliters of cell suspension (10 × 10^6 viable cells/ml) was inoculated in the cell compartment of each bioreactor to a final amount of 25 × 10^6 cells per bioreactor. The bioreactors were waggled carefully during the filling to allow even distribution.

Three different batches of cryopreserved differentiated HepaRG cells were inoculated into three bioreactors, and hepatic cell medium MIL600 supplemented with ADD670 was used during the whole culture period. Fresh human hepatocytes from donors 1 and 2 were inoculated into separate bioreactors on two different occasions, and hepatocytes from donor 3 were inoculated into two bioreactors run in parallel. Fresh human hepatocyte bioreactors were perfused with Heparmed Medium Vito 143 (Bioxchrom, Berlin, Germany), a modification of Williams Medium E specifically adapted for serum-free perfusion culture of liver cells by enrichment with amino acids, free
fatty acids, and trace elements. Before use, the medium was supplemented with 20 IU/l insulin, 5 mg/l transferrin, 3 μg/l glucagon (Biochrom, Berlin, Germany), 100,000 U/l penicillin, 100 mg/l streptomycin (Invitrogen), and 20 μg/ml gentamycin (PAA Laboratories GmbH, Pasching, Austria).

Bioreactor cultures were perfused with 12 ml of medium, recirculating at a flow rate of 3 ml/min. The temperature in the bioreactor chamber was maintained at 37°C, and bioreactors were oxygenated through the integrated gas capillaries with a gas mix of 95% air and 5% CO2 at a gas flow velocity of 5 ml/min per bioreactor. Before and between experiments, the medium flow rate was set to 0.6 ml/h, and the standard operation mode with continuous medium feed (open-system perfusion) was used.

**Metabolism Studies in Bioreactors.** Metabolite profiles of 14C-AZD6610 and 14C-diclofenac were assessed at culture days 5 and 6, respectively, by adding 1 ml of substance solution (120 μM) through the sampling port close to the bubble trap (Fig. 1), giving a final concentration of 10 μM (0.6 and 1.0 μCi/ml for 14C-AZD6610 and 14C-diclofenac, respectively) and a final medium volume of 12 ml. The medium in the bubble trap was mixed a few times before starting the recirculation of the medium (t = 0 h). The metabolism studies were performed at continuous medium perfusion, but without feeding medium into the perfusion circuit (closed-system perfusion). Samples (300 μl) were taken at 0, 2, 6, and 22 h. The bioreactor circuit was rinsed after each experiment with 50 ml of culture medium (single-pass perfusion) and then reset to the standard operation mode with continuous medium feed (open-system perfusion). Samples were frozen directly after collection. Before analysis, the samples were thawed and mixed with an equal volume of ACN. The samples were placed in the freezer (−20°C) for 20 min and then centrifuged for 20 min at 4000g and 4°C. The supernatant was mixed with an equal amount of water and analyzed by LC/MS (Waters QToF MS Xevo).

In addition to the metabolism studies in the bioreactor, 14C-AZD6610 and 14C-diclofenac were injected in a bioreactor system without cells to assess potential nonspecific binding to the bioreactor system. Samples were analyzed at 0, 0.25, 2, 4, 6, and 22 h and did not indicate any such nonspecific binding.

**Bioanalytical Equipment and Methods.** The LC/MS analyses were run on a Waters QToF MS Xevo operating under positive electrospray ionization conditions in extended dynamic range mode. The cone voltage was 15 V for 14C-diclofenac and 40 V for 14C-AZD6610. All other MS conditions were the same for both compounds. The m/z range was 80 to 1200 with an acquisition time of 0.1 s, the low collision energy was 6 V, and the high collision energy ramped from 20 to 30 V. Data were collected in centroid mode. The voltages were set to 0.5 kV for the capillary, 25 V for the sampling cone, and 4 V for the extraction cone. The source block temperature was 120°C, and the electrospray desolvation heater was 450°C. A nitrogen cone gas flow of 10 l/h was used, and the desolvation gas was set to 900 l/h. Leucine-enkephaline was used as lock mass (m/z 556.2771) for internal calibration at a concentration of 2 ng/μl and a flow rate of 10 μl/min. The Acquity ultra-performance liquid chromatography (UPLC) system (Waters) consisted of a column manager.
set at 40°C, an auto sample manager, and a binary solvent manager operating at a flow rate of 0.7 ml/min. Chromatographic separations were performed on the UPLC system by using an Acquity UPLC BEH C18 column (3.0 × 150 mm, 1.7 μm). The mobile phases consisted of A (water with 0.1% formic acid) and B (CAN). The LC gradient profile for 14C-diclofenac was as follows: 7% B during 0 to 1 min, a linear increase from 7 to 59% B during 1 to 25 min and 59 to 86% B during 25 to 30 min, then back to 7% B during 30 to 31 min. An equilibration time of 7 min at 7% B was allowed before injection of the next sample, and the injected sample volume throughout the study was 30 μl. The LC/MS and LC/radioactivity monitoring (RAM) analysis was performed separately within the same day. A third LC/MS system was used for the structural characterization of metabolites of AZD6610. The experimental set-up and data generated are given in the supplemental material.

Radioactivity Detection and Characterization of Metabolites. To quantify the formed metabolites, 30 μl of each sample was injected and separated by using the same systems and conditions as described above. All LC eluent was directed to a TopCount fraction collector (Gilson, Inc., Middletown, WI) and collected into scintillant coated 96-deepwell Lumaplates (PerkinElmer Life and Analytical Sciences, Groningen, The Netherlands). The collection time for each well was 6 s for 14C-diclofenac and 7.2 s for 14C-AZD6610. The plates were dried for 2 days, and radioactivity was measured using a TopCount NXT microplate scintillation counter (PerkinElmer Life and Analytical Sciences). The liquid scintillation counting TopCount files were imported to Laura 3.3.13.98c (LabLogic Systems Ltd., Sheffield, UK) to generate radiochromatograms. The raw data were collected as counts and transformed to cpm by the software. The radiochromatograms from each study sample were integrated for parent compound and separated metabolites. The basepoint, background, and peak integration parameters were set manually. The results are given as percentage of total detected radioactivity during the run. Identification of metabolites was based on the protonated molecule from full-scan MS acquisition, corresponding retention times for the MS peak, and a peak in the radio chromatogram, and the absence of the potential metabolite peak in the MS chromatogram of the 0-min sample. To facilitate the identification of metabolite M6 of AZD6610, MS and retention times were compared with the authentic standard.

Cell Damage Markers. Cell damage markers in fresh human hepatocytes and HepaRG cells cultured in the 3D bioreactor were measured in samples from the culture perfusate and/or the medium outflow. The samples were stored at −20°C and analyzed at the Laboratory of Clinical Chemistry at Sahlgrenska University Hospital, Gothenburg, Sweden. The release of aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) in the bioreactors was calculated on the basis of concentrations measured in the outflow bottle and in the perfusion circuit (Fig. 1), according to eq. 1:

\[ P / \Delta t = (C_0 - C_M) \times V_p / \Delta t + (C_d - C_{d-1}) \times V_p / \Delta t \quad (1) \]

where \( P \) is the total production rate, \( \Delta t \) is the time interval between two sampling time points, \( C_0 \) is concentration in the outlet vessel, \( C_M \) is medium blank concentration, \( V_0 \) is volume of medium in the outlet vessel, \( C_d \) is concentration in perfusate at the time point of sample taking, \( C_{d-1} \) is concentration in the previously taken sample from the perfusate, and \( V_p \) is recirculating volume.

Calculations. Some metabolites coeluted with other metabolites and could not be separated in the radiochromatogram. To estimate the contribution of each metabolite to the total peak area, a peak in the radiochromatogram, and the relative contribution of metabolite X, MX, of total radioactivity detected in the radiochromatographic peak was calculated on the basis of concentrations measured in the outflow bottle and in the perfusion circuit (Fig. 1), according to eq. 1:

\[ \% \text{ metabolite} (MX) = \frac{A_{MS \text{ metabolite} (MX)}}{A_{MS \text{ metabolites}}} \times \% \text{ radioactivity in radiochromatographic peak} \quad (2) \]

This calculation of metabolite proportions in the radiochromatographic peak is founded on the assumption that the MS responses of all metabolites are equal. Although the MS response is in fact unknown for all metabolites, the proportions calculated should be regarded as estimates, but nonetheless useful in the comparison of metabolite profiles between the different cell systems investigated.

In addition, the accurate mass measurement error is reported in ppm (Supplemental Tables S1 and S3) and calculated according to eq. 3:

\[ \text{mass error} = \frac{\text{accurate mass} - \text{exact mass}}{\text{exact mass}} \times 10^6 \quad (3) \]

Results

Identification of Enzymes Involved in AZD6610 Metabolism. The possible involvement of P450 and UGT enzymes in the metabolism of AZD6610 was studied in human liver microsomes and recombinant human P450 and UGT enzymes. The results showed that CYP4A11, CYP4F3B, and CYP2J2 were involved in the hydroxylation of AZD6610 to M6 (Fig. 2A), whereas CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP4F2, and CYP4F12 were inactive (data not shown). Furthermore, the results indicated that AZD6610 was not metabolized by CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP4F2, and CYP4F12.
the UGT1A3 was the main enzyme responsible for the glucuronidation of AZD6610 with minor contributions from UGT1A1, UGT1A6, UGT1A7, and UGT2B7 (Fig. 2B). UGT1A4, UGT1A8, UGT1A9, UGT1A10, UGT2B4 and UGT2B15 had little or no contribution to the formation of the glucuronide (data not shown). No metabolites or low amount of metabolites were detected when microsomes expressing P450s or UGTs were incubated without NADPH or UDP glucuronic acid.

**P450 and UGT mRNA Expression.** The mRNA expression of P450 and UGT isoenzymes involved in the metabolism of AZD6610 or diclofenac was analyzed in cryopreserved human hepatocytes, fresh human hepatocytes, and HepaRG cells on the same day as cell isolation or cell thawing (Fig. 3). Average mRNA expression levels in fresh human hepatocytes and HepaRG cells were compared with cryopreserved human hepatocytes. CYP2C8 and CYP2C9 were detected at low levels (0.02- and 0.3-fold, respectively) in HepaRG cells compared with cryopreserved human hepatocytes. The mRNA expression in fresh human hepatocytes was 0.2-fold for CYP2C8 and 1.8-fold for CYP2C9 of cryopreserved human hepatocytes. Furthermore, the mRNA expression of CYP4F3B was similar in all cell types, although a slightly lower expression was measured in HepaRG cells (0.6-fold) and fresh human hepatocytes (0.6-fold), compared with cryopreserved human hepatocytes. The mRNA of both CYP2J2 (0.4-fold) and CYP4A11 (0.01-fold) were expressed at lower levels in HepaRG cells compared with cryopreserved human hepatocytes. The mRNA expression level of UGT1A3 was similar in HepaRG cells and fresh and cryopreserved human hepatocytes, whereas the mRNA expression of UGT1A6 was 10-fold and UGT1A9 was 6-fold higher in HepaRG cells than in cryopreserved human hepatocytes. The mRNA expression of UGT2B7 was higher in fresh human hepatocytes (4-fold) and lower in the HepaRG cells (0.06-fold) than in cryopreserved human hepatocytes.

**Major Metabolic Pathways of AZD6610.** AZD6610 is metabolized to at least 13 metabolites and the most important metabolic pathways in primary human hepatocytes, HepaRG cells, and in vivo in humans are shown in Fig. 4. M3, M5, and M6 were formed via metabolic transformations of the hexyl side chain, whereas M10 was suggested to be the acyl glucuronide of AZD6610. The time profiles of AZD6610 depletion and formation of major metabolites are shown in five different hepatic in vitro systems in Fig. 5. The hydroxylated metabolite M6 was detected in cryopreserved human hepatocytes, fresh human hepatocytes, and HepaRG cells in suspensions on day 0 as well as in fresh human hepatocyte and HepaRG bioreactors on culture day 5. M5 and M3 were found in all three human hepatocyte systems. M3 was also found in HepaRG cell suspension, but was not detected in

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**Fig. 3.** mRNA expression of P450 and UGT genes in cryopreserved human hepatocytes (empty bars), fresh human hepatocytes (filled bars), and HepaRG cells (patterned bars). The expression was compared with cryopreserved human hepatocytes for all genes measured, and the bars represent the mean values ± S.D. of three different batches or donors. The levels of significant differences in mRNA expression in HepaRG cells and fresh human hepatocytes compared with cryopreserved human hepatocytes are provided. *, p < 0.05; **, p < 0.01.

**Fig. 4.** Tentative structures and suggested major metabolic pathways of AZD6610 in vivo in human, human hepatocyte, and HepaRG cells. Thin arrows indicate low metabolic activity, and thick arrows indicate high metabolic activity to give an overview of the significance of the metabolic pathways in different systems. Enzymes involved in the hydroxylation and glucuronidation of AZD6610 are shown. Stars denote the position of the 14C label.
HepaRG bioreactor. Furthermore, M5 was absent in both HepaRG cell suspension and HepaRG bioreactor. The glucuronide, M10, was formed in all five in vitro systems, but at much higher levels in HepaRG cells compared with human hepatic systems. M10 coeluted with the hydroxylated metabolite M11 in the radiochromatogram. However, the MS peak area of M11 was only 5% of the total M10 and M11 area in the HepaRG cells, whereas the MS peak area of M11 was approximately 50% of the total M10 and M11 area in the human hepatocytes. Furthermore, M7 coeluted with M8 and M9 and could not be separated in the radiochromatogram. The M7-M8-M9 radiochromatographic peak was detected in all five in vitro systems. The proposed structures of M7, M8, and M9 and the estimated contribution of each metabolite to the peak area in the radiochromatogram are presented in Supplemental Fig. S5 and Supplemental Table S2. The structural characterization of AZD6610 metabolites (Supplemental Figs. S1-S4), the LC/MS/RAM chromatograms (Supplemental Fig. S6), and the accurate mass of the protonated molecules and the mass errors (Supplemental Table S1) are available in the supplemental data. The time profiles of unidentified and minor metabolites are not shown in Fig. 5.

**Major Metabolic Pathways of Diclofenac.** Diclofenac is hydroxylated to hydroxydiclofenac or directly conjugated to diclofenac acyl glucuronide (Fig. 6) (Kumar et al., 2002; Sarda et al., 2012). In the second metabolic step, hydroxydiclofenac acyl glucuronide is formed via conjugation of hydroxydiclofenac or hydroxylation of diclofenac acyl glucuronide (Kumar et al., 2002; Sarda et al., 2012). The time profiles of diclofenac depletion and formation of major metabolites are shown in five different in vitro systems in Fig. 7. The clearance of diclofenac and the formation of hydroxydiclofenac acyl glucuronide were similar in all three suspension experiments performed on day 0 (Fig. 7, A–C), whereas diclofenac acyl glucuronide was detected only in HepaRG cell suspension and absent in fresh and cryopreserved human hepatocyte suspensions. Furthermore, the formation rates of hydroxydiclofenac were much higher in the human hepatocyte suspension compared with HepaRG cell suspension. In the HepaRG cell suspension experiment, the diclofenac acyl glucuronide peak in the radiochromatogram was wide and overlapped with the hydroxydiclofenac peak. However, to estimate the percentage of radioactivity of each metabolite of total radioactivity in
the sample calculations according to eqs. 2 and 3 were performed.

The bioreactor experiments were performed 6 days after cell inoculation, and the metabolite time profiles are shown in Fig. 7, D and E. Hydroxylated and glucuronidated metabolites were detected in both fresh human hepatocyte and HepaRG bioreactors, but the clearance of diclofenac and formation rates of metabolites were lower compared with suspension experiments. In addition, diclofenac acyl glucuronide, which was not detected in human hepatocyte suspension, was detected in human hepatocyte bioreactor.

The LC/MS/RAM chromatogram, the accurate mass of the protonated molecules, and the mass errors are shown in Supplemental Fig. S7 and Supplemental Table S3. The diclofenac LC/MS/RAM chromatogram shows two isomers of diclofenac acyl glucuronide and four isomers of hydroxyclofenac acyl glucuronide. The sum of respective isomers is presented in the graphs in Fig. 7. The time profiles of unidentified and minor metabolites are not shown in Fig. 7.

**Cell Damage Markers.** The release of ALAT and ASAT were measured in fresh human hepatocytes and HepaRG cells cultured in the bioreactor to determine cell quality. The release of ALAT and ASAT in fresh human hepatocytes bioreactors was low in donors 1 and 3, whereas donor 2 showed much higher ASAT and ALAT levels, indicating inferior quality (Table 2). Furthermore, the metabolite formation of AZD6610 and diclofenac in the bioreactor on culture days 5 and 6 was low in donor 2 and thus excluded from the results.

**Discussion**

The aim of this study was to investigate the drug metabolic pathways of two model substrates, AZD6610 and diclofenac, in fresh human hepatocytes, cryopreserved human hepatocytes, and HepaRG cells in suspensions and bioreactors. The in vitro results were compared with the metabolite profiles determined in vivo in human. One of the model substances, AZD6610, is metabolized mainly via hydroxylation to M6, followed by further oxidation to M5 and M3, which are found in feces and to some extent in urine in humans (unpublished results). In addition, the parent compound is circulating in plasma at significant levels, whereas low levels of the glucuronide M10 can be found in urine (unpublished results). All major human in vivo relevant AZD6610 metabolites were detected in fresh and cryopreserved human hepatocyte suspensions, and the hydroxylation pathway was found to be the major route of metabolism. In HepaRG cells, the glucuronidation pathway was favored with a considerably lower contribution of the hydroxylation pathways (Fig. 4). Thus, the main difference between primary human hepatocytes and HepaRG cells was the balance between the hydroxylation route, which was favored in human hepatocytes, and the glucuronidation route, which was favored in HepaRG cells.

The P450 enzymes (CYP2J2 and CYP4F3B) identified to be involved in the hydroxylation of AZD6610 to M6 were expressed at lower levels in HepaRG cells, which may be the reason for the difference in the capacity to produce the major metabolites in the hydroxylation pathway in the two cell systems. On the other hand, the high expression of UGT1A6 in HepaRG cells compared with primary human hepatocyte may explain the efficient glucuronidation pathway in these cells.

Enzymes involved in the main metabolic pathways of AZD6610 were not identified during early preclinical and clinical studies. None of the previously examined enzymes in P450 families 1, 2, and 3 were found to hydroxylate AZD6610. In this present study, CYP2J2 and enzymes in P450 family 4 were found to hydroxylate AZD6610 to M6. Only a few drugs, such as ebastine and fingolimod, have been reported to be substrates of these P450 enzymes (Hashizume et al., 2002; Kalsotra et al., 2004; Kalsotra and Strobel, 2006; Jin et al., 2011). The P450 enzymes identified to hydroxylate AZD6610 to M6 were CYP4A11 and CYP2J2, which are expressed mainly in liver and kidney (Palmer et al., 1993; Christmas et al., 2001; Bellamine et al., 2003), and CYP2J2, which is detected mainly in heart and to a lesser extent in liver and intestine (Wu et al., 1996). Enzymes in P450 family 4 are known to be involved in fatty acid ω-hydroxylation,
which is the first step in the formation of carboxylic acids, which can be excreted or further degraded via the \( \beta \)-oxidation pathway (Mortensen, 1992; Fer et al., 2008). It is noteworthy that the main metabolic pathway of AZD6610 seems to mimic the metabolism of fatty acids. After \( \omega \)-hydroxylation of AZD6610 at the methyl terminus of the hexyl side chain, the metabolite M6 is suggested to be oxidized further to the dicarboxylic acid M5 by dehydrogenases. Further metabolism of M5 to M3 is suggested to proceed via CoA conjugation and chain shortening via \( \beta \)-oxidation (Fig. 4).

Diclofenac is also a carboxylic acid with P450- and UGT-dependent metabolism. Essentially all major in vivo human metabolites were detected in the in vitro systems used in this study, except for diclofenac acyl glucuronide, which was not found in human hepatocyte suspension. It has been reported that 50% of the total dose in vivo in humans was excreted in urine and bile as 4-hydroxydiclofenac and 4-hydroxydiclofenac acyl glucuronide, whereas diclofenac acyl glucuronide accounted for 10 to 20% of the dose. The remaining dose was detected as other oxidative metabolites and their conjugates (Riess et al., 1978; Stierlin and Faigle, 1979; Stierlin et al., 1979). The initial hypothesis was that the clearance of diclofenac in vivo in humans was dominated by the formation of 4-hydroxydiclofenac catalyzed by hepatic CYP2C9 (Stierlin

**TABLE 2**

The release of ALAT and ASAT from fresh human hepatocytes and HepaRG cells during the first day of culture in bioreactors.

<table>
<thead>
<tr>
<th></th>
<th>Hepatocytes</th>
<th>HepaRG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BR 1</td>
<td>BR 1</td>
</tr>
<tr>
<td>ALAT ( \text{nkat/h/10}^7 \text{cells} )</td>
<td>0.18</td>
<td>0.09</td>
</tr>
<tr>
<td>ASAT</td>
<td>1.75</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>BR 2</td>
<td>BR 2</td>
</tr>
<tr>
<td>ALAT</td>
<td>1.08</td>
<td>0.12</td>
</tr>
<tr>
<td>ASAT</td>
<td>6.54</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>BR 3A</td>
<td>BR 3A</td>
</tr>
<tr>
<td>ALAT</td>
<td>0.20</td>
<td>0.12</td>
</tr>
<tr>
<td>ASAT</td>
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<td>0.31</td>
</tr>
<tr>
<td></td>
<td>BR 3B</td>
<td>BR 3B</td>
</tr>
<tr>
<td>ALAT</td>
<td>0.21</td>
<td>0.00</td>
</tr>
<tr>
<td>ASAT</td>
<td>1.88</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Fig. 7. The time profiles of diclofenac metabolism in cryopreserved human hepatocytes (A), fresh human hepatocytes (B), and HepaRG cells (C) in suspensions on day 0 and in fresh human hepatocytes (D) and HepaRG cells (E) on culture day 6 in bioreactors. The results are given as percentage of total detected radioactivity, and the data points are mean values ± S.D. (n = 3).
and Faigle, 1979; Transon et al., 1995; Kumar et al., 2006a,b). However, later studies revealed that the diclofenac acetyl glucuronide was hydroxylated via CYP2C8 in vitro (Kumar et al., 2002). Thus, the excreted 4-hydroxydiclofenac acetyl glucuronide may be formed via two separate pathways in vivo, and the glucuronidation pathway might play an important role in the clearance of diclofenac (Fig. 6). In the present study, a clear difference was observed comparing HepaRG cells and human hepatocytes. The diclofenac acetyl glucuronide level was high in HepaRG cell suspension and absent in human hepatocyte suspension. Previous studies have shown that CYP2C8 mRNA expression correlated well with CYP2C8 activity (Ohtsuki et al., 2012). Therefore, the low expression of CYP2C8 in differentiated HepaRG cells (Fig. 3) (Kanebratt and Andersson, 2008b; Anthérien et al., 2010) may result in a low capacity to hydroxylate the diclofenac acetyl glucuronide. In contrast, the higher expression of CYP2C8 in human hepatocytes might facilitate a rapid depletion of diclofenac acetyl glucuronide, the intermediate metabolite, in human hepatocytes. Although notable differences in the relative levels of hydroxydiclofenac were observed in HepaRG cells and human hepatocytes, the hydroxydiclofenac acetyl glucuronide was detected at similar levels in HepaRG cells and human hepatocytes, which may be explained by the two different pathways, which both end up in hydroxydiclofenac acetyl glucuronide.

Freshly isolated human hepatocytes are known to rapidly lose liver phenotypic functions, including drug metabolism capacity, in vitro (Rodriguez-Anton et al., 2002). A clear example is the decrease of hydroxydiclofenac in human hepatocytes by approximately 90% after 3-day culture in 2D compared with fresh hepatocytes. This is in sharp contrast to the high level of diclofenac metabolites detected in 7-day-old human hepatocytes cultured in bioreactors in the present study. The maintenance of the drug-metabolizing capacity in the bioreactor inoculated with human hepatocytes is a compelling feature of the 3D model and is consistent with previous findings (Zeilinger et al., 2002, 2011). HepaRG cells, on the other hand, are known to retain metabolic capacity for several weeks in both 2D and bioreactor cultures (Josse et al., 2008; Anthérien et al., 2010; Darnell et al., 2011). The detection of hydroxydiclofenac, diclofenac acetyl glucuronide, and hydroxydiclofenac acetyl glucuronide in fresh human hepatocyte and HepaRG bioreactors 7 days after cell inoculation clearly demonstrates that the P450 and UGT activities were maintained for at least 1 week. Moreover, the major in vivo intermediate metabolite, diclofenac acetyl glucuronide, which was absent in fresh human hepatocyte suspension, was detected in fresh human hepatocyte bioreactor. This may be explained by the more in vivo like situation in the bioreactor, where the acetyl glucuronide can be effluxed into the medium and escape further metabolism. However, the detection of the acetyl glucuronide could also be explained by a decrease in CYP2C8 activity over time. In future studies, determination of CYP2C8 expression and activity in the bioreactor should be considered to study the stability of the enzyme.

In general, the clearance of substrates and formation rates of metabolites were lower in bioreactors compared with suspensions. Nevertheless, the relative levels of the metabolites in suspension and 3D experiments were similar. However, an important experimental difference between the two in vitro systems must be kept in mind. In the cell suspension, metabolites were analyzed from both the medium and lysed cells, whereas only the medium was analyzed in the bioreactor experiments. The levels of metabolites retained in the cells cultured in the bioreactor were not analyzed, and the metabolite profile may be different from that detected in the medium. Glucuronides are generally too polar to pass membranes via passive diffusion, thus the detection of glucuronides in the bioreactor medium indicates an active efflux of conjugated metabolites, from cells cultured in the bioreactor back to the circulating medium (Zamek-Gliszczynski et al., 2006; Lagas et al., 2010).

In conclusion, this study describes the biotransformation pathways in HepaRG cells and human hepatocytes of two drug substances, which are metabolized by both P450 and UGT enzymes. The proportion between relevant biotransformation pathways of each investigated drug was clearly different in the various cell systems. The human in vivo metabolite profile of AZD6610 was best represented by primary human hepatocytes, whereas all major diclofenac metabolites were detected in HepaRG cells. The lower P450 activity and higher UGT activity in HepaRG cells compared with human hepatocytes are important findings that should be considered when using HepaRG cells in drug metabolism studies. However, several drugs have to be studied to further evaluate the differences between HepaRG cells and human hepatocytes in their capacity to metabolize drugs. Moreover, the metabolite profiles in cryopreserved and fresh human hepatocytes were almost the same for both compounds. It is noteworthy that all major human in vivo metabolites of diclofenac were detected in human hepatocyte bioreactor, whereas one major human in vivo metabolite was not found in human hepatocyte suspension. The liver bioreactor using both fresh human hepatocytes and HepaRG cells retained biotransformation capacity for at least 1 week. Thus, the incubation time can be increased from a few hours in suspension to several days in 3D cultures, which opens up for detection of metabolites from slowly metabolized drugs.

Acknowledgments

We thank Annika Janefeldt and la Hultman for the identification of UGT enzymes involved in the formation of the AZD6610 glucuronide; and Lisa-Mari Nilsson, Carl Jorns, and Helen Zemack for technical support in the isolation of human hepatocytes from liver tissues.

Authorship Contributions

Participated in research design: Darnell, Ulvestad, Weidolf, and Andersson.

Conducted experiments: Darnell, Ulvestad, and Ellis.

Performed data analysis: Darnell and Weidolf.

Wrote or contributed to the writing of the manuscript: Darnell, Ulvestad, Weidolf, and Andersson.

References


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