Primary Human Hepatocyte Spheroids as an In Vitro Tool for Investigating Drug Compounds with Low Hepatic Clearance

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ABSTRACT

Characterizing the pharmacokinetic properties of drug candidates represents an essential task during drug development. In the past, liver microsomes and primary suspended hepatocytes have been extensively used for this purpose, but their relatively short stability limits the applicability of such in vitro systems for drug compounds with low metabolic turnover. In the present study, we used three-dimensional primary human hepatocyte spheroids to predict the hepatic clearance of seven drugs with low to intermediate clearance in humans. Our results indicate that hepatocyte spheroids maintain their in vivo–like phenotype during prolonged incubations, allowing to monitor the depletion of parent drug for 7 days. In contrast, attempts to increase the relative metabolic capacity by pooling hepatocyte spheroids resulted in an immediate fusion of the spheroids followed by hepatocellular de-differentiation processes, demonstrating limited applicability of the pooling approach for quantitative pharmacokinetic studies. The hepatic clearance values obtained from incubations with individual spheroids were in close correlation with the clinical reference data, with six out of seven drug compounds being predicted within a 3-fold deviation and average fold and absolute average fold errors of 0.57 and 1.74, respectively. In conclusion, the hepatocyte spheroid model enables accurate hepatic clearance predictions for slowly metabolized drug compounds and represents a valuable tool for determining the pharmacokinetic properties of new drug candidates as well as for mechanistic pharmacokinetic studies.

SIGNIFICANCE STATEMENT

Traditional in vitro systems often fail to predict the hepatic clearance of slowly metabolized drug compounds. The current study demonstrates the ability of primary human hepatocyte spheroids to provide accurate projections on the hepatic clearance of drug compounds with low and intermediate clearance.

Introduction

Appropriate absorption, distribution, metabolism, and excretion (ADME) properties are crucial for selecting drug candidates and avoiding late-stage failures of drug development programs. Therefore, new chemical entities routinely undergo in vitro high-throughput ADME screenings at early drug discovery stages to identify and eliminate compounds with undesirable pharmacokinetic properties, such as short half-life and poor bioavailability. During lead optimization, medicinal chemists further improve the metabolic stability and other pharmacokinetic properties of lead compounds (Obach et al., 1997; Kratochwil et al., 2017). Consequently, a large number of compounds entering preclinical development display low or no turnover in metabolic stability assays typically conducted using liver microsomes in the presence of nicotinamide adenine dinucleotide phosphate (Di and Obach, 2015).

Although prolonged metabolic stability is a highly desirable pharmacokinetic attribute allowing for a once daily dosing regimen, the characterization of such low clearance compounds is extremely challenging for ADME scientists during the later preclinical development, since common in vitro systems as used in screening assays are no longer applicable. The preclinical testing is further challenged by the fact that not only slowly metabolized compounds but also compounds eliminated by nonoxidative metabolism (e.g., phase II conjugation reactions by uridine 5′-diphospho-glucuronosyltransferases) and pathways beyond hepatic metabolism (e.g., transporter-mediated permeability or extrahepatic metabolism) are preferably selected by the microsomal screening approach (Argikar et al., 2016). For instance, uridine 5′-diphospho-glucuronosyltransferase-mediated metabolism can be covered in microsomal incubations by adding corresponding cofactors, whereas microsomal fractions lack cytosolic enzymes and are likewise not applicable for compounds with low (rate-limiting) active and/or passive permeability (Kusuhara and Sugiyama, 2009; Camenisch, 2016). The use of primary hepatocytes can partly meet these challenges, as they express the entirety of hepatic drug-metabolizing enzymes and transporters, yet primary hepatocytes rapidly de-differentiate in suspension or 2D monolayer development, and the activity of enzymes and transporters begins to decrease within a few hours (Di and Obach, 2015). For drug compounds with low metabolic clearance in vitro, this period is too short to achieve sufficient depletion of parent drug compound that would allow for reliable intrinsic clearance (CLint) estimations (Chan et al., 2013; Kratochwil et al., 2017).

Recently, three-dimensional spheroid cultures of primary human hepatocytes have been established allowing the culture of phenotypically

ABBREVIATIONS: AAFE, absolute average fold error; ADME, absorption, distribution, metabolism, and excretion; AFE, average fold error; CLh, hepatic organ clearance; CLint, intrinsic clearance; Eh, hepatic extraction ratio; IVIVE, in vitro–in vivo extrapolation; MPCC, micropatterned coculture; P450, cytochrome P450; UPLC, ultra-performance liquid chromatography; UPLC-MS/MS, UPLC–tandem mass spectrometry.
stable hepatocytes for several weeks (Lin and Chang, 2008; Messner et al., 2013; Bell et al., 2016). Extensive characterization studies by our laboratory revealed that primary human hepatocyte spheroids maintain their morphology, viability, hepatocellular function, and the activity of drug-metabolizing enzymes over time, closely resembling hepatocytes in vivo (Bell et al., 2016, 2017; Vorrink et al., 2017). Moreover, we have successfully applied hepatocyte spheroids for hepatotoxicity screenings and mechanistic investigations as well as for the modeling of liver diseases (Hendriks et al., 2016; Kozyra et al., 2018; Vorrink et al., 2018; Hendriks et al., 2019; Hurrell et al., 2020).

In the present study, we established primary human hepatocyte spheroids as a novel in vitro-in vivo extrapolation (IVIVE) tool for predicting the hepatic clearance of low and intermediate turnover drugs. After the functional validation of hepatocyte spheroids, the \( CL_{\text{int}} \) of seven drugs with low and intermediate clearance, which are metabolized by different cytochrome P450 (P450) enzymes, was determined in hepatocyte spheroids from three different donors and compared with clinical metabolizing enzymes and transporters.

Materials and Methods

Cell Culture. Cryopreserved primary human hepatocytes were obtained from BioreclamationIVT (USA) or KalyCell (France). Donor information of hepatocyte lots are summarized in Supplemental Table 1. Hepatocytes were thawed according to the manufacturer’s instruction and hepatocyte spheroids were generated as described previously (Bell et al., 2016). In brief, hepatocytes from individual donors were seeded in culture media (Williams’ E medium (Life Technologies, Thermo Fisher Scientific) supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 100 mM dexamethasone, 5.5 μg/mL transferrin, 6.7 mg/mL sodium selenite, and 10% fetal bovine serum) in 96-well ultra-low attachment plates (Corning, USA) at a density of 1500 viable cells per well. After cell aggregation on day 5, the media was replaced by serum-free culture media, and drug exposures were initiated on day 7.

Media-Loss Assay. The in vitro \( CL_{\text{int}} \) of seven drugs with low \{apixaban (CYP3A4), carbamazepine (CYP3A4), clozapine (CYP1A2), elamipretide (CYP2D6)\} and intermediate \{nortriptyline (CYP2D6), risperidone (CYP2D6), venlafaxine (CYP2D6/CYP2C19)\} clearance was estimated during up to 7 days. Hepatocyte spheroids were incubated in 100 μL serum-free culture media containing either 1 μM apixaban, 1 μM carbamazepine, 1 μM clozapine, 1 μM nortriptyline, 1 μM risperidone, or 1 μM venlafaxine at 37 °C and 5% CO2 in a humidified incubator. Nominal drug concentrations were at least 10-fold below the reported Michaelis-Menten constant (Km) (Ring et al., 1996; Limet and Olesen, 1997; Fogelman et al., 1999; Venkatakrishnan et al., 1999; Ya-sui-Furukori et al., 2001; Cazali et al., 2003; Wang et al., 2010) to avoid saturation of drug-metabolizing enzymes (Obach, 2001). The DMSO concentration was adjusted to 0.2% in all procedures and UPLC-MS/MS instrumentation validated and certified by the manufacturer’s instructions.

UPLC-MS/MS/MS Analysis. Test drugs were analyzed using sample preparation procedures and UPLC-MS/MS instrumentation validated and certified for therapeutic drug monitoring purposes. Samples were mixed with equal volumes of cold acetonitrile/methanol (90:10) containing internal standard and were mixed for 5 seconds, frozen for 10 minutes at −20 °C, and centrifuged at a speed of 3100 × g for 15 minutes at 4 °C, and drug concentrations were quantified in the supernatant by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS).

Results

Viability and Functionality of Hepatocyte Spheroids. Hepatocyte spheroids have previously been shown to maintain their phenotype and the activity of drug-metabolizing enzymes over several weeks (Bell et al., 2016), enabling chronic drug exposures for hepatotoxicity

\[
CL_{\text{int}} = \frac{-k_e \times 10^6 e_{\text{pred, obs}} \times 120 \times 10^6 \text{cells} \times g \text{ liver}}{25.7 \times g \text{ bodyweight}} \quad (1)
\]

where \( k_e \) is the elimination rate constant representing the slope of the linear regression from the in-transformed percentage of remaining parent drug versus incubation time. Scaling factors for hepatocellularity and liver weight were taken from Smith et al. (2008) and Davies and Morris (1993), respectively. Incubation binding was taken into account by determining parent drug concentrations in media at early time points (i.e., 10 minutes after addition of the dosing solution) representing unbound initial drug concentrations in serum-free media. The hepatic organ clearance \( (CL_{\text{org}}) \) was predicted using the well-stirred liver model according to eq. 2 (Pung and Rowland, 1977):

\[
CL_{\text{org}} = \frac{Q_h \times f_{\text{us}} \times CL_{\text{int}}}{Q_h + f_{\text{us}} \times CL_{\text{int}}} \quad (2)
\]

where \( Q_h \) is the hepatic blood flow (20.7 mL/min/kg) and \( f_{\text{us}} \) is the unbound fraction in blood (Supplemental Table 2). Clinical pharmacokinetic parameters used to derive the clinical reference \( CL_{\text{org}} \) data as well as corresponding literature references are provided in Supplemental Table 2. Clinical \( CL_{\text{org}} \) values were derived from the total oral plasma clearance, taking into account the oral bioavailability and the fractional contribution of renal drug clearance assuming only hepatic and renal drug elimination and a human body weight of 70 kg as described in the Supplemental Material. The classification of low and intermediate clearance was derived based on the corresponding hepatic extraction ratio \( (E_{\text{h}}) = (CL_{\text{h}} - CL_{\text{us}})/CL_{\text{h}} \) in humans, where low \( (CL_{\text{h}} < 2.3 \text{ mL/min/kg}) \) and intermediate hepatic clearance \( (6.2 < CL_{\text{h}} < 14.5 \text{ mL/min/kg}) \) refers to \( E_{\text{h}} < 0.3 \) or \( E_{\text{h}} < 0.7 \), respectively (Benet and Zhu-Amaros, 1995).

The statistical significance of parent drug depletion over time was determined using an F-test (GraphPad Prism 9.0.1, Suite, US). Slopes of the concentration-time profiles were considered to be significantly nonzero if a \( P \) value of <0.05 was obtained. The accuracy of \( CL_{\text{org}} \) predictions was assessed by calculating the average fold error (AFE) and absolute average fold error (AAFE) according to eq. 3 and eq. 4, respectively:

\[
\text{AFE} = \left( \frac{\sum_{i=1}^{n} |y_{\text{pred},i} - y_{\text{obs},i}|}{\sum_{i=1}^{n} y_{\text{obs},i}} \right) \times 100 \quad (3)
\]

\[
\text{AAFE} = \left( \frac{\sum_{i=1}^{n} |y_{\text{pred},i} - y_{\text{obs},i}|}{\sum_{i=1}^{n} y_{\text{obs},i}} \right) \times 100 \quad (4)
\]

where \( p \) and obs are the predicted and observed \( CL_{\text{org}} \) values, respectively, and \( N \) is the number of data points.

Cell Viability. The viability of hepatocyte spheroids was assessed by determining the cellular ATP content using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instructions. Luminescence per spheroid was measured on a Microbeta LumijET 2400 Microplate Counter (Perkin Elmer).

Albumin Secretion. The secretion of albumin by hepatocyte spheroids was determined by quantifying the albumin concentration in the cell culture medium using the Quanti LSys Reagent (Quagen). RNA was reverse-transcribed into cDNA using SuperScript III reverse transcriptase and analyzed by reverse transcription polymerase chain reaction using a TaqMan Universal mix and Taqman probes (Supplemental Table 3) on a 7500 Fast Real-Time PCR system. Data analysis was performed using the \( 2^{-\Delta\Delta C_{T}} \) method and TATA-binding protein as housekeeping gene.

CYP3A4 Protein Expression. Total protein was extracted from hepatocyte spheroids using RIPA Lysis and Extraction buffer (Thermo Fisher Scientific) supplemented with Complete protease inhibitor (Roche, Switzerland). The protein content was determined using a Bradford assay, and 25 μg of protein was separated using a 10% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad Laboratories) and transferred onto an AmershamPortran membrane (GE Healthcare Life Sciences, UK). The membrane was incubated with anti-CYP3A4 (1:1000, Cypex, UK) and antivinculin (1:10,000, Abcam, UK) followed by goat anti-rabbit IgGhorseradish peroxidase (1:10,000, DAKO, Denmark) and was visualized using Super Sig-

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In contrast, the CYP3A4 protein expression did not decrease in spheroids and CYP3A4 protein detectable by Western blot after 5 and 7 days (Fig. 2E). The expression of CYP3A4, which was analyzed as a representative P450 enzyme, was detected during 7 days. Similarly, the protein expression of polypeptide 1B1 was detected during 7 days. Furthermore, the mRNA expression of major hepatic P450 enzymes A and B during 7 days, whereas the cellular ATP content in pooled spheroids decreased about 2-fold in pooled spheroids as compared with individual spheroids entirely fused, forming solid and compact aggregates (Fig. 2A). In addition, the cellular ATP content indicated stable cell viability of pooled spheroids from donor A and B during 7 days, whereas the cellular ATP content in spheroids from donor C decreased by about 40% within 7 days after pooling (Fig. 2C). Furthermore, the mRNA expression of major hepatic P450 enzymes and uptake transporters was analyzed during 7 days after pooling (Fig. 2D), indicating a rapid decrease in CYP3A4, CYP2C8, CYP2C9, and CYP1A2 mRNA expression. In contrast, the CYP2C19, CYP2D6, and organic cation transporter 1 mRNA expression decreased less than 2-fold, and no change in the mRNA expression of organic anion transporting polypeptide 1B1 was detected during 7 days. Similarly, the protein expression of CYP3A4, which was analyzed as a representative P450 enzyme, remained unaltered after pooling the spheroids with hardly any CYP3A4 protein detectable by Western blot after 5 and 7 days (Fig. 2E). In contrast, the CYP3A4 protein expression did not decrease in spheroids from the same donor, which were cultured in individual wells.

**In Vitro CL_{hu} Incubations and Prediction of CL_{hu}** To examine the feasibility of CL_{hu} predictions, seven test drugs with reported low to intermediate hepatic clearance were incubated with individual hepatocyte spheroids, and the disappearance of parent drug was monitored over time (Fig. 3). The low clearance drugs apixaban, carbamazepine, and clozapine (reported CL_{hu} in humans ranging from 0.110 to 3.55 mL/min/kg, Table 1) were incubated with spheroids from donors A, B, and C during 7 days without medium change to allow for sufficient removal of parent drug from the incubations. Significant depletion of three out of four low clearance drugs was observed in hepatocyte spheroids from all three donors, and concentration-time profiles are shown in Fig. 3, A–D. Exception was carbamazepine, for which no significant depletion of parent drug was measured in hepatocyte spheroids from any of the tested donors. Although not significant, a small decline in parent drug concentrations over time was detected in incubations with hepatocyte spheroids from donors A and C, and the obtained in vitro data are shown in Table 1, yet carbamazepine was excluded for the subsequent comparison with clinical reference data and further statistical tests. In contrast, no depletion of carbamazepine was observed in donor B (data not shown), whose concentration-time profile was not considered for the predicted CL_{hu} value presented in Table 1.

In addition, the intermediate clearance drugs nortriptyline, risperidone, and venlafaxine (reported CL_{hu} in humans ranging from 6.68 to 8.79 mL/min/kg, Table 1) were investigated in hepatocyte spheroids from donor C. All three drug compounds are predominately eliminated by the highly polymorphic CYP2D6 enzyme (Fogelman et al., 1999; Venkatakrishnan et al., 1999; Yasui-Furukori et al., 2001). To facilitate the comparison with clinical CL_{hu} reference data, incubations were conducted in donor C only, which was identified to inhere the CYP2D6 extensive metabolizer (wild-type) genotype (Supplemental Table 1). Similar to previous incubations, parent drug depletion was observed for all three test compounds over time.
yet, the in vitro half-life was generally markedly lower than for the investigated low clearance drugs (Table 1). Accordingly, incubations with the intermediate clearance drugs could be terminated already after 3–4 days.

Next, we performed CL\text{h} predictions based on the in vitro CL\text{int} data and compared the obtained values with clinical reference data (Table 1). The predicted CL\text{h} values ranged from 0.434 to 1.64 mL/min/kg and from 3.00 to 5.59 mL/min/kg for drugs with low and intermediate clearance, respectively. The direct comparison with clinical reference data indicated a close correlation between predicted and observed values with an AFE of 0.57, an AAFE of 1.74, and six out of seven drug compounds predicted within a 3-fold deviation when excluding carbamazepine (Fig. 4).

To investigate potential changes in the metabolic activity after the pooling of hepatocyte spheroids, we monitored the depletion of the low clearance drugs apixaban, carbamazepine, clozapine, and olanzapine in pooled spheroids from donors A, B, and C during 7 days. The drug compounds were cleared from the media to a higher extent in all incubations with pooled spheroids as compared with individual spheroid incubations (substrate depletion-time profiles are shown in Supplemental Fig. 1). Yet, after taking the hepatocyte number into account, the CL\text{int} data derived from pooled spheroids were lower, notably underestimating the hepatic clearance of the majority of the investigated compounds. The fold deviation between predicted and observed CL\text{h} data ranged from 0.1 to 0.6, and the AAFE increased to 4.95 for the four test drugs (Table 1).

Discussion

Anticipating the clearance of slowly metabolized drugs is particularly challenging, as common in vitro systems feature short-term stability and do not allow to reliably determine the clearance of such compounds (Di and Obach, 2015). Here, we established primary human hepatocyte spheroids as a new IVIVE tool for predicting the hepatic clearance of compounds with low and intermediate metabolic turnover. Hepatocyte spheroids demonstrated phenotypical stability during 7 days allowing for extended incubations to quantify the disappearance of parent drug from the culture media. Using this experimental setup, we were able to determine the in vitro CL\text{int} of six low and intermediate clearance drugs from individual spheroids and successfully predicted the hepatic
clearance of these compounds within a 3-fold deviation from the clinical reference data (AAFE of 1.74).

The demands for in vitro ADME test systems undergo fundamental changes as current drug discovery programs generate compounds with uncommon physicochemical properties from unprecedented chemical space (Tu et al., 2013). Nonoxidative metabolism and transporter-mediated pathways like sinusoidal uptake or renal and biliary secretion are often involved in the elimination of such compounds. These processes can largely be covered in profiling assays by using more holistic in vitro systems such as primary hepatocytes or cytosolic fractions (Sahi et al., 2010). Furthermore, static hepatic clearance models such as the Extended Clearance Model have been developed, allowing the integration

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**Fig. 3.** Substrate depletion-time profiles in hepatocyte spheroids. (A–D) Depletion of compounds with low hepatic clearance in individual hepatocyte spheroids from donors A, B, and C. (E–G) Depletion of compounds with intermediate hepatic clearance in individual hepatocyte spheroids from donor C (data obtained in two independent experiments). Parent drug concentrations were quantified in cell culture media, and CL_{int} was estimated from the linear phase of parent drug depletion as described in Materials & Methods. Data points represent mean values of duplicates ± S.D. A, donor A; O, donor B; ◇ and □, donor C.
Comparison of predicted and observed CLh data. Data points represent mean CLh values ± S.D. obtained using hepatocyte spheroids from donors A, B, and C (apixaban, carbamazepine, clozapine, olanzapine) or mean values ± S.D. obtained using hepatocyte spheroids from donor C in two independent experiments (nortriptyline, risperidone, venlafaxine). Literature references for clinical CLh values are provided in Supplemental Table 2.

![Fig. 4. Comparison of predicted and observed CLh data. Data points represent mean CLh values ± S.D. obtained using hepatocyte spheroids from donors A, B, and C (apixaban, carbamazepine, clozapine, olanzapine) or mean values ± S.D. obtained using hepatocyte spheroids from donor C in two independent experiments (nortriptyline, risperidone, venlafaxine). The solid line is the line of unity and dotted lines represent 3-fold deviations. api, apixaban; clo, clozapine; nor, nortriptyline; ola, olanzapine; ris, risperidone; ven, venlafaxine. The use of commercial long-term in vitro liver systems for pharmacokinetic studies continuously increases, and most often micropatterned cocultures (MPCCs) of primary human hepatocytes with stromal cells are applied. MPCC systems likewise feature long-term stability and have been successfully used for IVIVE of low turnover compounds (Chu et al., 2013; Hultman et al., 2016; Umehara et al., 2020). These studies yielded overall a comparable accuracy with 70%–92% of compounds...
being predicted within a 3-fold deviation, whereby our spheroid model predicted 86% of compounds within a 3-fold deviation. Nevertheless, it has to be noted that the selection of compounds differed between studies. In particular, Umehara et al. (2020) included a diverse set of uptake transporter and non-P450 substrates, whereas our compound set consisted of P450 substrates. A face-to-face comparison of a harmonized set of compounds and the same hepatocyte lots would be required to directly compare the performance of hepatocyte spheroid and MPCC models. Similarly, another recent study by Kanebratt et al. (2021) applied hepatocyte spheroids to predict the clearance of compounds with low turnover. Despite introducing an additional empirical scaling factor to account for systemic underprediction, their study achieved fewer accurate predictions as indicated by AFE and AAFe values of 0.53 and 2.7, respectively, whereas our study obtained AFE and AAFe values of 0.57 and 1.74, respectively, without applying empirical scaling factors. Furthermore, the study by Kanebratt et al. (2021) indicated a trend toward more pronounced deviation between predicted and observed clearance data for drug compounds with higher clearance, which was not evident in our study. Interestingly, the authors pooled three spheroids per incubation to increase the metabolic capacity and observed comparable CLint data using pooled and individual hepatocyte spheroids. Our attempts to improve the cell-to-media volume ratio provided markedly lower CLint values in pooled spheroids (Table 1). Follow up investigations on the effect of pooling spheroids indicated merging of the individual spheroids within 24–48 hours, which was accompanied by reduced albumin secretion as well as reduced mRNA expression of major hepatic drug-metabolizing enzymes and uptake transporters as well as reduced CYP3A4 protein expression (Fig. 2). The fusion of spheroids likely introduced major rearrangements in the cellular structures resulting in de-differentiation and entire loss of the hepatocellular phenotype. We did not investigate whether the hepatocellular phenotype reverses over time (like during normal spheroid formation), however, hepatocytes in the center of the fused spheroid structure are likely not sufficiently supplied with oxygen and nutrients due to the larger spheroid diameter (Lin and Chang, 2008) and will be susceptible to early cell death. Consequently, we recommend to avoid pooling of spheroids, but to perform extended incubations with individual spheroids to allow for sufficient parent drug depletion. As an alternative, plates with integrated microwells could be considered (Wassmer et al., 2020).

The good performance of our model might result from the ability of hepatocyte spheroids to continuously secrete albumin at in vivo rate (Fig. 1). The presence of albumin (or other protein) in the medium is assumed to support the cellular uptake, a process known as protein-mediated uptake. The underlying mechanism is not fully understood and current hypotheses were reviewed in detail elsewhere (Bowman and Benet, 2018; Bietieh et al., 2019). In disagreement with the free-drug hypothesis (Pang and Rowland, 1977), different studies observed that cellular uptake rates decreased less than expected in the presence of plasma protein when considering the unbound drug concentration, and this effect was increased for compounds with higher plasma protein binding (Miyauchi et al., 2018; Poulin and Haddad, 2018; Bowman et al., 2019). Furthermore, underprediction of drug clearance by in vitro approaches was reported to be proportional to the extent of plasma protein binding (Liang et al., 2020). In line with these observations, a recent study applying MPCC reported superior clearance predictions using hepatocyte donors with high albumin secretion rate (Da-Silva et al., 2018), and likely both MPCC and spheroids models profit from the integrated albumin secretion. On the other hand, the increasing concentrations of albumin over time complicate the estimation of intrinsic clearance, as the unbound fraction of drug in the media changes during the incubation. In the present study, initial unbound drug concentrations in the media were determined from supernatant sampled from the assay plate shortly upon applying the dosing solution, assuming complete absence of protein in the media. With regard to the very low albumin concentrations in the media observed after 7 days and the rather small degree of plasma protein binding of the investigated compounds (fraction unbound in plasma ≥0.05, Supplemental Table 2), no effects of albumin on the fraction unbound in the media and obtained CLint estimates are expected for our dataset. To account for the time-dependent increase of albumin for drugs with extensive plasma protein binding, measured drug concentrations in the media could be corrected with the fraction unbound for the respective albumin concentration (estimated using plasma protein binding data).

In conclusion, we demonstrated that primary human hepatocyte spheroids remain phenotypically stable for up to 7 days without medium change allowing for extended incubations to accurately predict the hepatic clearance of drug compounds with low to intermediate hepatic clearance. Hepatocyte spheroids represent a valuable IVIVE tool and are expected to significantly facilitate the characterization of slowly metabolized compounds to improve IVIVE during pharmaceutical development.

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Authorship Contributions

Participated in research design: Riede, Wollmann, Molden, Ingelman-Sundberg.

Conducted experiments: Riede, Wollmann.

Performed data analysis: Riede, Wollmann.

Wrote or contributed to the writing of the manuscript: Riede, Wollmann, Molden, Ingelman-Sundberg.

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