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Use of HμREL Human Coculture System for Prediction of Intrinsic Clearance and Metabolite Formation for Slowly Metabolized Compounds

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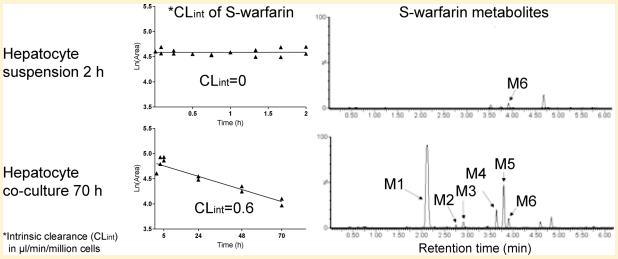
Supporting Information

ABSTRACT: Design of slowly metabolized compounds is an important goal in many drug discovery projects. Standard hepatocyte suspension intrinsic clearance (CL_{int}) methods can only provide reliable CL_{int} values above 2.5 μL/min/million cells. A method that permits extended incubation time with maintained performance and metabolic activity of the in vitro system is warranted to allow in vivo clearance predictions and metabolite identification of slowly metabolized drugs. The aim of this study was to evaluate the static HμREL coculture of human hepatocytes with stromal cells to be set up in-house as a standard method for in vivo clearance prediction and metabolite identification of slowly metabolized drugs. Fourteen low CL_{int} compounds were incubated for 3 days, and seven intermediate to high CL_{int} compounds and a cocktail of cytochrome P450 (P450) marker substrates were incubated for 3 h. In vivo clearance was predicted for 20 compounds applying the regression line approach, and HμREL coculture predicted the human intrinsic clearance for 45% of the drugs within 2-fold and 70% of the drugs within 3-fold of the clinical values. CL_{int} values as low as 0.3 μL/min/million hepatocytes were robustly produced, giving 8-fold improved sensitivity of robust low CL_{int} determination, over the cutoff in hepatocyte suspension CL_{int} methods. The CL_{int} values of intermediate to high CL_{int} compounds were at similar levels both in HμREL coculture and in freshly thawed hepatocytes. In the HμREL coculture formation rates for five P450-isoform marker reactions, paracetamol (CYP1A2), 1-OH-bupropion (CYP2B6), 4-OH-diclofenac (CYP2C9), and 1-OH-midazolam (3A4) were within the range of literature values for freshly thawed hepatocytes, whereas 1-OH-bufuralol (CYP2D6) formation rate was lower. Further, both phase I and phase II metabolites were detected and an increased number of metabolites were observed in the HμREL coculture compared to hepatocyte suspension. In conclusion, HμREL coculture can be applied to accurately estimate intrinsic clearance of slowly metabolized drugs and is now utilized as a standard method for in vivo clearance prediction of such compounds in-house.



KEYWORDS: *HμREL coculture, slowly metabolized drugs, low CL_{int}, clearance prediction, P450 activity, metabolite formation*

INTRODUCTION

The constant challenges to decrease the decline of R&D productivity and to reduce attrition rate of candidate drugs are key factors that every pharmaceutical company is aiming for. Integrating absorption, distribution, metabolism, and excretion properties in drug discovery has improved the prediction success of human pharmacokinetics and is being underlined by the reduction of pharmacokinetic-related attrition contributing to around 40% of development terminations in the 1980s,[1] to 10% in more recent estimations.[2]

Human in vitro metabolism and preclinical disposition data is used to predict patient pharmacokinetics, and a common goal in many cases is to develop drugs with low metabolic turnover in order to achieve once daily administration.[3] The prediction of in vivo clearance from in vitro data is challenging and frequently described as giving an underprediction even when scaling factors or regression lines are applied.[4−7] The increasing number of low clearance drugs within the pharmaceutical industry, about 30% for Pfizer and AstraZeneca,[3] makes the in vivo clearance prediction even more challenging, due to the lack of or very low measurable disappearance rates of the compound in the currently used in vitro systems. Metabolism studies in human liver microsomes are well established as cost-effective and permitting high throughput.[8] During the past decade cryopreserved human hepatocytes have become increasingly robust, which has enabled the establishment of their routine use in drug discovery. Hepatocytes provide both phase I and phase II metabolizing enzymes, as well as the presence of transporters, which together enable more comprehensive elucidation of drug clearance and metabolism.[9−11] For the suspension hepatocyte clearance assay, incubation times of maximum 4 h are recommended to utilize the cells while enzyme activity is stable.[3,12] The ability to accurately determine an intrinsic

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Table 1. Calculated in Vitro CL_{int} in HμREL Coculture (Mean ± SD, n = 2 for Intermediate to High CL_{int} and n = 3 for Low CL_{int}), Predicted in Vivo CL_{int}, and Fold Difference between Observed and Predicted CL_{int}

	main responsible enzyme(s)	fu_b	fu_{inc}	in vitro CL_{int}	predicted in vivo CL_{int}	obsd human in vivo clearance	obsd CL_{int} in vivo	fold difference in vivo/coculture
				Intermediate to High CL_{int}				
imipramine	CYP2C9 > 2D6 > 3A4	0.074	0.59	19.6 ± 1.4	18.2	13.5[a]	31.8	1.7
sildenafil	CYP3A4/2C9/2C19	0.056	0.66	13.1 ± 2.2	15.0	6[a]	17.7	1.2
verapamil	CYP3A4	0.080	0.70	16.8 ± 5.9	19.8	13.3[a]	127	6.4
irbesartan	CYP2C9	0.010	0.52	10.3 ± 1.1	4.6	3.9[a]	10.8	2.3
clozapine	CYP3A4 > 2C8/2D6	0.044	0.35	16.5 ± 1.7	19.2	5.2[a]	8.43	0.4
naloxone	UGTs	0.60	0.90	32.3 ± 4.1	86.1	23[b]	329	3.8
7-OH-coumarin	UGTs	na[c]	na	84.7 ± 5.1	na	na	31.8	na
				Low CL_{int}				
prazosin	CYP	0.043	0.69	4.4 ± 1.3	4.91	2.85[a]	5.11	1.0
diflunisal	UGT	0.0030	0.50	4.6 ± 1.3	1.06	0.18[a]	0.33	0.3
lorazepam	UGT	0.099	0.72	1.1 ± 0.4	3.32	1.2[a]	2.06	0.6
oxaprozin	P450, UGT	0.0010	0.66	2.5 ± 0.4	0.24	0.07[a]	0.13	0.5
diazepam	CYP2C19 > 3A	0.016	0.63	1.2 ± 0.3	1.09	0.51[a]	0.92	0.8
(S)-warfarin	CYP2C9	0.0070	0.95	0.6 ± 0.1	0.17	0.06[a]	0.06	0.4
paracetamol	UGT	0.72	0.93	0.5 ± 0.2	5.22	5.0[b]	6.67	1.3
furosemide	UGT	0.020	0.86	1.8 ± 0.7	1.40	1.7[a]	3.66	2.6
disopyramide	CYP3A4	0.67	0.83	0.3 ± 0.1	3.16	0.90[b]	0.94	0.3
metoprolol	CYP2D6	0.79	0.92	1.0 ± 0.3	8.61	13.3[a]	39.7	4.6
AZ1	3A4, UGT	0.021	0.893	5.6 ± 1.6	3.43	4.7	15.0	4.4
AZ2		0.26	0.938	0.3 ± 0.1	1.40	2.1	2.32	1.7
ketoprofen	UGT	0.0087	0.87	5.9 ± 1.0	1.13	1.2[a]	1.19	1.0
quinidine	CYP3A4	0.12	0.77	0.6 ± 0.5	1.65	4.0[a]	5.03	3.0

[a]Reference 4. [b]Reference 36. [c]Not available.

clearance (CL_{int}) value is determined by the assay's capacity to show metabolic turnover that is greater than the inherent variability of the assay.[13] With an incubation time of 4 h human hepatocyte CL_{int} values as low as 2.5 μL/min/million cells can be determined.[3] However, this is not sufficient for slowly metabolized drugs. Different strategies to overcome the challenge of accurately predicting in vivo clearance of low clearance compounds, as well as generating in vivo relevant metabolites and investigating multiple metabolic transformations for such compounds, have evolved during the past couple of years.[3] These strategies can be broken into two general approaches: (1) serial incubations of the same compound solution or (2) extension of the cell performance over a longer period of time. In the first method, the relay method, the supernatant is transferred every 4 h to freshly thawed hepatocytes to increase the incubation time up to 20 h or longer.[14] The other approach utilizes cells or systems which aim at maintaining enzyme activity over time, e.g., monolayer cultures of plated cryopreserved hepatocytes or HepaRG cells, as well as cocultures such as HepatoPac and HμREL[15−17] and more recently hepatocyte cultures in 3D-spheroid formats.[18] These new culture systems have shown maintained enzymatic activity for up to 7 days. Bonn and co-workers[15] recently evaluated the performance of plated human hepatocytes, HepaRG cells and HμREL cocultures of human hepatocyte and stromal cells to accurately determine CL_{int} values below 1 μL/min/million hepatocytes and predict human in vivo clearance. In this recent study, the HμREL coculture of human hepatocyte and stromal cells was superior to the other in vitro systems due to a higher level of reproducibility and the ability to run incubations up to 72 h or longer.[15]

Another aspect in addition to in vivo clearance prediction is the capacity of the in vitro system to produce in vivo relevant metabolites and represent the metabolic pathways in humans, which is in line with the recommendations of both FDA (2008)[19] and the EMA (2013).[20] The knowledge of which metabolites are formed is to support selection of relevant toxicity species but also is important earlier in the drug discovery process for the design of new chemical entities. A recent paper showed that while incubations in cryopreserved human hepatocytes showed a good predictability of the metabolic pathways seen in human plasma, they tended to only have the ability to detect the first transformation step in the pathway.[21]

In this present study, the static HμREL coculture was further evaluated for its robustness in producing low CL_{int} values and its utility for in vitro in vivo clearance predictions for slowly metabolized drugs. To validate the HμREL coculture as an in vivo clearance prediction method for slowly metabolized drugs, a compound set of 14 low CL_{int} drugs and 7 intermediate to high CL_{int} drugs was included. The stability of phase I and phase II enzyme activities in the HμREL coculture, for the most important cytochrome P450s (P450s) and UDP-glucuronosyltransferases (UGTs), were studied and compared with in-house or literature data of freshly thawed hepatocytes. In vivo clearance data was available for 20 compounds, and the in vivo clearance was predicted applying the regression line approach.[6] An additional aim of this study was to investigate the possibility to perform metabolite identification in combination with CL_{int} determination for slowly metabolized drugs.

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MATERIALS AND METHODS

Material and Chemicals. Cocultures of the 5-donor plateable hepatocyte pool (lot 1410235, XenoTech) and nonparenchymal stromal cells (stromal cell type and ratio of hepatocyte per stromal cell are proprietary information) in type I collagen coated 96-well plates were purchased from HμREL (North Brunswick, NJ, USA). Test compounds imipramine, sildenafil, verapamil, irbesartan, clozapine, naloxone, 7-OH-coumarin, ketoprofen, AZ1 ((2*R*,3*R*)-3-(6-((azetidin-1-ylsulfonyl)methyl)-2-(2,3-difluorobenzylthio)pyrimidin-4-ylamino)butane-1,2-diol), diflunisal, prazosin, oxaprozin, furosemide, diazepam, lorazepam, metoprolol, (*S*)-warfarin, quinidine, paracetamol, disopyramide, and AZ2 ((*R*)-3(4-(1-((*S*)-2,3-dihydoxyl[13C3]propanoyl)-1,2,3,6-tetrahydropyridin-4-yl)-3,5-difluorophenyl)-5-(([15N]isooxazol-3-yloxy)methyl)-oxazolidin-2-one) were obtained as dimethyl sulfoxide (DMSO) stock solutions from AstraZeneca compound management team (Gothenburg, Sweden). Midazolam, diclofenac, phenacetin, bufuralol, bupropion, 1-OH-midazolam, 4-OH-diclofenac, and 1-OH-bufuralol were obtained from Sigma-Aldrich, Sweden, and OH-bupropion was purchased from Toronto Research Chemicals Inc., Canada. LC−MS standard volume marker (5,5-diethyl-1,3-diphenyl-2-iminobarbituric acid (DDIBA)) was obtained as dimethyl sulfoxide (DMSO) stock solution from AstraZeneca compound management team (Gothenburg, Sweden). Acetonitrile of LC−MS grade was from Fischer Scientific, U.K., formic acid 98/100% from UN1779, Fischer Scientific, U.K., and Milli-Q water Q-POD from Millipore. A 10-donor pool of cryopreserved human hepatocytes (lot IRK or LYB) was used for the hepatocyte suspension assay, obtained from Bioreclamation IVT (Brussels, Belgium). Leibovitz's L-15 medium, without phenol red, with L-glutamine (Gibco 21083027), used for the hepatocyte suspension assay, was purchased from Invitrogen (Stockholm, Sweden). HμREL PlatinumHeps maintainance medium and HμREL PlatinumHeps dosing medium were delivered from HμREL.

HμREL Coculture. Plates with coculture of primary human hepatocytes and stromal cells were shipped in maintenance medium, at 37 °C from HμREL (North Brunswick, NJ, USA). Number of seeded viable hepatocytes was 30,000 hepatocytes per well in 96-well plates. The cells were cocultured for 6 days prior to arrival at AstraZeneca R&D Gothenburg, Sweden, where the cells were left to acclimatize in HμREL PlatinumHeps maintenance medium overnight in an incubator at 37 °C in humidified atmosphere containing 95% air and 5% CO_2.

Compounds for CL_{int} and P450 Activity. The evaluation of the HμREL coculture system was carried out using three test sets of compounds covering metabolism via different CYP and UGT enzymes (Table 1). To evaluate clearance of slowly metabolized drugs in HμREL coculture, test set 1 with 14 compounds (ketoprofen, diflunisal, prazosin, oxaprozin, furosemide, diazepam, lorazepam, metoprolol, (*S*)-warfarin, quinidine, paracetamol, disopyramide, AZ1, and AZ2) with low human in vivo clearance (<5 mL/min/kg) or no measurable CL_{int} in hepatocyte suspension were included (Table 1). In test set 2, 7 compounds (imipramine, sildenafil, verapamil, irbesartan, clozapine, naloxone, and 7-OH-coumarin) with higher human in vivo clearance (>5 mL/min/kg) or measurable CL_{int} in hepatocyte suspensions were utilized to compare the CL_{int} in HμREL coculture and human hepatocyte suspension (Table 1). To study the specific activity of different P450 enzymes expressed in the HμREL system, test set 3, a cocktail of midazolam (3 μM), diclofenac (10 μM), phenacetin (30 μM), bupropion (40 μM), and bufuralol (5 μM), was used and the formation of 1-OH-midazolam, 4-OH-diclofenac, paracetamol, OH-bupropion, and 1-OH-bufuralol was measured.

Assessment of Test Compound Solubility. Prior to the in vitro assay the solubility in aqueous buffer media is determined for all test compounds as described below. The thermodynamic solubility of the compound is measured in a shake-flask approach starting from 10 mM DMSO solutions. The DMSO is evaporated and the dried compounds are equilibrated in glass vials in aqueous phosphate buffer (0.1 M, pH 7.4) for 24 h at 25 °C under constant stirring. The portion with the dissolved compound is then separated from the remainder through a double centrifugation with a tip wash in between, this to ensure that no residues of the dried compound are interfering. The solutions are diluted with purified water before quantification using UPLC/MS/MS. In each run three QC samples are incorporated to ensure the quality of the assay. The samples are compared to a standard of the test compound of known concentration dissolved in DMSO.

Compound Incubation and Sampling in HμREL Coculture. Test compounds were dissolved in DMSO at 10 mM, then diluted in 50% acetonitrile to a concentration of 50 μM, and finally diluted to 2 μM in dosing medium. All steps were performed at room temperature, and the 2 μM test compounds were preheated at 37 °C before start of incubation. Prior to assay, maintenance medium was removed from the cells, followed by a washing step with 100 μL of preheated blank dosing medium without serum. Compound incubation was initiated by addition of 50 μL of dosing medium and 50 μL of dosing medium containing 2 μM test compound. The final test concentration was 1 μM. Plates were kept without shaking in the incubator during the experiments, at 37 °C in humidified atmosphere containing 95% air and 5% CO_2.

For intermediate to high CL_{int} compounds, incubations were performed on culture day 7 (experimental day 1) and repeated on culture day 10 (experimental day 4). Samples of 45 μL were taken from duplicate wells at 5, 20, 40, 60, 120, and 180 min. Low CL_{int} compounds were incubated for 70 h starting on culture day 7 (experimental day 1). Samples of 45 μL were taken from separate wells in duplicates for each time point at 1, 3, 5, 24, 48, and 70 h. The intermediate to high CL_{int} compounds were run at 2 different occasions (validations 1 and 2), and the low CL_{int} compounds were run at 3 different occasions (validations 1, 2, and 3). All samples were quenched with 180 μL of ice-cold acetonitrile containing 0.8% formic acid and 15 nM DDIBA as bioanalytical volume marker. Samples were placed in the freezer (−20 °C) for 20 min and were then centrifuged at 4 °C for 20 min at 3220*g*. Then, clear aliquots of supernatant (50 μL) were diluted in twice the amount of water (100 μL) and kept in the freezer until analysis of parent compound using LC−MSMS.

Compound Incubation and Sampling in Hepatocyte Suspension. Data from suspended hepatocytes were from historical in-house data generated with a standard automated hepatocyte assay at a minimum of three occasions. The 10-donor pool of cryopreserved human hepatocytes (lots IRK and LYB) were thawed and washed in Leibovitz's L-15 medium. The cells were thawed in prewarmed (37 °C) medium and subsequently washed in two steps in room temperature

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medium, whereafter the amount of cells and viability were determined by the trypan blue exclusion method. The cell suspension was diluted to 1 million cells/mL (viability >80%) and transferred into round-bottom 96-well plates (247.5 μL/well). The test compounds were dissolved in DMSO and further diluted in 50% acetonitrile with final concentration of 0.1% DMSO and 1% acetonitrile in the incubation. Incubation was performed at 37 °C with agitation at a frequency of 13 Hz. The hepatocytes were preincubated for 15 min, and the reaction was initiated by addition of one test compound/well giving a final concentration of 1 μM and a final incubation volume of 250 μL/well. Ten samples evenly distributed over 120 min were withdrawn from each well during incubation. At termination, 15 μL of medium was quenched in 45 μL of ice-cold acetonitrile, containing 0.8% formic acid and the internal standards alprazolam (100 nM), caffeine (200 nM), and tolbutamide (100 nM). The samples were centrifuged for 30 min at 4 °C and 3220*g*. Clear aliquots of supernatant were diluted in equal amounts of water and kept at 4 °C until quantification of parent using LC−MSMS.

Bioanalysis. The test compound and bioanalytical internal standard in all samples were analyzed by high performance liquid chromatography connected to a triple quadrupole tandem mass spectrometer employing electrospray ionization and multiple reaction monitoring. A typical bioanalytical setup was an Acquity ultraperformance liquid chromatography (UPLC) system coupled to a Waters Xevo TQ MS mass spectrometer with an atmospheric pressure electrospray interface (Waters, Zellik, Belgium). Separation was performed on a CSH C18 column Waters Acquity UPLC HSS T3 column (50 mm × 2.1 mm, 1.8 μm). A gradient composed of eluent A (H_2O + 0.1% formic acid) and eluent B (acetonitrile +0.1% formic acid) was used. For the first 0.3 min, isocratic conditions were applied with 99.8% eluent A, then a linear gradient increased the organic phase from 0.2% to 95% during 1 min until 1.3 min, followed by a plateau of 5% eluent A for 0.5 min before going back to 99.8% eluent A. For paracetamol, a slightly modified gradient was applied with a first shallow linear gradient from 99.8 to 85% eluent A ramping during 0.3 to 1.8 min, followed by an additional steep linear gradient from 85% to 5% eluent A performed during 0.2 min and with a plateau of 5% eluent A for 0.5 min before going back to 99.8% eluent A. Flow rate was 1 mL/min. The column temperature was set to 40 °C and sample temperature to 10 °C. Waters TargetLynx version 4.1 SCN 905 was used for LC−MSMS system control and data analysis. Optimization of each compound was performed using MassLynx/IntelliStart. The positive and/or negative ionization mode was established using the calculated monoisotopic mass of the compound, the MS/MS data was acquired by selecting the $[M + H]^+$ or $[M - H]^-$ ions, and the declustering potential and collision energy were optimized via the system's step functions. A chromatographic test was performed before analysis. The metabolites formed from the cocktail were quantified using an eight point standard curve. Mass transitions, structures, and additional information for each compound can be found in Tables SI 1 and 2.

Sampling for Metabolite Identification in HμREL Coculture. After removing the samples for CL_{int} determination, samples for metabolite identification at 5, 24, and 70 h were prepared for quinidine, (*S*)-warfarin, metoprolol, paracetamol, lorazepam (only 70 h), and oxaprozin (only 70 h) from the incubation plates by the addition of 165 μL of ice-cold acetonitrile. The plates were placed in the freezer for 20 min (−20 °C) and then centrifuged for 20 min, 4 °C, 4000*g*. The supernatant (100 μL) was transferred to a new 96-well plate and thereafter diluted with an equal volume of water (100 μL) before analysis.

Incubation and Sampling for Metabolite Identification in Hepatocyte Suspension. For the hepatocyte suspension incubations a 10-donor pool of cryopreserved human hepatocytes was used (lot IRK), obtained from Celsis In vitro Technologies (Brussels, Belgium). Leibovitz's L-15 medium, without phenol red, with L-glutamine (Gibco 21083-027), was purchased from Invitrogen (Stockholm, Sweden). The hepatocytes were taken from −150 °C, thawed at 37 °C, and thereafter washed twice with Leibovitz's L-15 medium. After dilution to 1 million cells/mL with Leibovitz's L-15 medium, 245 μL of the hepatocyte suspension was added per well in a 96-deep-well plate. After preincubation the reaction was started by addition of 5 μL of quinidine, (*S*)-warfarin, metoprolol, or paracetamol (final concentration in incubation was 4 μM). After 2 h, the incubation was stopped with addition of 3 parts of ice-cold acetonitrile and thereafter centrifuged for 20 min, 4 °C, 4000*g*, and then the supernatant (100 μL) was transferred to a new 96-well plate and diluted with an equal amount of water (100 μL) before analysis.

Analysis of Metabolite Identification Samples. Analysis was performed using UPLC (ultraperformance liquid chromatography) combined with high resolution mass spectrometry. Chromatographic separations were performed on an Acquity UPLC BEH C18 column with dimensions 2.1 × 100 mm and 1.7 μm particle size (Waters, U.K.). The LC system consisted of a Waters Acquity UPLC binary Solvent Manager, Sample Manager, and Column Manager. The mass spectrometer used was a Synapt G2 Q-TOF (Waters, U.K.) with an electrospray interface (ESI). The software used for instrument control (UPLC system and mass spectrometer), acquisition, and data evaluation was MassLynx (version 4.1, Waters, U.K.). The mobile phases used for analysis were A, 0.1% formic acid, and B, 100% acetonitrile. The UPLC gradient used for quinidine, (*S*)-warfarin, lorazepam, and oxaprozin started with 5% B with a linear increase to 70% B in 6 min, and thereafter B was immediately increased to 90% and retained for 6.7 min. For metoprolol, the gradient started with 5% B with a linear increase to 40% in 5 min, and thereafter B was immediately increased to 90% and retained for 6.7 min. For paracetamol, the gradient started with 1% B with a linear increase to 25% in 5 min, and thereafter B was immediately increased to 90% and retained for 6.7 min. Flow rate was 0.8 mL/min, and the column oven was set to 45 °C and the sample tray temperature to 10 °C. Specific mass spectrometer interface conditions were cone voltage 20 V, capillary voltage 0.5 kV, source temperature 120 °C, desolvation temperature 450 °C, desolvation gas flow 800 L/h, and detector voltage 2200 V. The Q-TOF mass spectrometer was calibrated with sodium formate in the positive mode with a mass window of 50−1200 Da, and leucine enkephalin was used as lock mass for the acquisitions. Positive ion mass spectra were acquired with mass range 80−1200 Da in centroid mode with a scan time of 0.1 s. Different software for metabolite identification combined with manual extraction of expected metabolites, both phase I and phase II, was used to find all metabolites formed in the system.

CALCULATIONS

Calculations of CL_{int}. Calculation of CL_{int} was based on the substrate disappearance rate. The LC−MS peak areas were

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used for the calculations, and a linear plot of ln area vs time was created to visualize the data. The slope was calculated for each data set, and the half-life ($T_{1/2}$) and CL_{int} were calculated using the slope, number of viable hepatocytes (30 000) seeded to each well, and the incubation volume of 100 μL (eq 1 and 2).

$$T_{1/2} = (\ln 2)/-\text{slope} \quad (1)$$

$$CL_{int} = \frac{\ln 2 \times 1000}{T_{1/2}\ (\text{min}) \times \text{cell concn (million cells/mL)}} = \mu\text{L/min /million cells} \quad (2)$$

Each CL_{int} value was calculated from log−linear regression through 12 data points (1, 3, 5, 24, 48, and 70 h in duplicates). As a quality criterion to effectively distinguish a low CL_{int} value from no measurable turnover, a comparison of fits was conducted in GraphPad Prism 6.01 (GraphPad Software, Inc., Suite, USA) using extra sum-of-squares *F*-test, in which the null hypothesis is set to slope = zero. The null hypothesis is not rejected (slope = zero) if the 95% confidence interval slope value is found ranging over the span of zero. This indicates that the CL_{int} value does not differ significantly from zero ($P > 0.05$) and no reliable CL_{int} value can be determined. CL_{int} values are only considered reliable if *P* value < 0.05. The individual CL_{int} curves can be found in Figures SI 1−21.

In Vivo Scaling. The in vivo CL_{int} was predicted from in vitro CL_{int} by applying eq 3. The physiological human scaling factors were 120 million cells/g liver, 1680 g liver weight, and 70 kg body weight. The binding of drugs in blood (fu_b) was measured as previously described by ref 22 or obtained from relevant scientific literature, and incubational binding values (fu_{inc}) were measured according to ref 23 or predicted from lipophilicity data according to what was previously described by ref 24.

$$\text{in vivo } CL_{int} = \frac{CL_{int} \times \frac{120 \times 10^6 \text{ cells}}{\text{g liver}} \times \frac{24\text{g liver}}{\text{kg}} \times fu_b}{fu_{inc}} \text{mL} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \quad (3)$$

The regression line approach was used for the prediction of in vivo CL_{int} as described previously.[6] The regression line coefficients were achieved from data on human hepatocyte incubations in suspension with slope and intercept at 0.7500 and 0.6532, respectively.

Observed human in vivo CL_{int} was calculated by applying the well-stirred model[11,25] (eq 4) using published human clearance data (Table 1).

$$CL_{int,\text{in vivo}} = \frac{CL_b}{\left(1 - \frac{CL_b}{Q_H}\right)} \quad (4)$$

where Q_H is hepatic blood flow (20 mL/min/kg) and CL_b is hepatic blood clearance.

Calculations of Metabolite Formation Rate. The formation rate of formed metabolites was calculated as pmol/min/million hepatocytes using the metabolite concentrations at 60 min and 30 000 hepatocyte per well.

RESULTS

CL_{int} in HμREL Coculture and Cryopreserved Human Hepatocytes. The evaluation of the HμREL coculture system was carried out using three test sets of compounds covering metabolism routes via a range of different CYP and UGT enzymes (Table 1). To evaluate clearance of slowly metabolized drugs in HμREL coculture, test set 1 with 14 compounds with low human in vivo clearance (<5 mL/min/kg) or not measurable CL_{int} in hepatocyte suspension were included (Table 1). The compounds were incubated for 70 h starting on culture day 7 at three independent validation occasions 1, 2, and 3. The CL_{int} data from the low CL_{int} compound set was consistent among the three different validations, and the coefficient of variation was on average 33% with a range of 16−84%, where quinidine was an outlier (84%) and increased the average variation. Oxaprozin, furosemide, diazepam, metoprolol, and lorazepam had CL_{int} values between 1 and 2.5 μL/min/million hepatocytes, whereas (*S*)-warfarin, quinidine, paracetamol, disopyramide, and AZ2 had CL_{int} values below 1 μL/min/million hepatocytes (Figure 1, Table 1). However, CL_{int} values of 0.2 μL/min/million hepatocytes for disopyramide and AZ2 could only be quantified reliably at one occasion, whereas for the other two occasions the data was too noisy for the quantification of such a low value (Figure 1). In HμREL coculture it was possible to robustly determine CL_{int} values of 0.3 μL/min/million hepatocytes and above, whereas CL_{int} values below 0.3 μL/min/million hepatocytes were not always reproducible. Thus, the cutoff value for this system is set to 0.3 μL/min/million hepatocytes. Examples of substrate depletion plots of slowly metabolized drugs are presented in Figure 2.

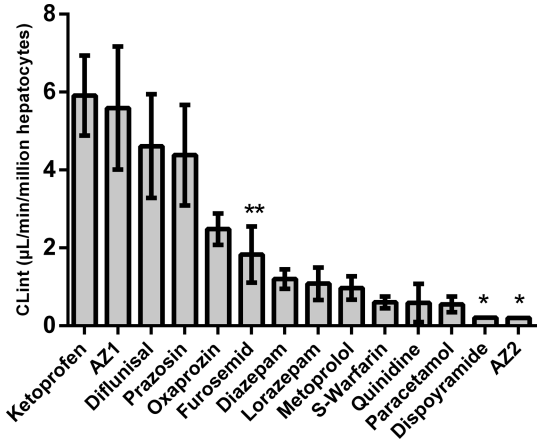

Figure 1. CL_{int} values for slowly metabolized compounds in HμREL coculture from different validation experiments incubated for 3 days. Means ± SD ($n = 3$; *$n = 1$, **$n = 2$).

In test set 2, 7 compounds with higher human in vivo clearance (>5 mL/min/kg) or measurable CL_{int} in hepatocyte suspension were utilized to compare the CL_{int} in HμREL coculture and human hepatocyte suspension (Table 1). The compounds were incubated in HμREL coculture for 3 h on culture day 7 (experimental day 1) and culture day 10 (experimental day 4) in validations 1 and 2 (Figure 3) and additionally for 2 h in hepatocyte suspension at 3 different occasions. The CL_{int} values from the HμREL coculture were reproducible between different experimental days and validations, though in general slightly lower on experimental day 4 than on day 1. The coefficient of variation in CL_{int} values between culture day 7 (experimental day 1) and culture day 10 (experimental day 4) was estimated to be 24% and 10% in validations 1 and 2, respectively. The coefficient of variation in

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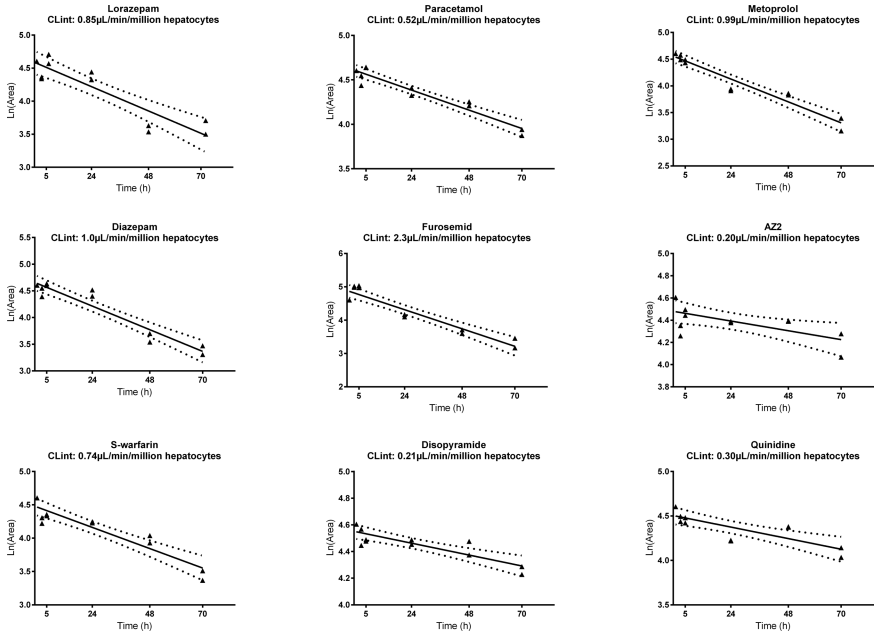

Figure 2. Depletion plots of slowly metabolized drugs with CL_{int} values < 3 µL/min/million cells in HµREL coculture.

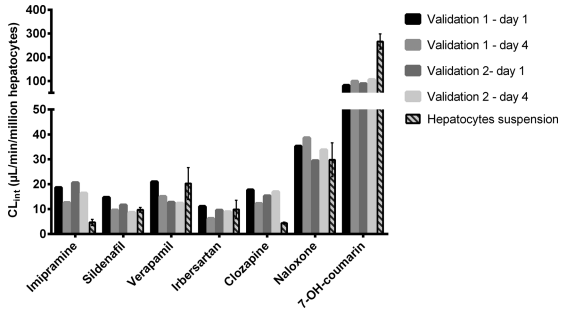

Figure 3. Comparison of CL_{int} values in HµREL coculture at culture day 7 (experimental day 1) and day 10 (experimental day 4) and between validations 1 and 2. CL_{int} values in HµREL coculture are also compared with CL_{int} values in human hepatocyte suspension. Each bar represents calculations from one CL_{int} curve with 12 data points in HµREL coculture and means ± SD from at least three CL_{int} curves in human hepatocyte suspension.

CL_{int} values between validations 1 and 2 was estimated to 15%. In total, the variation between both experimental days and validation sets was 19%.

The data produced in HµREL coculture was generally in agreement with data obtained from experiments using cryopreserved human hepatocytes in suspension. However, imipramine and clozapine had 4-fold lower CL_{int} values in the hepatocyte suspension. In contrast, the CL_{int} values observed for 7-OH-coumarin were 2-fold higher in hepatocyte suspension (Figure 3).

In Vitro in Vivo Clearance Scaling. For in vivo clearance scaling, in vitro and in vivo parameters for 20 of the compounds investigated were obtained from in-house data and the literature (Table 1). The scaling was performed as described in Material and Methods, and 2-fold and 3-fold differences between observed in vivo clearance and predicted in vivo clearance were calculated for all 20 compounds and for the 14 slowly metabolized compounds. For slowly metabolized drugs, 50% and 71% of the predicted values were within 2-fold and 3-fold, respectively (Table 1, Figure 4). The outcome when including all 20 compounds in the calculation was very similar to 45% within 2-fold and 70% within 3-fold of observed in vivo clearance (Table 1, Figure 4).

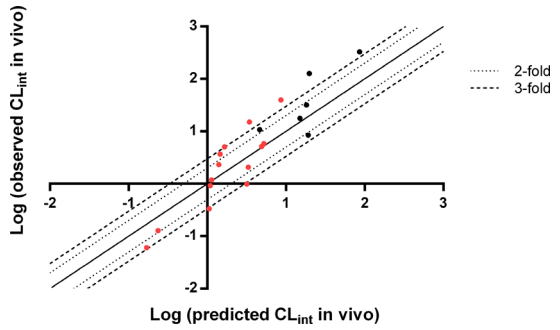

Figure 4. In vitro in vivo correlation of low CL_{int} (red dots) and intermediate to high CL_{int} (black dots) compounds in HµREL coculture.

P450-Specific Activity in HµREL Coculture. To study the activity of the important P450 enzymes expressed in the HµREL coculture, test set 3, a cocktail of P450 substrates, was incubated for 3 h on culture day 7 (experimental day 1). The formation rates of 1-OH-midazolam (3A4), 4-OH-diclofenac (CYP2C9), paracetamol (CYP1A2), 1-OH-bufuralol (CYP2D6), and OH-bupropion (CYP2B6) are shown in Figure 5. The hydroxylation of diclofenac and phenacetin to 4-OH-diclofenac and paracetamol, respectively, was quite extensive (>40 pmol/min/million hepatocytes), whereas the formation of 1-OH-midazolam was more moderate (20 pmol/min/million hepatocytes). For bupropion and bufuralol the hydroxylation was in the lower range (<5 pmol/min/million hepatocytes). The coefficients of variation for metabolite formation between the three different validation sets were on average <10% (1-OH-midazolam 7%, 4-OH-diclofenac 6%, paracetamol 5%, 1-OH-bufuralol 6%, OH-bupropion 13%). The profiles of metabolite formation over time can be found in Figure SI 22.

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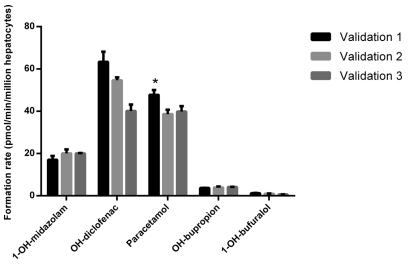

Figure 5. Comparison of the formation rates of 1-OH-midazolam (CYP3A4), 4-OH-diclofenac (CYP2C9), paracetamol (CYP1A2), 1-OH-bufuralol (CYP2D6), and OH-bupropion (CYP2B6) in HμREL coculture at three different occasions. The results are given as means ± SD (n = 2). *Paracetamol formation rate for one replicate was achieved in a different experiment due to analytical problems during validation 1.

Metabolite Identification of Low Turnover Compounds. Samples for metabolite identification were taken at 5, 24, and 70 h for quinidine, (*S*)-warfarin, metoprolol, and paracetamol and at 70 h for lorazepam and oxaprozin at the same incubation conditions as for the CL_{int} determination in the HμREL coculture. In addition, quinidine, (*S*)-warfarin, metoprolol, and paracetamol were incubated for 2 h in hepatocyte suspension to compare the metabolites formed in suspension and HμREL coculture. Metabolite identification was performed using high resolution mass spectrometry, and verification of the metabolites was based on high mass accuracy. All metabolites detected in hepatocyte suspensions were also observed in the HμREL coculture. For quinidine, (*S*)-warfarin, and paracetamol, more metabolites were detected in the HμREL coculture compared to hepatocyte suspension incubations (Table 2, Figure 6), despite the fact that the substrate concentrations were 4 μM in hepatocyte suspension compared to 1 μM in HμREL coculture. The number of metabolites found in the HμREL coculture varied from two metabolites for lorazepam (M1, M2) and paracetamol (M1, M2) up to six metabolites for (*S*)-warfarin (M1, M2, M3, M4, M5, M6) (Table 2, Figure 6). In hepatocyte suspension the number of metabolites varied from one metabolite for (*S*)-warfarin (M6) and paracetamol (M1) up to four metabolites for quinidine (M1, M3, M4, M5) (Table 2, Figure 6). Regarding phase I metabolism, mainly hydroxylations and dealkylations were seen in the HμREL coculture and in hepatocyte suspensions, whereas phase II metabolism represented by glucuronic acid conjugation was mainly observed in the HμREL coculture. The metabolites found were in agreement with the main responsible enzymes for the compounds, with the exception for paracetamol, where no UGT mediated metabolite could be found, and for (*S*)-warfarin, where minor amounts of glucuronides could be found. For quinidine, (*S*)-warfarin, metoprolol, and paracetamol, all

Table 2. Identified Metabolites, Based on High Mass Accuracy Data, of Quinidine, (*S*)-Warfarin, Metoprolol, Paracetamol in Hepatocyte Suspension at 2 h and HμREL Coculture at 5, 24, and 70 h and Lorazepam and Oxaprozin in HμREL Coculture at 70 h

						HμREL coculture (MS area)		
compound	mass trace (M + H)	mass shift	transformation	t_R (min)	hep suspension 2 h[a]	5 h[b]	24 h[b]	70 h[b]
quinidine	325.1904			2.44				
M1	341.1847	(+16)	hydroxylation	1.85	6	29	67	64
M2	357.1790	(+32)	hydroxylations	2.08	0	0	2	11
M3	341.1826	(+16)	hydroxylation	2.46	11	10	17	13
M4	341.1855	(+16)	hydroxylation	2.62	21	2	8	32
M5	343.2021	(+18)	hydroxylation, reduction	2.83	15	0	5	19
(*S*)-warfarin	309.1118			4.60				
M1	309.1265	(+0)		2.19	0	18	298	699
M2	501.1331	(+192)	hydroxylation, glucuronidation	2.75	0	1	1	9
M3	501.1393	(+192)	hydroxylation, glucuronidation	2.91	0	0	4	23
M4	325.1070	(+16)	hydroxylation	3.64	0	6	60	65
M5	325.1070	(+16)	hydroxylation	3.79	0	0	132	192
M6	311.1270	(+2)	reduction	3.90	20	15	23	35
Metoprolol	268.1896			2.97				
M1	284.1849	(+16)	hydroxylation	1.94	38	8	31	68
M2	254.1748	(−14)	demethylation	1.99	36	35	107	313
M3	268.1540	(+0)	demethylation, hydroxylation	2.05	68	15	173	442
paracetamol	152.0669			2.23				
M1	184.0593	(+32)	hydroxylations	1.51	2	12	83	42
M2	194.0805	(+42)		3.70	0	33	58	92
lorazepam	321.0190			3.80				18
M1	497.0513	(+176)	glucuronidation	3.02				44
M2	497.0513	(+176)	glucuronidation	3.06				60
oxaprozin	294.1132			4.93				55
M1	486.1401	(+192)	hydroxylation, glucuronidation	2.46				30
M2	486.1401	(+192)	hydroxylation, glucuronidation	2.84				58
M3	470.1428	(+176)	glucuronidation	4.05				20

[a]Substrate concentration was 4 μM in hepatocyte suspension. [b]Substrate concentration was 1 μM in HμREL coculture.

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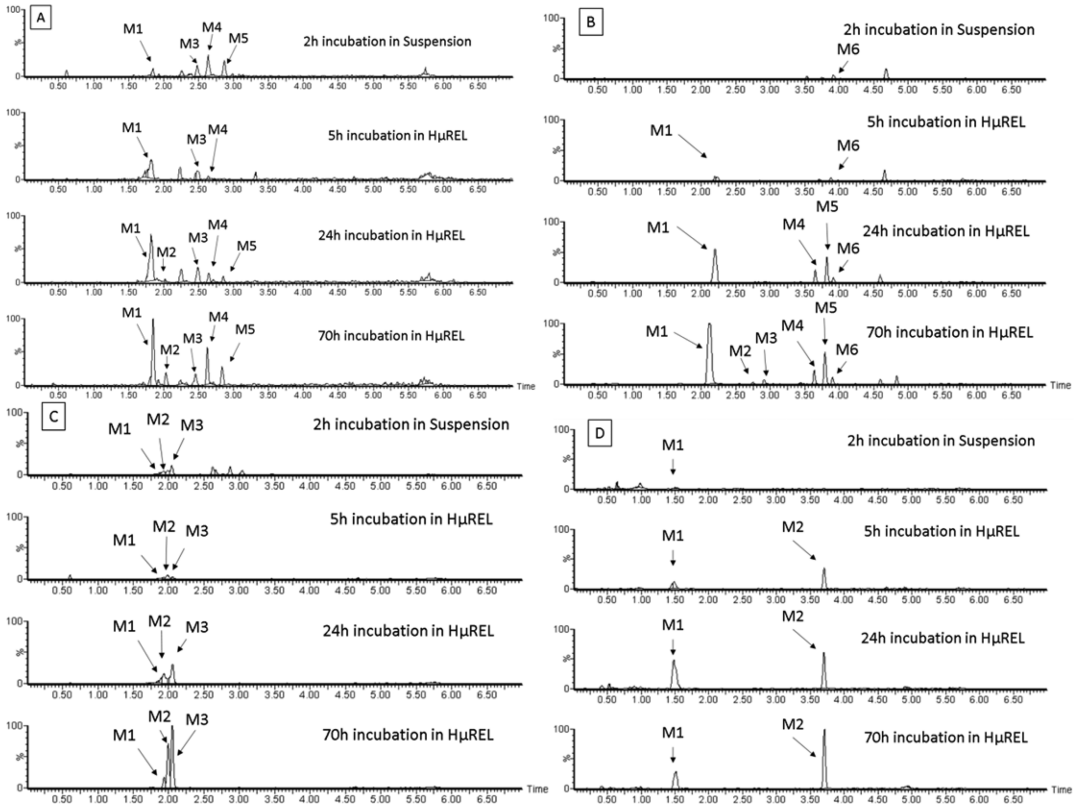

Figure 6. UPLC–MS chromatograms showing the formation of metabolites from incubations in hepatocyte suspension (substrate concentration 4 μM and time point 2 h) and in HμREL coculture (substrate concentration 1 μM and time points 5, 24, and 70 h) for quinidine (A),(*S*)-warfarin (B), metoprolol (C), and paracetamol (D).

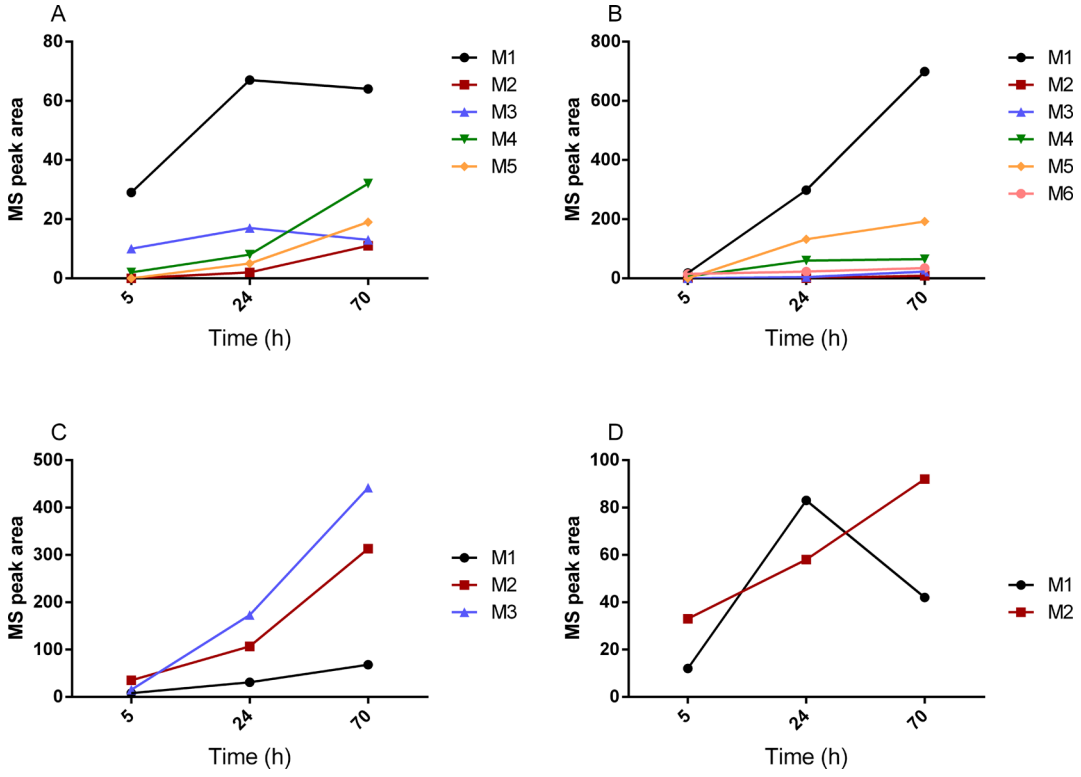

Figure 7. Kinetics of metabolite formation from quinidine (A), (*S*)-warfarin (B), metoprolol (C), and paracetamol (D) in the HμREL coculture.

metabolites found in the HμREL coculture (except M1 for paracetamol) increased in amount by time and the kinetics of the individual metabolite formation could be followed (Figure 7). Due to different ionization properties between the

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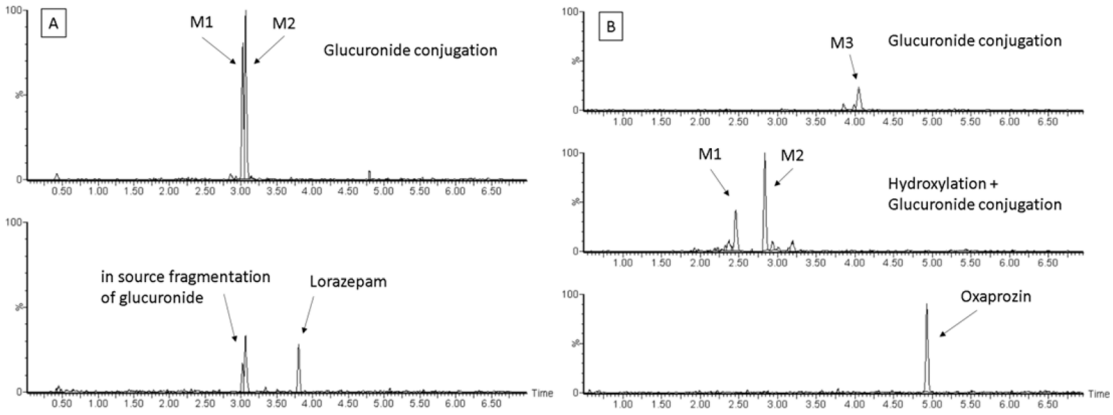

Figure 8. UPLC−MS chromatograms showing parent and metabolite formation from incubations in HμREL coculture for 70 h at a substrate concentration of 1 μM with lorazepam (A) and oxaprozin (B). Direct conjugation with glucuronide is observed for both lorazepam and oxaprozin. A combination of hydroxylation and glucuronide conjugation is also seen for oxaprozin.

metabolites, no quantitative measurement of the kinetics could be performed. In HμREL coculture, both direct glucuronidation and glucuronidation following an initial hydroxylation were observed for lorazepam and oxaprozin, which is in agreement with the main enzymes responsible for the metabolism of the two compounds (Figure 8).

DISCUSSION

Design of slowly metabolized compounds is an important goal in many drug discovery projects. The major finding of the present study was that HμREL coculture of human hepatocytes and stromal cells maintains activity of important drug metabolizing enzyme over time, facilitating clearance predictions of slowly metabolized drugs. Clearance scaling using the well-stirred model gave prediciton accuracy of the human intrinsic clearance for 50% of the drugs within 2-fold and 71% of the drugs within 3-fold of the clinical values. CL_{int} values as low as 0.3 μL/min/million hepatocytes were robustly produced, giving up to 8-fold improvement for CL_{int} determination for low CL_{int} compounds, compared to standard hepatocyte suspension CL_{int} methods. In addition, it was possible to detect metabolites in the HμREL coculture that were not detected in hepatocyte suspension.

Recently, it has been shown that metabolic activity is maintained in cocultures, 3D spheroids, and bioreactors for several days to weeks.[17,18,26−28] However, it is not always clear how the activity is changed compared to freshly isolated or thawed primary human hepatocytes. The present study demonstrated that the CL_{int} in HμREL coculture was comparable to data from hepatocyte suspension for compounds with intermediate to high CL_{int}. This observation highlights the stable metabolic capacity of the hepatocytes in the coculture, evaluated on the first and last experimental days, i.e., culture days 7 and 10, which agrees with performance of freshly thawed hepatocytes in suspension. The enzyme activities were stable between the culture days and between different HμREL experiments. A 5-donor plateable batch was used in the HμREL coculture, whereas a 10-donor batch was utilized in the suspension experiments. Although different batches were utilized, the comparison confirms that the cocultured hepatocytes maintain important phase I and phase II enzyme activities at similar levels as freshly thawed hepatocytes, during the whole culture period and during the 3 days incubation with low CL_{int} compounds. In addition, the specific enzyme activity of the most important P450s was assessed by measuring the formation of paracetamol (CYP1A2), OH-bupropion (CYP2B6), 4-OH-diclofenac (CYP2C9), 1-OH-bufuralol (CYP2D6), and 1-OH-midazolam (CYP3A4) on culture day 7 (start day of experiment) and the enzyme activities were at similar levels in all three experiments. The formation rates in HμREL coculture on culture day 7 compared to the same hepatocyte batch 4 h after plating were 2-fold higher for paracetamol and 7- and 2-fold lower for OH-bupropion and 1-OH-midazolam, respectively (the formation rates 4 h after plating were obtained from Xenotech product datasheet). Although the formation rates for OH-bupropion and 1-OH-midazolam were lower in the HμREL coculture, they were still in the range of observed formation rates at K_m (calculated as $V_{max}/2$) in freshly thawed cryopreserved human hepatocytes from 16 different donors.[29] The formation of 4-OH-diclofenac was also within the formation rates at K_m observed in the hepatocytes from the 16 different donors.[29] Paracetamol formation rate in HμREL coculture was close to the median value in freshly thawed hepatocytes from 9 different donors,[30] whereas 1-OH-bufuralol formation rate was 20-fold lower compared to formation rates at K_m (calculated as $V_{max}/2$) in a 10-donor pooled hepatocyte batch.[31] Thus, among the major P450 enzymes tested, merely CYP2D6 activity was lower in HμREL coculture, whereas CYP1A2, CYP2B6, CYP2C9, and CYP3A4 activities were on comparable levels with freshly thawed human hepatocytes published elsewhere. The maintained enzyme activity at high level is crucial for enabling clearance and metabolite formation of slowly metabolized drugs.

Bonn and co-workers recently compared the CL_{int} of slowly metabolized drugs in plated primary hepatocytes, HepaRG cells, and HμREL cocultures of human hepatocytes and stromal cells.[15] The in vivo clearance prediction of nine drugs having CL_{int} values ranging from undetermined to 38 μL/min/million cells in hepatocyte suspension was within 3-fold for 78% of the drugs for plated hepatocyte and HμREL coculture, whereas 50% was within 3-fold for HepaRG cells. The HμREL coculture provided a larger reproducibility than the other two systems. In addition, the superior longer incubation time in the HμREL coculture, 70 h or longer, compared with 8 h in plated hepatocytes, has been important for some candidate drugs that have been tested in parallel with the validation compounds in the present study (data not shown). The in vivo clearance

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prediction for most of the 8 drugs ((*S*)-warfarin, diazepam, disopyramide, metoprolol, AZ1, ketoprofen, sildenafil, and imipramine) that were included in this and Bonn et al.'s study was similar, although different hepatocyte batches were used, further confirming the reproducibility of the HμREL coculture.[15] Additionally, it is critical to an accurately low CL_{int} determination that the test compound is completely dissolved under assay conditions, to not influence the results through precipitation over time or slow dissolution. In this study all test compounds were confirmed soluble in the internal solubility assay at the tested concentration (1 μM), thus confirming the quality of the reported CL_{int} values.

Two other systems have previously been evaluated for clearance prediction of slowly metabolized drugs, HepatoPac coculture of hepatocytes and fibroblasts and the relay method with hepatocytes in suspension.[14,16,32] The outcome of the in vivo clearance prediction for HepatoPac and the relay method was similar compared to the result in the present study utilizing HμREL coculture. For low clearance drugs, the in vivo clearance prediction was reported within 3-fold for 9 of 10 (90%) drugs in HepatoPac and for 9 of 11 (82%) drugs in the relay method,[14,32] as compared to 10 of 14 (71%) drugs in the present HμREL coculture study. However, an accurate comparison is difficult since the number of compounds is limited in the studies with only a few overlapping compounds. Nevertheless, it appears as if all three methods can be utilized to address low clearance challenges for slowly metabolized drugs. However, when comparing different low CL_{int} methods in-house, HμREL coculture, which is delivered ready to use on culture day 6, was more straightforward to use compared to the relay method. Further, cocultures with maintained enzyme and transporter activity enable induction, drug transport, and toxicity evaluations, which may not be possible in the relay method.

In this study, HμREL coculture has only been validated for human in vivo clearance predictions. However, the confidence in the in vivo clearance prediction can be increased by including at least two preclinical species, normally rats and dogs,[32] and a validation of HμREL coculture with rat and dog hepatocytes may further improve in vivo clearance predictions. Di and co-workers (2013) showed that when applying the relay method with cryopreserved human, rat, and dog hepatocytes, the best human in vivo prediction was obtained when both rats and dogs demonstrated strong in vivo in vitro correlation for a certain compound.[32]

In addition to clearance prediction of slowly metabolized drugs, the relay method and different cocultures are superior to suspended hepatocytes and microsomes in predicting human in vivo metabolites.[13,33,34] An extensive evaluation with 27 drugs in HepatoPac coculture showed that the identified major human metabolites (>10% of dose) increased on average from 64 to 82% for excretory metabolites and 53 to 75% for circulatory metabolites when the drugs were incubated for 7 days in the coculture compared to 2 h in suspended human hepatocytes.[33,35] To challenge HepatoPac, the relay method was performed on three of the drugs, capromorelin, CP-122721, and ziprasidone, for which only 50% of the human-relevant metabolites were captured in the HepatoPac. The success rate for detection of major in vivo metabolites for these three drugs was increased from 50% to 75% with the relay method.[34]

In the present HμREL coculture study, the remaining cells and medium, after removal of the CL_{int} medium samples at 5, 24, and 70 h, were analyzed for formed metabolites and compared with metabolite formation during a 2 h incubation in suspended hepatocytes. Both phase I and phase II metabolism could be detected and followed over time, and an increased number of metabolites as well as higher amounts of metabolites from slowly metabolized drugs were observed in the HμREL coculture compared to hepatocyte suspension. The formation over time can be used as support when identifying unknown metabolites from background peaks, but due to different ionization properties between the metabolites it cannot be used for quantitation of CL_{int} values. The possibility to analyze metabolites formed at the same time and conditions as the CL_{int} determinations secures the relevance of the data collected with respect to drug kinetics. Structural information gained from the metabolite identification using high mass accuracy data in combination with fragmentation data can be used for the design of new compounds and guide the design toward less reactive compounds. Prior to exposing humans for the first time to a drug, it is essential to secure that all major human metabolites are covered in the preclinical species used for in vivo toxicology studies. The cross-species comparison including hepatocytes from preclinical toxicity species and human is crucial to judge if it is safe to enter clinical trials. The use of the relay method and cocultures with different preclinical species, e.g., from Hepregen and HμREL, have a great potential to increase the in vitro generation of major human metabolites, thus reducing the risk of not covering human metabolites in toxicity studies.[13]

In conclusion, we showed that the HμREL coculture is a robust and reliable method for human in vivo clearance prediction and metabolite detection of slowly metabolized drugs. HμREL coculture has similar phase I and phase II enzyme activity as freshly thawed hepatocytes for at least 10 days, which enables a range of in vitro studies to predict the human in vivo situation. Prediction of induction, drug−drug interaction, metabolites, drug transport, and toxicity could all be improved in a system with polarized human hepatocytes that maintains enzyme activity at a high level.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.6b00396.

Additional information for each compound, individual CL_{int} curves, and profiles of P450 specific metabolite formation over time (PDF)

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‡I.H. and C.V.: equal contribution.

Notes

The authors declare no competing financial interest.

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suspended hepatocytes. Furthermore, Constanze Hilgendorf and Richard Thompson are gratefully acknowledged for fruitful discussions and input on this manuscript.

■ ABBREVIATIONS USED

CL_{int}, intrinsic clearance; CYP, cytochrome P450; fu_b, binding of drugs in blood; fu_{inc}, incubational binding; $T_{1/2}$, half-life; UGT, UDP-glucuronosyltransferase; P450, cytochrome P450

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