Impact of Intracellular Concentrations on Metabolic Drug-Drug Interaction Studies

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Abstract. Accurate prediction of drug-drug interactions (DDI) is a challenging task in drug discovery and development. It requires determination of enzyme inhibition in vitro which is highly system-dependent for many compounds. The aim of this study was to investigate whether the determination of intracellular unbound concentrations in primary human hepatocytes can be used to bridge discrepancies between results obtained using human liver microsomes and hepatocytes. Specifically, we investigated if Kp uu could reconcile differences in CYP enzyme inhibition values (K i or IC 50). Firstly, our methodology for determination of Kp uu was optimized for human hepatocytes, using four well-studied reference compounds. Secondly, the methodology was applied to a series of structurally related CYP2C9 inhibitors from a Roche discovery project. Lastly, the Kp uu values of three commonly used CYP3A4 inhibitors—ketoconazole, itraconazole, and posaconazole—were determined and compared to compound-specific hepatic enrichment factors obtained from physiologically based modeling of clinical DDI studies with these three compounds. Kp uu obtained in suspended human hepatocytes gave good predictions of system-dependent differences in vitro. The Kp uu was also in fair agreement with the compound-specific hepatic enrichment factors in DDI models and can therefore be used to improve estimations of enrichment factors in physiologically based pharmacokinetic modeling.

KEY WORDS: drug-drug interaction; intracellular bioavailability; physiologically based pharmacokinetic modeling; scaling factor; unbound drug concentrations.

INTRODUCTION

Accurate predictions of drug-drug interactions (DDIs) are a challenging task during drug development because the relevant inhibitor concentration is not directly accessible (1, 2). Predictions of DDIs depend on in vitro parameters such as the half-maximal inhibitory concentration (IC 50) or the inhibition constant (K i) (3). These in vitro values are then used in physiologically based pharmacokinetic (PBPK) models to simulate DDIs in vivo (4).

For predictions of metabolic DDIs with CYP enzymes, K i or IC 50 values for a given compound are typically determined in liver microsomes or in hepatocytes. There is a growing interest in using cryopreserved human hepatocytes (HH) instead of human liver microsomes (HLM) for preclinical DDI assessment. In the microsomal setting, the drug concentration available to interact with the enzyme is equal to the unbound drug concentration in the incubation medium (i.e., the nominal incubation concentration, Cinc, corrected for fraction of unbound drug in the liver microsomes (fu,mic)) (5). However, the HH model may be a more physiologically relevant system than HLM, because it takes into account multiple processes that influence enzyme inhibition. In the hepatocyte setting, compounds need to reach the cell interior where the CYP enzymes are located, a process that may involve passive permeability and active transport. Subsequent to this, the intracellular unbound drug concentration (Cu,cell) may be further influenced by metabolic clearance, protein binding, and partitioning into organelles (e.g., lysosomal trapping) or cellular membranes (5–8). These factors can all contribute to inconsistencies between the Cu,cell and Cinc, leading to differences in the Kapp or IC50app values measured in HLM and HH. Therefore, careful consideration must be given to which in vitro system is appropriate for testing enzyme inhibition.

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Determination of $C_{u,cell}$ is especially challenging if active transport is involved and is therefore classified as a low-confidence parameter in PBPK modeling (9). The US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) recommended the $[I] / K_i$ ratio (where $[I]$ is the inhibitor concentration) as a parameter in static DDI predictions (10). However, the determination of $[I]$ is not currently standardized. The surrogates commonly used are the average or maximum unbound concentration in blood or at the inlet to the liver, or the maximum total concentration in the circulation at steady state (10–12). In addition to the different methods to estimate $[I]$, the determination of $K_i$ also varies. For example, $K_{i,app}$—based on determinations in cryopreserved HH suspended in human plasma—has been proposed (13). Such systems aim to reduce $in vitro$ to $in vivo$ translational uncertainties by more closely mimicking the $in vivo$ situation, building protein binding and intracellular bioavailability considerations into the $in vitro$ system.

Strategies for more direct estimations of $C_{u,cell}$ recently emerged (14–18). These include the parallel determination of hepatocellular drug accumulation at steady state ($K_p$) and the unbound fraction of drug in the cell ($f_{u,cell}$) which are combined to calculate the unbound partition coefficient $K_{pu}$. $K_{pu}$ is defined as the ratio between unbound drug concentration in the cell interior and the unbound drug concentration in the cell exterior at steady state and can also be derived from kinetic parameters (17).

We recently introduced the term intracellular bioavailability ($F_{ic}$), defined as the ratio between the intracellular unbound concentration, which is available to elicit effects inside the cell, and the extracellular concentration. In the present work, $F_{ic}$ is equal to $K_{pu}$, as no protein was added to the incubation media, and therefore, the term $K_{pu}$ has been used for simplicity. $F_{ic}$ or $K_{pu}$ account for active and passive mechanisms and predict target engagement and phenotypic responses in cells (19). We have shown that this parameter reflects transporter effects in cell lines transfected with single transporters. Further, we have seen differences in the $K_{pu}$ values for freshly isolated, plated HH, and suspended HH. This is explained by the altered clearance in the two systems (7).

Given this strong influence of culture formats on $K_{pu}$, we hypothesized that $K_{pu}$ could be used to reconcile the differences in measurement obtained from different human-relevant experimental systems. We therefore first investigated a series of reference CYP inhibitors (saquinavir, neflinnavir, enoxacin, and clarithromycin) in HLM and HH, previously shown to have differences in $K_{i,app}$ in rat liver microsomes and rat hepatocytes (20,21). Second, we applied an optimized assay for a series of investigational compounds from a drug discovery program that were all identified as CYP2C9 inhibitors. Finally, $K_{pu}$ was determined for a series of commonly used CYP inhibitors of the azole antifungal family—ketoconazole, itraconazole, and posaconazole. These compounds were chosen because they require hepatic enrichment factors (referred to as “hepatic uptake value” in the SimCYP software), to reconcile $in vitro$ and $in vivo$ $K_i$ values (11,22). We reasoned that these scaling factors represent $K_{pu}$ at the site of DDI.

**METHODS**

**Chemicals**

Compounds were retrieved from the in-house stock at the Roche laboratories at their highest available purity and dissolved at 10 mM in DMSO or, if lower, at their highest solubility. DMSO stocks were kept at −20°C.

**Compound Selection**

The literature was screened for a validation set of compounds with reported discrepancies between cellular and microsomal IC$_{50}$ or $K_i$ values. Enoxacin, clarithromycin, saquinavir, and neflinnavir were identified as suitable candidates (20). The method was then applied to an internal compound set from Roche (hereafter RO compounds) consisting of a structurally related series known to inhibit CYP2C9, and for which information on IC$_{50}$ in HLM and HH was available. As a third compound set, three members of the azole antifungal family—ketoconazole, itraconazole, and posaconazole—were identified as compounds with reported $in vitro$ $K_i$ values that differ from the $K_i$ values reported in models predicting $in vivo$ data.

**Cell Culture**

A pooled HH batch (BioreclamationIVT, LiverPool 10-donor HH, product no. X008001, Lot RBR) was used in the CYP inhibition assay and for determination drug uptake in suspended HH. After thawing, the cells were suspended in InVitroGro CP medium at the specified concentration in each assay (product no. Z99029, BioreclamationIVT) and used immediately for experiments.

**CYP Inhibition Assay in HLM and HH**

For determination of CYP2C9 inhibition, 180 μL of HLM 0.2 mg/mL (BD Gentest, Cat no. 452117, pool from 150 donors of mixed gender) plus substrate (5 μM diclofenac for CYP2C9 and 5 μM midazolam for CYP3A4) was added to a deep 96-well plate together with the test compound or reference inhibitor (concentration range from 0.008 μM to 1 μM). After the addition of 20 μL of 10 mM NADPH, the plate was incubated for 5 min. The reaction was stopped by adding 200 μL of acetonitrile containing an internal standard. After centrifugation of 10 min at 6200×g, the concentration of the substrate in the samples was determined by LC-MS (Sciex API4000, see S6 for LC-MS parameters).

For HH, 50 μL of a suspension of cryopreserved HH (3 × 10$^6$ cells/mL) were added to a 96-well round bottom plate and pre-incubated for 20 min in Williams E medium (without FBS). Fifty microliters of a × 3 concentrated test compound-solution and 50 μL of × 3 concentrated substrate (diclofenac for CYP2C9, midazolam for CYP3A4 and tacrine for CYP1A2) were added and the plate was incubated on a shaker at 37°C, 5% CO$_2$, and 900 rpm. Incubations were stopped after 5 min by transferring 100 μL of the suspension to a fresh plate and adding 100 μL of acetonitrile containing the internal standard and then analyzed as above.
Where available, IC50 values were compared to values found in the literature (Table S1).

**Intracellular Unbound Drug Accumulation Ratio (Kpuu)**

Kpuu was determined as previously described (7,18) using Eq. (1):

$$K_{puu} = K_p \frac{f_{u,cell}}{f_{u,medium}}$$  \hspace{1cm} (1)

where Kp is the steady-state cellular uptake, and fu,cell the fraction of unbound compound in the cell, determined as described below. The fraction unbound of the compound in the medium (fu,medium) was equal to 1 as no serum proteins or other drug binding components were added to the incubation media.

**Steady-State Cellular Uptake (Kp)**

The Kp in suspended HH was measured similarly to previous reports with some modifications (7,18). Briefly, cells were rinsed twice with pre-warmed containing 10 mM HEPES. One hundred microliters of cell suspension (5 x 10^6 cells/mL) was added to a conical 96-well plate, and 100 µL of 1 µM drug solutions in Hank’s buffered salt solution (HBSS) buffered with 10 mM HEPES was added (resulting in a 0.5 µM final concentration). All experiments were performed in triplicates on up to three independent occasions. Full-time curves were established at 1, 3, 7.5, 15, 30, and 45 min to determine Kp at equilibrium. For single time point measurements, the incubation time was matched to the CYP inhibition assays. A sample of the medium was removed after centrifugation in a pre-cooled centrifuge at 4°C (100×g, 5 min), and ten-fold diluted with a mixture of acetonitrile and water (60:40) containing the internal standard for determination of Cmedium. The remaining supernatant was removed by aspiration and cells were washed twice with ice-cold buffer. The intracellular compound was extracted using the acetonitrile to water to internal standard mixture for recovery and stability calculations. Incubation time was 4 h at 37°C and 900 rpm for all compounds except itraconazole which reached equilibrium only after 24 h (Fig. S4). The unbound fraction in the cell homogenate (fu,hom) was determined according to Eq. (3):

$$f_{u,hom} = \frac{C_{buffer}}{C_{hom}}$$  \hspace{1cm} (3)

and the fraction of unbound compound in the cell (fu,cell) was calculated by correcting for homogenate dilution according to Eq. (4):

$$f_{u,cell} = \frac{1}{D(1/f_{u,hom} - 1) + 1}$$  \hspace{1cm} (4)

where the dilution constant D was calculated using Eq. (5), and assuming the Vcell to be equal to 6.5 µL/mg protein (23). Phom is the protein concentration of the cell homogenate quantified using the BCA assay.

$$D = 1/(V_{cell}\times P_{hom})$$  \hspace{1cm} (5)

The lower detection limit was reached for determination of fu,cell below 0.01%, and the binding was assumed to be linear at the concentrations used, based on previous results (18).

For determination of the fraction unbound to microsomes (fu,mic), HLM at 0.2 mg/mL (equivalent concentration to CYP inhibition assay; BD Gentest, Cat no. 452117, pool from 150 donors of mixed gender) were used instead of cell homogenate and calculated in analogy to fu,hom (Eq. 3).

**Determination of Molecular Properties**

Molecular properties of literature compounds were determined using ADMET predictor (Simulations Plus, version 7.2) and cross-checked to values published in the public databases DrugBank (25) and PubChem (http://pubchem.ncbi.nlm.nih.gov). Molecular properties (logD, charge, PSA) of RO compounds were calculated using Roche proprietary in silico tools.

**Statistical Analysis**

All statistical analyses were performed in Graph-Pad Prism (version 7.04).

**RESULTS**

**Method Optimization**

Four reference compounds of different chemical character were studied first. The physicochemical properties of these
drugs are summarized in Table I. Enoxacin and clarithromycin are poorly metabolized, hydrophilic drugs while saquinavir and nelfinavir are extensively metabolized, lipophilic drugs (26). All four have been previously shown to have a clear difference in their $K_{i,app}$ values for liver microsomes and suspended hepatocytes in the rat (Table S1). This made these compounds suitable for investigation in human liver microsomes (HLM) and hepatocytes (HH). The fraction unbound of the four compounds to HLM ($f_{u,mic}$) was determined using equilibrium dialysis and compared to $f_{u,mic}$ in rat liver microsomes (Fig. 1a, Table S3). $K_{puu}$ in suspended HH was determined by combining $K_p$ and $f_{u,cell}$ (Table I). $K_{puu}$ in rat hepatocytes was derived from $K_p$ values from the literature (Table S3). Enoxacin and clarithromycin had $K_{puu}$ values above 1, indicating that they accumulated in HH. In contrast, saquinavir and nelfinavir had $K_{puu}$ values below 1 (Fig. 1b).

Next, we used the $f_{u,mic}$ and $K_{puu}$ values in human and rat to scale apparent $K_i$ (or $IC_{50}$) values determined in microsomes and hepatocytes, respectively. Before scaling, these apparent values revealed a significant discrepancy between hepatocytes and microsomes, with the differences ranging up to 22-fold (Fig. 1c). To obtain corrected $K_i$ or $IC_{50}$, we multiplied apparent $K_i$ or $IC_{50}$ in microsomes with $f_{u,mic}$ and apparent $K_i$ or $IC_{50}$ in hepatocytes with $K_{puu} (= K_p \times f_{u,cell})$. After applying these corrections, $K_{i,app}$ or $IC_{50,app}$ values could largely be reconciled (i.e., ~2-fold differences). Nelfinavir—the most lipophilic compound in the

| Compound | MW (g/mol) | Charge | LogD$_{7.4}$ | $f_{u,mic}$ | $f_{u,cell}$ | $K_p$ | $K_{puu}$ |
|----------|------------|--------|-------------|-------------|--------------|------|----------|
| Enoxacin | 320.32     | Zwitter| −1.1        | 0.53 ± 0.13 | 1.0 ± 0.0    | 7.4 ± 1.1 | 7.4 ± 0.2 |
| Clarithromycin | 747.95 | Basic   | 1.6         | 0.40 ± 0.08 | 0.10 ± 0.04 | 25.9 ± 2.1 | 2.7 ± 0.4 |
| Saquinavir | 670.84 | Neutral | 3.5         | 0.15 ± 0.07 | 0.00030 ± 0.00006 | 109 ± 12 | 0.030 ± 0.002 |
| Nelfinavir | 567.78 | Neutral | 4.6         | 0.04 ± 0.02 | 0.00025 ± 0.00005 | 956 ± 312 | 0.24 ± 0.03 |

**Table I.** Major Physicochemical Properties and Results in HLM and Suspended HH. $K_p$, $f_{u,cell}$, and $f_{u,mic}$ in Rat Liver Microsomes and Hepatocytes Derived from the Literature Are Summarized in S3. Transporter and Enzyme Substrates Are Indicated in Table S7

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**Fig. 1.** a Comparison of $f_{u,mic}$ in human vs. rat liver microsomes. Rat values are derived from Brown et al. (20). b Comparison of $K_{puu}$ in human vs. rat hepatocytes. c Log fold difference of apparent and corrected (unbound) $IC_{50}$ determined in human liver microsomes and human hepatocytes or $K_i$ determined in rat microsomes and rat hepatocytes. The dotted lines represent a 2-fold difference. Error bars represent standard deviations. Apparent and corrected $K_i$ or $IC_{50}$ values are presented in Tables S1 and S2. Enox, E: enoxacin; Clar, C: clarithromycin; Saq, S: saquinavir; Nel, N: nelfinavir
hydrophilic properties (Table II).

They are all acidic at pH 7.4, with logD values indicating

secondary amide, and aromatic substituents including (iso-

a common core containing a sulfonamide and a

compared. The compounds of this series are known to inhibit
diclofenac as substrate. The IC₅₀ values of RO1, RO3, and

Kpuu as Scaling Factor of IC₅₀ for an RO Discovery Series
Inhibiting CYP2C9

We further applied our approach to an internal compound set (RO compounds). The nine compounds in this
discovery series were outliers in the internal screening
processes in which IC₅₀ values of HH and HLM were
compared. The compounds of this series are known to inhibit
CYP2C9. These nine compounds are structurally related, with
a common core structure containing a sulfonamide and a
secondary amide, and aromatic substituents including (iso-
thiazoles, benzothiophenes, pyridines, or furanes (Fig. 2a).
They are all acidic at pH 7.4, with logD values indicating
hydrophilic properties (Table II).

First, we determined the IC₅₀,app in HH and HLM using
diclofenac as substrate. The IC₅₀ values of RO1, RO3, and
RO4 were identical for HH and HLM, while the other
compounds differed up to 12-fold (RO9) (Fig. 2b), with, a
root-mean-squared error (RMSE) of 9.4 for IC₅₀,hep,app
compared to IC₅₀,mic,app. As in the previous section, we next
determined Kpuu and fu,mic taking care to use the same
batches of hepatocytes and microsomes as for the inhibition
experiments, respectively (Table II). All parameters were
matched to the IC₅₀ assay.

We then used the Kpuu and fu,mic values as scaling factors
to determine IC₅₀,corr. Correction using Kpuu or fu,mic alone
did not improve the correlations (Fig. 2c and d). After
combining Kpuu and fu,mic to give IC₅₀,hep,corr and IC₅₀,mic,corr
respectively, the linear correlation between the IC₅₀ values
improved, giving and R² of 0.92 and a 10-fold reduction in
RMSE from 9.4 to 0.9 (Fig. 2e).

Because the compounds were analogous to each other, we
also analyzed the possible influence of substituents on
Kpuu. For instance, in five of the nine compounds, the R2
substituent was kept constant while the R1 substituent varied.
However, no systematic influence of the change in substituent

Table II. Properties of the RO Discovery Compound Series and Results in HLM and Suspended HH

| Compound | MW (g/mol) | Charge | LogD₇.₄ | PSA | pKa | P_app (× 10⁻⁶ cm/s) | f_u,mic | f_u,cell | Kp | Kpuu |
|----------|------------|--------|---------|-----|-----|---------------------|--------|---------|----|------|
| RO01     | 385.24     | Acidic | 0.7     | 123 | 5.41| 10                  | 0.42   | 0.0149  | 24.0| 0.357|
| RO02     | 432.34     | Acidic | 1       | 153 | 3.14| 2                   | 0.36   | 0.00130 | 47.3| 0.0613|
| RO03     | 410.26     | Acidic | −1      | 147 | 2.91| 9                   | 0.41   | 0.017   | 17.9| 0.30  |
| RO04     | 424.29     | Acidic | −0.7    | 147 | 2.80| 13                  | 0.45   | 0.02    | 27.6| 0.46  |
| RO05     | 452.75     | Acidic | 1.1     | 153 | 2.76| 2                   | 0.23   | 0.0021  | 18.5| 0.039|
| RO06     | 415.29     | Acidic | 0.4     | 130 | 2.80| 3                   | 0.36   | 0.0039  | 61.6| 0.24  |
| RO07     | 503.41     | Acidic | 0.9     | 175 | 3.17| 4                   | 0.84   | 0.00392 | 41.9| 0.16  |
| RO08     | 390.28     | Acidic | −0.1    | 125 | 2.66| 22                  | 0.41   | 0.009   | 22.2| 0.21  |
| RO09     | 454.38     | Acidic | −0.3    | 162 | 2.49| 2                   | 0.97   | 0.0075  | 11.7| 0.088|
could be observed. We conclude that the compound series was too small to allow a proper substituent analysis; this underscores the importance of experimental analysis of small series of compounds.

**Kp\textsubscript{uu} in IVIVE and DDI Models of the Azole Antifungal Family**

Finally, we investigated if Kp\textsubscript{uu} could improve predictions of DDI models that are based on in vitro K\textsubscript{i} values. Compounds of theazole family are commonly used as inhibitors of CYP3A4 in preclinical and clinical DDI studies (2,27). A concentrative cellular uptake of ketoconazole, itraconazole, and posaconazole has been widely described, but uptake mechanisms are not fully elucidated (28–31). This concentrative uptake requires correction of K\textsubscript{i} (which is typically determined in HLM) in order to match in vivo K\textsubscript{i} values in DDI models. Therefore, compound-specific correction factors (referred to as “hepatic enrichment factor” or “hepatic uptake scalar”) have been introduced to improve the IVIVE in several models, which are summarized in Fig. 3a. We therefore investigated if Kp\textsubscript{uu} values measured in suspended HH agreed with these correction factors (Table III and Fig. 3b). The Kp\textsubscript{uu} values were high, ranging from 7 to 38, in line with the previously described concentrative uptake. Interestingly, the Kp\textsubscript{uu} values were within 2-fold of the empirically determined correction factors in the DDI models, indicating that Kp\textsubscript{uu} is a major contributor to the “hepatic enrichment factor” for theazole series.

**DISCUSSION**

In this work, we evaluated the potential of Kp\textsubscript{uu} as scaling factor for CYP enzyme inhibition studies, using three experimental setups.

For the first set of compounds, we investigated if the determination of intracellular unbound concentrations could be used to reconcile differences in potency in CYP inhibition observed between microsomes and hepatocytes. CYP enzymes can be considered as targets or off-targets facing the cell interior. We have previously shown that F\textsubscript{i,c} or Kp\textsubscript{uu} can explain differences in potency between isolated intracellular targets in biochemical high-throughput screening assays and the corresponding cellular assays (19). Drug response for intracellular targets in a variety of subcellular compartments including the cytosol, endosomes, and the nucleus has been successfully predicted, using our methodology for determination of F\textsubscript{i,c}, F\textsubscript{cyto}, or F\textsubscript{endo} (19,32). Intracellular unbound drug concentrations are determined by combining the cellular accumulation ratio (Kp) with the fraction of unbound compound in the cell (f\textsubscript{u,cell}). f\textsubscript{u,cell} is determined using the homogenization method which relies on the assumption that the degree of unspecific binding of drugs is not altered upon homogenization of cells (7,18).

In the first series of experiments, we used reference compounds with previously reported differences in K\textsubscript{i} of CYPs in rat liver microsomes and rat hepatocytes, as well as Kp, f\textsubscript{u,mic} and f\textsubscript{u,cell} values in the rat (20).

Our experimental human f\textsubscript{u,cell} values differed more than 100-fold for the two lipophilic drugs compared to those in the rat study that was calculated based on lipophilicity and cellular volume (33) (saquinavir 0.14 vs. 0.0003 and nelfinavir 0.035 vs. 0.0002, rat vs. human, respectively). There was a better agreement for the two more hydrophilic compounds (enoxacin 0.99 vs. 1.00 and clarithromycin 0.86 vs. 0.10 in rat vs. human, respectively).

The homogenization/membrane dialysis method has been compared with the temperature and lipophilicity methods for estimating the f\textsubscript{u,cell} of a limited series of structural unrelated compounds by Riede et al. (15). In their study, the homogenization method gave generally lower values than the

**Table III. Properties and Results for the Azole Antifungal Series and Results in HLM and Suspended HH**

| Compound     | MW (g/mol) | Charge | LogD\textsubscript{7.4} | f\textsubscript{u,mic} | f\textsubscript{u,cell} | Kp          | Kp\textsubscript{uu} |
|--------------|------------|--------|------------------------|-----------------------|------------------------|-------------|---------------------|
| Ketoconazole | 531.43     | Neutral | 3.7                    | 0.32 ± 0.12           | 0.004 ± 0.001          | 1659 ± 479  | 6.9 ± 1.1           |
| Itraconazole | 705.63     | Neutral | 4.9                    | 0.21 ± 0.13           | 0.003 ± 0.002          | 2717 ± 1573 | 7.5 ± 6.3           |
| Posaconazole | 700.78     | Neutral | 4.4                    | 0.21 ± 0.11           | 0.005 ± 0.003          | 6931 ± 1235 | 37.6 ± 12.8         |

**Fig. 3.** Correlation of IVIVE correction factors with Kp\textsubscript{uu}. a Overview of correction factors used for theazole antifungal compounds in in vivo DDI studies. b Illustration of hepatic uptake scalars in rat and human DDI models (error bars represent range) vs. in vitro Kp\textsubscript{uu} in suspended HH (error bars represent SD).
temperature and lipophilicity methods. This suggests an overestimation of intracellular binding by the latter two. However, a large variability (up to 13-fold differences) between laboratories has been observed (7,15,17,34,35). $f_{\text{u,cell}}$ is dependent on accurate dilution factors for scaling $f_{\text{u,hom}}$ to $f_{\text{u,cell}}$ (Eq. 4 and 5). In the comparative study by Riede et al. (15), very low cell concentrations (130,000 cells/mL) were used, in comparison to 10 million cells/mL in our studies or 50 million cells/mL by Riccardi et al. (34,35). Such experimental differences may not allow a reliable comparison between laboratories, but in general, all experimental setups agree on significantly lower $f_{\text{u,cell}}$ for highly lipophilic drugs as compared to calculations based on logD. This indicates the importance of using a cellular matrix with relevant lipid and protein binding sites of drugs for the determination of $f_{\text{u,cell}}$ (8). Moreover, no significant inter-specifics variability in $f_{\text{u,cell}}$ has been observed in a recent comparative study (34). Therefore, for the four reference compounds, the methodological differences were more significant than the expected inter-specifics variability in $f_{\text{u,cell}}$ and we used our experimental $f_{\text{u,cell}}$ values for determination of $K_{\text{puu}}$ in both rat and human. On the other hand, inter-specific variability is expected in $K_{\text{p}}$ values, which can be influenced by active uptake and elimination processes. Therefore, the specific $K_{\text{p}}$ in rat or human hepatocytes was used for determination of $K_{\text{puu}}$ (Table S3). For the four reference compounds, $K_{\text{p}}$ for the human and rat hepatocytes followed the same rank order and were in the same order of magnitude, with slightly higher values in rat hepatocytes for three out of the four compounds. It should be noted that clarithromycin is a weak base that can be subject to lysosomal trapping. Using pH partitioning theory (18), the calculated cytosolic $K_{\text{puu}}$ (or $F_{\text{cyt}}$) in human hepatocytes would result in 1.3, instead of the experimental value of 2.7, which does not account for subcellular localization of the drug.

Two of the four reference compounds, enoxacin and clarithromycin, displayed a $K_{i}$ ratio ($K_{\text{mic}}/K_{\text{hep}}$) or $IC_{50}$ ratio and $K_{\text{puu}}$ above one, indicating a concentrative uptake of the compounds in hepatocytes. By contrast, the $K_{i}$ ratio or $IC_{50}$ ratio and $K_{\text{puu}}$ were less than one for saquinavir and nelfinavir, suggesting that the lower potency in rat hepatocytes was driven by poor access to the intracellular compartment (and to the CYP enzyme) as opposed to the microsomes where the CYP enzyme is freely exposed in the medium. For three out of the four compounds, $K_{i}$ or $IC_{50}$ values in liver microsomes and hepatocytes were in good agreement after applying relevant $K_{\text{puu}}$ and $f_{\text{u,mic}}$ as scaling factors (Fig. 1c) (34–36). For nelfinavir, scaling $K_{i}$ or $IC_{50}$ values with $K_{\text{puu}}$ and $f_{\text{u,mic}}$ resulted in a larger discrepancy rather than an improvement. Interestingly, nelfinavir was identified as an outlier also in our previous $K_{\text{puu}}$ studies in MDCK cells, suggesting the involvement of unknown active processes that are not captured by the $K_{\text{puu}}$ methodology (7). Nelfinavir is very lipophilic, poorly soluble, and a substrate of active efflux (P-gp)—these properties suggest that the compound is prone to nonspecific binding and that there are significant confounding effects occurring in the hepatocyte experiment. Indeed, poor mass balance (~50%) was observed for nelfinavir and the similarly lipophilic saquinavir in the hepatocyte experiments. As the mass balance was recovered in the presence of the CYP inhibitor ABT, this confirms that the loss of compound is due to hepatic metabolism (Fig. S4). The hepatocyte metabolism of nelfinavir and saquinavir has been reported previously in a study that identified these two compounds as outliers. This was based on their intrinsic clearance in microsomes being much higher than in hepatocytes, compared to other compounds with similar clearance mechanisms (37).

It should be noted, however, that in our studies, the hepatocyte metabolism of nelfinavir and saquinavir was not a limiting factor for establishing the concentration equilibrium, since $K_{p}$ values were unaffected by the presence or absence of ABT (Fig. S4). Furthermore, the use of albumin or an increase in temperature of wash buffers from 4 to 37°C (38,39) did not change the results significantly (Fig. S5), which precludes the likelihood that non-specific binding was affecting the results. Thus, the discrepancy in the $K_{\text{corr}}$ values of nelfinavir could not be explained by CYP-mediated metabolism or non-specific binding and is likely driven by other factors. Despite the discrepancy for nelfinavir, we overall obtained a significant harmonization of the $K_{i}$ ratios between microsomes and hepatocytes, suggesting the validity of this approach and indicating the value of further elaboration.

In the next set of experiments, we therefore harmonized the experimental conditions between the CYP inhibition experiments and the $K_{\text{puu}}$ experiments. Instead of comparing $K_{\text{puu}}$ to CYP inhibition constants from the literature, we determined $IC_{50}$ in HH and HLM with methodologies as similar as possible to each other to generate a consistent dataset. As a result, the $K_{\text{puu}}$ and $f_{\text{u,mic}}$-corrected $IC_{50}$ values for the discovery series of CYP2C9 enzyme inhibitors were in excellent agreement for HH and HLM (Fig. 2e). We conclude that the best agreement between the two systems is obtained by applying simultaneous correction factors for both systems, along with taking into consideration differences in assay setup and inter-batch variability.

Given the significance of $K_{\text{puu}}$ in the two series of in vitro experiments, we next evaluated if it could also be used as a scaling factor in in vivo studies. More specifically, we investigated if $K_{\text{puu}}$ could be used to estimate the empirical hepatic uptake scalar required in PBPK models of ketoconazole, itraconazole, and posaconazole as CYP inhibitors. Many models use such empirically determined scalars (Fig. 3a). However, others avoid them because the uptake mechanism of theseazole compounds are not mediated via classic hepatic uptake transporters such as the OATP or OCT transporter family (29,40). $K_{\text{puu}}$ is a measure of all combined effects in a cell and provides a mechanistic tool for estimation of uptake scalars without the need for elucidating the underlying factors contributing to the net drug disposition in the cell. We found that $K_{\text{puu}}$ values in suspended HH were in good agreement with the empirically determined hepatic uptake scalars in the models for both rat and human (Fig. S3). In the case of itraconazole, the three major metabolites are equally or more potent inhibitors of CYP3A4 than the parent compound, which is a complicating factor in PBPK modeling (28,40,41). The measurement of $K_{\text{puu}}$ of the parent compound alone was however sufficient for recovering the hepatic uptake scalars. Therefore, we believe that $K_{\text{puu}}$ can be rationally integrated into prospective PBPK modeling approaches. The use of HH instead of its rat counterpart reduces the potential effects of inter-species differences in drug transporter expression and metabolic enzyme sensitivity to inhibition. It is also the most relevant in vivo model system for making extrapolations to humans. The relevance of $K_{\text{puu}}$ to in vivo studies is further supported by a recent study in which a series of four statins was assessed for in vitro and in vivo liver enrichment (35); in vivo liver-to-plasma, $K_{\text{puu}}$ in rat and
human were in good agreement with in vitro K_{puu} obtained in cryopreserved suspended hepatocytes. Similar scenarios are anticipated for, e.g., rifampicin, which has an empirical hepatic uptake scalar of 16.9 (42) and for bosentan, with a hepatic uptake scalar of 5 to 6 (43).

Determination of K_{app} directly in HH can be an alternative to the incorporation of hepatic uptake scalars. However, most K_{i} screens are based on HLM. To make our approach more generally applicable, we suggest the use of K_{corr} values, and account for intracellular unbound concentrations in the target cells in any tissue of interest (Fig. 4). Our approach is not limited to the liver and can be applied on any cell of interest with an intracellular target, including studies of DDIs in intestinal enterocytes.

CONCLUSION

In summary, our results indicate that K_{puu} in HH is an easy-to-interpret in vitro parameter that can be used as a human-relevant scaling factor to bridge the differences of experimental systems such as liver microsomes and hepatocytes and gives a mechanistic understanding of any result discrepancies (Fig. 4). Since K_{puu} is the net result of all processes that affect the intracellular unbound drug concentration in HH, no prior knowledge of uptake and elimination processes is required. Indeed, very high or low K_{puu} values can indicate that active cellular processes are playing a significant role in drug disposition. Therefore, K_{puu} can be used as a decision tool (e.g., if the K_{puu} is lower than 0.5 or higher than 2) for more detailed investigations. Very lipophilic compounds have been found to be more challenging for assessment of K_{puu} and future work should focus on this compound class. Further, K_{puu} has been shown to reflect hepatic uptake scalars used in literature for the triazole antifungal family and we believe that the use of K_{puu} is an approach that can be pursued to provide a mechanistic understanding of scalars used in PBPK models that predict drug exposure and DDI potential.

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