Intracellular Drug Concentrations and Transporters: Measurement, Modeling, and Implications for the Liver

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Intracellular concentrations of drugs and metabolites are often important determinants of efficacy, toxicity, and drug interactions. Hepatic drug distribution can be affected by many factors, including physicochemical properties, uptake/efflux transporters, protein binding, organelle sequestration, and metabolism. This white paper highlights determinants of hepatocyte drug/metabolite concentrations and provides an update on model systems, methods, and modeling/simulation approaches used to quantitatively assess hepatocellular concentrations of molecules. The critical scientific gaps and future research directions in this field are discussed.

Intracellular drug concentrations are fundamentally important to drug efficacy and toxicity as well as for understanding and accurately predicting drug interactions and intersubject variability in drug response (either on-target or off-target effects). Intracellular drug concentrations are difficult to quantify directly in humans. Therefore, blood or plasma drug concentrations are typically used as a surrogate measure under the assumption that unbound drug concentrations in the systemic circulation mirror intracellular unbound drug concentrations at the site of action. This assumption is based on the free-drug hypothesis, i.e., that unbound drug concentrations on either side of a membrane are in thermodynamic equilibrium.[1] However, this assumption may not be valid for many drugs, especially those that are poorly permeable (e.g., charged or polar compounds), actively transported, or extensively metabolized *in vivo*. Uncertainty regarding the actual unbound intracellular concentration of drugs makes it difficult to predict *in vivo* effects that depend on interactions between the unbound drug and intracellular targets.

The efficacy of drugs for which the target site is the liver will be directly affected by unbound hepatic drug concentrations. For example, the liver is the target organ for statins. Statin efficacy is influenced by hepatocellular statin concentrations, which may be modulated by hepatic transport and/or metabolism processes.[2] Regardless of the location of the target site, the ability of a drug to exert a sustained pharmacologic effect may be limited owing to rapid removal from the systemic circulation by eliminating organs (e.g., liver). The hepatic clearance of drugs depends on the intracellular unbound concentration at the site of metabolism or transport. In addition, intracellular unbound concentrations of drugs/metabolites in relevant tissues may be important parameters for drug-induced toxicities. For instance, inhibition of the bile salt export pump (BSEP/*ABCB11*) is one hypothesized

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mechanism of drug-induced liver injury.[3] Because BSEP mediates bile acid efflux across the canalicular membrane, knowledge of unbound hepatocyte concentrations of BSEP inhibitors is critical for predicting BSEP-related cholestasis in humans.[4] Similarly, accurate predictions of drug–drug interactions (DDIs) caused by inhibition or induction of metabolic enzymes require knowledge of unbound hepatocyte concentrations of the potential perpetrators. If the perpetrators are substrates for hepatic uptake transporters, cytochrome P450 (CYP)-mediated DDIs are often underpredicted on the basis of unbound plasma concentrations of inhibitors.[5]

This white paper highlights the state of the art of the determinants of intracellular drug concentrations. The discussion includes (i) a review of fundamental concepts regarding factors that influence drug concentrations in hepatocytes, (ii) an overview of important hepatic transporters and examples of how these proteins may affect hepatocyte drug concentrations, (iii) experimental and modeling approaches that may be used to estimate intracellular drug concentrations, and (iv) current challenges and recommendations regarding the determination of unbound drug concentrations in hepatocytes during the process of drug development.

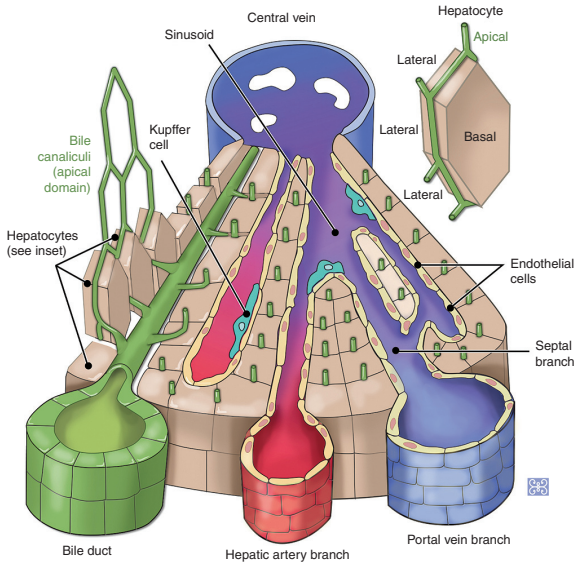

Figure 1 Microanatomy of the liver. Hepatocytes, bile ductules, and sinusoids are represented. The structure of a hepatocyte is depicted with its apical, basal, and lateral sides. The sinusoid is depicted to highlight the fact that the blood is not well mixed in the sinusoid.

FACTORS THAT INFLUENCE INTRACELLULAR DRUG DISPOSITION IN THE LIVER

The intracellular unbound drug concentration in hepatocytes is influenced by hepatic physiology and the physicochemical properties of a drug. In contrast to the common simplification of its role, the liver is not merely a well-stirred compartment. At the organ level, drug concentrations in the hepatic microvasculature may vary between different parts of the liver depending on the mixing of portal and venous blood (**Figure 1**), and zonal differences in metabolism and biliary excretion. Thus, hepatocytes may be exposed to different drug concentrations depending on their location in the sinusoid. The rate and extent of drug movement into hepatocytes are affected by local concentrations in the sinusoid and may be limited by either permeability across the basolateral membrane or hepatic blood flow.[6] Plasma protein and tissue binding may affect intracellular unbound drug concentrations.[1] Within the hepatocyte, hepatocellular drug exposure is determined by (i) the rate and extent of uptake into the hepatocyte and (ii) the rate and extent of removal from the cell (**Figure 2**). Several processes are involved in intracellular drug exposure, including passive diffusion (described by the passive diffusion clearance, CL_{diff}), transporter-mediated basolateral uptake (intrinsic basolateral uptake clearance, $CL_{act,uptake}$) and efflux (intrinsic basolateral efflux clearance, $CL_{act,efflux}$), biliary excretion (intrinsic biliary excretion clearance, CL_{bile}), and hepatic metabolism (intrinsic metabolic clearance, CL_{met}; **Figure 2a**,**b**).[7,8] The impact of these processes on intracellular drug exposure is described in more detail below.

In addition to transport and metabolism processes, which determine overall cellular drug exposure, intracellular binding and partitioning (described by the unbound fraction in the liver, $f_{u,liver}$, and the cellular partition coefficient based on total concentrations, K_p) and sequestration into subcellular organelle compartments (e.g., lysosomes or mitochondria) can affect the fraction of drug that is available for intracellular pharmacological action, metabolism, and excretion (**Figure 2a**). However, under steady-state conditions, unbound cytosolic drug concentration is determined by the intrinsic uptake and elimination clearances, and any intracellular binding and partitioning will affect the unbound drug fraction (because the total intracellular concentration is increased) but will have no effect on unbound cytosolic drug concentration.[1]

$K_{puu,liver}$ is defined as the steady-state liver-to-sinusoidal blood partition coefficient for unbound drug, typically under the assumption that the liver is a "well-stirred" homogeneous compartment. As such, unbound liver concentration refers to unbound concentration in the cytosol. $K_{puu,liver}$ is a function of CL_{diff}, $CL_{act,uptake}$, $CL_{act,efflux}$, CL_{bile}, and CL_{met}. CL_{diff} is driven by transmembrane concentration gradients and is linked closely to the drug's physicochemical properties, such as lipophilicity (typically described by the octanol-water partition coefficient (logP/logD)), polarity, molecular size, and degree of ionization at physiological pH (pK_a). Higher CL_{diff} values are generally observed for smaller, more lipophilic, un-ionized compounds. For compounds for which CL_{diff} is much greater than other intrinsic clearance values, $K_{puu,liver}$ approximates 1. In such cases, intracellular hepatic concentrations will be determined primarily by the metabolic and/or biliary excretion clearances. If metabolic or efflux clearance values are larger than CL_{diff} and hepatic uptake is determined primarily by CL_{diff}, then $K_{puu,liver}$ can be <1. By contrast, for compounds with low CL_{diff}, transporter-mediated $CL_{act,uptake}$ will have a greater impact on intracellular concentration and $K_{puu,liver}$ can be >1.[9,10] The impact of altered transport on the intracellular concentration and exposure of

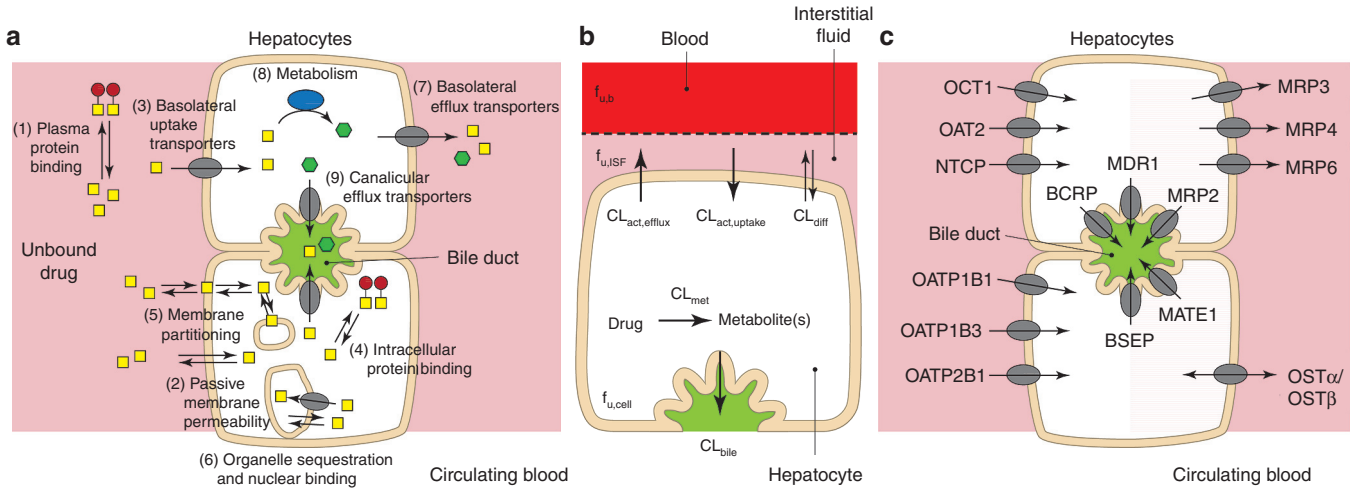

Figure 2 Factors affecting intracellular drug concentrations in the hepatocyte. (**a**) Processes affecting intracellular drug concentrations are depicted. (1) Drug in the blood that is not associated with blood cells or plasma proteins can enter the hepatocyte (2) through passive diffusion or (3) via active uptake mediated by basolateral uptake transporters. Within the hepatocytes, (4,5) the drug can bind to intracellular structures (e.g., proteins, DNA, and membranes) or (6) partition into subcellular organelles such as mitochondria or lysosomes via a combination of carrier-mediated transport and/or passive diffusion driven by the electrochemical membrane potential and the pH gradient. Based on the free-drug hypothesis, only the unbound drug in the hepatocyte can undergo (7) efflux back to sinusoidal blood via the action of basolateral efflux transporters, (8) enzymatic biotransformation, or (9) excretion into the bile mediated by canalicular efflux transporters. (**b**) Passive and active clearance processes affecting hepatocyte intracellular concentrations. $CL_{act,efflux}$, intrinsic active efflux clearance; $CL_{act,uptake}$, intrinsic active uptake clearance; CL_{bile}, intrinsic biliary excretion clearance; CL_{diff}, passive diffusion clearance; CL_{met}, intrinsic metabolic clearance; $f_{u,b}$, unbound fraction of drug in the blood; $f_{u,cell}$, unbound fraction of drug in cell; $f_{u,ISF}$, unbound fraction of drug in interstitial fluid. (**c**) Membrane localization of key uptake and efflux transporters that may affect hepatocyte intracellular concentrations. The schematic representation is limited to transporters expressed in the plasma membrane that have previously shown effects on drug disposition and toxicity and could potentially modulate unbound drug concentrations in the liver.

these types of compounds is summarized in **Table 1** and is discussed in more detail below (refs. 76–98, cited in the table, can be found in the **Supplementary Data** online).

It should be noted that the partition coefficient K_p based on total concentrations can deviate from unity even in the absence of transporter and metabolism effects. In particular, for lipophilic compounds, partitioning among cellular membranes and other cell structures can result in a K_p value that is orders of magnitude greater than unity. In addition, electrochemical and pH gradients across the plasma membrane and internal (organelle) membranes can result in preferential subcellular distribution of drug molecules based on charge and ionization potential. For example, lysosomal pH is generally 1–2 units lower than the cytosol pH. As a consequence, drugs with weakly basic properties that are predominantly uncharged in the cytosol will become charged upon entering the lysosome, effectively trapping these compounds because of the limited permeability of the charged molecular species. Such pH-driven lysosomal sequestration is saturable, is dependent on adenosine triphosphate (ATP)(because the pH gradient is maintained by membrane-bound ATPases), and can result in several-fold increases in K_p.[11–13] Similarly, the inner mitochondrial membranes possess a negative transmembrane electrical potential relative to the cytosol, which can result in the trapping of positively charged drug molecules.[11,14,15] Sequestration within lysosomes and mitochondria can translate to considerable local drug concentrations in these organelles and potentially cause changes in on-target or off-target effects within hepatocytes, which may have pharmacological and/or toxicological implications that cannot be predicted based solely on systemic concentrations.

Given that drug concentrations in each subcellular compartment can vary as discussed above, it is noteworthy that the definition of "intracellular drug concentrations" is obscure. In this white paper, the term "intracellular drug concentrations" denotes unbound drug concentrations in the cytosol (also referred to as unbound hepatocellular drug concentrations) and the term "subcellular drug concentrations" signifies drug concentrations in various subcellular compartments.

IMPACT OF TRANSPORTERS AND ENZYME–TRANSPORTER INTERPLAY ON HEPATIC DRUG CONCENTRATIONS

The liver is the major organ responsible for drug metabolism and excretion. Not surprisingly, membrane transporters that influence hepatic intracellular concentrations can have a major impact on the overall disposition of drugs as well as their efficacy and toxicity. The cellular localization of key human hepatic transport proteins is depicted schematically in **Figure 2c**. Transporters in the basolateral membrane mediate the uptake of substrates from blood into the hepatocyte and/or efflux of substrates in the opposite direction; transporters in the canalicular membrane mediate the excretion of substrates into bile; and transporters in the intracellular membranes sequester substrates in subcellular compartments within hepatocytes. The examples below and summarized in **Table 1** demonstrate how these transport proteins have the potential to affect intracellular and systemic (plasma) exposure of substrates, including endogenous compounds, drugs, and/or metabolites.

Table 1 Examples of studies in which membrane transporters have been shown to alter hepatocellular drug concentrations

Transporter (gene)	Localization and directionality	Substrate	Effect on intracellular concentrations[a]	Effect on plasma exposure[a]	Observation[b]	Model system
MRP2 (*ABCC2*)	Canalicular efflux	^{99m}Tc-Mebrofenin	↑	NA	Prolonged and increased hepatobiliary exposure in patients with genetically impaired MRP2 function[23]	Humans *in vivo*
		7–Hydroxymethotrexate	↑	↑	Hepatic accumulation of 7-hydroxymethotrexate[26]	Mrp2$^{-/-}$ mice
		Valproate glucuronide	↑	NA	Inhibition by probenecid increased hepatic exposure[27]	Isolated perfused rat livers
MRP3 (*ABCC3*)	Basolateral efflux	7–Hydroxymethotrexate	↑	↑	Pronounced accumulation of 7-hydroxymethotrexate[26]	Mrp2$^{-/-}$/Mrp3$^{-/-}$ mice
		Valproate glucuronide	↑	NA	Inhibition by probenecid increased hepatic exposure[27]	Isolated perfused rat livers
		Unconjugated bile acids	↓	↑	Induction by ANIT decreased hepatic concentrations[29]	Mice
BCRP (*ABCG2*)	Canalicular efflux	Rosuvastatin	(↑)	NA	Reduced-function polymorphisms increased hepatic efficacy[24,25,76]	Humans *in vivo*
		Nitrofurantoin	↑	NA	RNA interference knockdown reduced *in vitro* biliary clearance[77]	Sandwich-cultured rat hepatocytes
OCT1 (*SLC22A1*)	Basolateral uptake	Metformin	(↓)	↔	Reduced-function polymorphisms limited the pharmacological effect[20]	Humans *in vivo*
		Metformin	↓	↔	Reduced hepatic concentrations[18]	Oct1$^{-/-}$ mice
OATP1B1 (*SLCO1B1*)	Basolateral uptake	Methotrexate	(↓)	↑	Reduced-function polymorphisms decreased gastrointestinal toxicity and increased plasma concentrations[78]	Humans *in vivo*
		Methotrexate	↓	↑	Decreased hepatic concentration and increased plasma exposure[70]	Oatp1a$^{-/-}$/1b$^{-/-}$ mice
		Gadoxetate disodium (MRI contrast agent)	↓	NA	Inhibition by lapatinib decreased hepatic parenchymal enhancement[17]	Humans *in vivo*
		Pravastatin	↓	↑	Decreased hepatic concentrations[79]	Oatp1a$^{-/-}$/1b$^{-/-}$ mice
MATE1 (*SLC47A1*)	Canalicular efflux	Metformin	↑	↑	Increased hepatic concentrations[80]	Mate1$^{-/-}$ mice
			NA	↔	Reduced-function polymorphic variants enhanced glucose-lowering effects of metformin[81]	Humans *in vivo*

ANIT, alpha-naphthyl isothiocyanate; AUC, area under the concentration–time curve; BCRP, breast cancer–resistance protein; MATE, multidrug and toxin extrusion protein; MRI, magnetic resonance imaging; MRP, multidrug-resistance protein; NA, data are not available; OATP, organic anion–transporting polypeptide; OCT, organic cation transporter.

[a]Up and down arrows indicate increased and decreased intracellular concentrations (or plasma AUC), respectively. Left–right arrows indicate no significant change. Parentheses denote examples for which the effect on intracellular concentrations is not directly measured but instead implied from pharmacological or toxicological observations. [b]Refs. 76–98 are listed in **Supplementary Data** online.

Decreased hepatocyte concentrations due to impaired hepatic uptake

At a given plasma concentration, impaired function of a basolateral uptake transport protein as a result of a genetic polymorphism or a DDI would be expected to decrease hepatocyte concentrations of a drug if its hepatic uptake depends solely, or primarily, on that basolateral uptake transporter. For example, gadoxetate disodium, an extracellular magnetic resonance imaging (MRI) contrast agent, is transported by organic anion–transporting polypeptide 1B1 (OATP1B1/*SLCO1B1*), OATP1B3 (*SLCO1B3*), and Na^+-taurocholate–cotransporting polypeptide (*SLCO10A1*) into hepatocytes.[16] Lapatinib, an inhibitor of OATP1B1, significantly decreases hepatic parenchymal enhancement of gadoxetate disodium–enhanced MRI scans, potentially compromising the ability to diagnose liver tumors using this imaging agent.[17] Similarly, the glucose-lowering effects of metformin would be expected to be dependent on its hepatic exposure. Metformin is a substrate for the basolateral uptake organic cation transporter 1 (OCT1/*SLC22A1*) and 2 (OCT2/*SLC22A2*), expressed in liver and kidney, respectively. Hepatic metformin concentrations were approximately eight-fold lower in Oct1-knockout mice, as compared with wild-type mice, despite similar concentrations of metformin in the plasma, consistent with the hypothesis that OCT1 controls intracellular

hepatocyte concentrations of metformin.[18] However, increased systemic concentrations due to decreased clearance or altered distribution and elimination pathways, in conjunction with impaired hepatic uptake, may result in little net change in hepatic drug exposure (area under the concentration–time curve $(AUC)_{liver}$). For example, in one study, metformin hepatic exposure was decreased only modestly (knockout-to-wild-type ratio of ~0.6) in $Oct1^{-/-}/Oct2^{-/-}$ mice.[19] In humans, reduced-function genetic polymorphisms of OCT1 could also decrease the therapeutic response of metformin, although the clinical relevance of these variants remains to be established in large-scale studies.[20]

Increased hepatocyte concentrations due to impaired canalicular excretion and/or basolateral efflux

Hepatic drug/metabolite exposure is expected to increase asymptotically with increasing impairment of hepatic excretory transport for compounds that are substrates for efflux transporters.[21] This relationship has been supported by data generated in isolated perfused rodent liver studies, in which liver, perfusate, and biliary concentrations of anionic substrates were measured under various scenarios of impaired efflux transport. The hepatobiliary imaging agent ^{99m}Tc-mebrofenin, an iminodiacetic acid analog, is taken up efficiently into hepatocytes by OATP1B1 and OATP1B3 and preferentially excreted into bile by the canalicular multidrug-resistance protein 2 (MRP2/*ABCC2*); ^{99m}Tc-mebrofenin is also a substrate for the basolateral efflux transporter MRP3 (*ABCC3*).[22] Humans with impaired MRP2 function (Dubin–Johnson syndrome) exhibit increased and prolonged hepatic exposure to ^{99m}Tc-Mebrofenin and other iminodiacetic acid analogs.[23] Some statins are also substrates for MRP2 and breast cancer–resistance protein (BCRP/*ABCG2*). Impaired statin excretion from the hepatocyte due to reduced canalicular transport would be expected to result in higher intracellular exposure and increased efficacy.[2] In fact, patients expressing at least one reduced-function *ABCG2* variant 421C>A (rs2231142) allele were more likely to achieve the low-density lipoprotein (LDL) cholesterol target after rosuvastatin treatment, as compared with simvastatin treatment, but this difference between treatments was not significant for carriers of the reference allele.[24] In addition, carriers of the 421C>A allele exhibited increased plasma AUC values and higher peak plasma concentrations of rosuvastatin.[25] However, because BCRP is also expressed in the intestinal epithelium, this effect may be related to increased intestinal absorption in addition to decreased biliary clearance.

Under normal conditions, canalicular MRP2 functions in a complementary manner with basolateral MRP3 and MRP4 (*ABCC4*) to maintain low intracellular concentrations of endogenous and exogenous organic anions. Altered function of these efflux transporters due to genetic mutations, disease state alterations, or DDIs may affect hepatocyte exposure to substrates. For example, the toxic metabolite of methotrexate, 7-hydroxymethotrexate, accumulated extensively in the livers of $Mrp2^{-/-}$ mice; accumulation was even more pronounced in $Mrp2^{-/-}/Mrp3^{-/-}$ mice.[26] Probenecid-mediated inhibition of these same transporters was postulated to be responsible for the increased hepatocyte concentrations of valproate glucuronide observed in isolated perfused rat livers following probenecid and valproate coadministration.[27]

Decreased hepatocyte concentrations due to increased basolateral efflux

Induction of basolateral efflux transporters in disease states such as cholestasis or nonalcoholic fatty liver disease[28] may result in decreased hepatocyte exposure to substrates. Although clinical observations are limited, animal studies have shown altered hepatic exposure as a result of induced basolateral efflux transporters. Mice treated with the cholestatic agent alpha-naphthyl isothiocyanate exhibited increased plasma concentrations and decreased hepatic concentrations of unconjugated bile acids, consistent with increased hepatic basolateral Mrp3 expression;[29] however, interpretation of this finding is complicated by parallel decreases in the expression of basolateral uptake transporters that may transport bile acids.

Increased hepatocyte concentrations due to transporter-mediated intracellular sequestration

Membrane transporters are increasingly recognized as important determinants of local drug concentrations in subcellular domains and organelles. For example, cellular toxicities of antiviral agents have been linked to local accumulation in mitochondria as a result of transport via equilibrative nucleoside transporters (*SLC19*).[30] Other transport proteins may be involved in the subcellular localization of drugs,[31,32] but data in hepatocytes are lacking.

Effects of drug-metabolizing enzymes and their interplay with transporters

Many drugs undergo extensive metabolism in the hepatocyte via the action of cytochrome P450s (CYPs; phase I metabolism) and/or conjugation by uridine 5′-diphospho–glucuronosyltransferases, sulfotransferases, and/or glutathione *S*-transferases (phase II metabolism). These enzymes are highly enriched in the liver and are involved in the elimination, detoxification, or activation of various endo- and xenobiotics.[33] Importantly, there is significant overlapping of substrate specificity between hepatic enzymes and transporters. For instance, some drugs, such as atorvastatin and repaglinide, are substrates of both CYP enzymes and OATP1B uptake transporters.[34] Most phase II conjugates are substrates of canalicular (e.g., MRP2, BCRP) and/or basolateral (e.g., MRP3) efflux transporters, which mediate the active transport of these metabolites into the bile or blood, respectively.[35] Thus, the interplay between hepatic transporters and enzymes is a complex process that can modulate systemic and hepatocyte concentrations of drugs and metabolites, as well as endobiotics.[36]

In general, basolateral uptake and efflux transporters regulate the extent of intracellular drug accumulation. Enzymes and canalicular efflux transporters modulate cellular concentrations via metabolism and biliary excretion. Studies of isolated perfused rat livers with digoxin, a substrate for rat hepatic

Oatp1a4, P-glycoprotein (P-gp), and Cyp3A, have demonstrated the interplay between metabolism and transport in determining hepatic digoxin exposure.[37] Rifampin reduced hepatic exposure of digoxin to Cyp3A by inhibiting basolateral uptake mediated by Oatp1a4. By contrast, quinidine slightly decreased hepatic exposure of digoxin and significantly increased the levels of the digoxin metabolite Dg2 and the ratio of Dg2 to digoxin in the liver. These findings are consistent with inhibition of P-gp-mediated canalicular excretion of digoxin, which results in increased availability to and, subsequently, increased metabolism by Cyp3A.[37] Erythromycin, a commonly used probe for CYP3A4 activity, is also a substrate for MRP2 and P-gp. In a study in cancer patients, a common reduced-function variant of MRP2 (24C>T; rs717620) was found to be associated with increased erythromycin metabolism on the basis of the erythromycin breath test. This effect was attributed to increased hepatic residence time of erythromycin due to a reduction in MRP2-mediated biliary secretion.[38] Collectively, these data are in agreement with results from physiologically based pharmacokinetic (PBPK) modeling studies demonstrating that decreased uptake transporter activity may decrease the rate (and thus extent) of metabolism, assuming that hepatic uptake is the major contributor to systemic elimination. By contrast, decreased activity of biliary transporters would be expected to increase the rate of metabolism under linear kinetic conditions due to competition for intracellular substrate concentrations between biliary efflux transporters and enzymes.[39]

In summary, studies illustrating the effect of transporters and enzyme–transporter interplay on hepatic exposure of drugs and their metabolites are often conducted using *in vitro* cultured hepatocytes, *in situ* isolated perfused livers, or transporter-knockout animals. Data from these model systems may be confounded, for example, by *in vitro* conditions that are relatively simplistic as compared with the complexity of the *in vivo* system, species differences, and the possibility that knockout models may show compensatory alterations in metabolic and/or transport pathways. Generally, data that directly demonstrate transporter effects and enzyme–transporter interplay in humans are sparse owing to the technical challenges involved in directly measuring intracellular and bile concentrations of drugs/metabolites *in vivo* in human livers. The interaction (e.g., inhibition or induction) with transporters/enzymes in other organs, such as the gastrointestinal tract or kidney, coupled with the often nonselective inhibitory profiles of coadministered medications, may complicate the interpretation of *in vivo* clinical data. In this regard, PBPK modeling may be useful to interpret and predict such complex transporter effects and enzyme–transporter interplay as well as the impact on hepatic exposure of drugs and metabolites under different scenarios.[39]

EXPERIMENTAL METHODS FOR MEASURING INTRACELLULAR DRUG CONCENTRATIONS

The *in vitro*, *in situ*, and *in vivo* models that potentially can be applied to estimate unbound hepatocyte concentrations of drugs and metabolites are summarized in **Table 2**, along with the major applications and limitations of these models. In most cases, these models cannot provide direct estimation of unbound hepatic drug concentrations in humans, but the data generated can be applied as input kinetic parameters for mechanistic modeling to predict intrahepatic concentrations. With the availability and application of analytical tools that accommodate femtoliter-level sample volumes and submicron-scale image resolution, direct measurements of intracellular concentrations are becoming more realistic. Current and future experimental methodologies that provide qualitative or quantitative measurements of intracellular drug concentrations are summarized in **Table 3** and discussed below. These include indirect methods, which require a modeling approach to estimate the amount of intracellular drug, and direct methods, which provide quantification of drug in defined cellular or subcellular volumes. To date, many of these techniques are not established for the intracellular measurement of drug molecules in hepatocytes. However, published examples have been provided that illustrate potential future applicability to drugs and metabolites.

Indirect methods

Tissue homogenization. The amounts of drug extracted from tissue homogenate and a corresponding blood sample have been used to describe drug partitioning, to differentiate active vs. passive tissue uptake, and to derive pharmacokinetic parameters for compartmental models, including uptake and efflux clearances. Although convenient, this approach fails to acknowledge the various compartments (e.g., bile and sinusoidal blood), cell types, and subcellular organelles within which drug molecules often distribute heterogeneously. Therefore, determination of hepatic intracellular drug concentrations using this approach may be misleading.[40] This has been addressed in part by tissue homogenate dilution, fractionation, and equilibrium dialysis to determine subcellular localization and intracellular unbound drug concentrations using brain and liver samples.[11,41]

Fraction unbound in hepatocytes. The unbound fraction of drugs in hepatocytes is generally determined using equilibrium dialysis techniques, followed by quantification of drug concentrations via standard analytical techniques, such as high-performance liquid chromatography–tandem mass spectrometry, in hepatocytes where metabolic activity has been inhibited.[42] This methodology is applicable to hepatocyte experiments with compounds that are metabolically cleared and whose entry into hepatocytes is not limited by hepatic uptake.[43] However, for compounds for which entry into the hepatocyte is limited by permeability, intracellular unbound fractions cannot be determined using this methodology. Intracellular unbound hepatocyte concentrations in the presence of an active transport mechanism have been estimated by simultaneously fitting experimental data determined across a range of drug concentrations and time points.[44] This modeling approach is discussed in more detail below. Alternatively, intracellular unbound drug concentrations have been quantified *in vitro* through parallel measurements of intracellular bound and

total drug concentrations in cultured cell lines[13] and in suspended[9] and sandwich-cultured hepatocytes.[41]

Tomography imaging. Whole-body, noninvasive imaging methods, such as single-photon emission computed tomography and positron emission tomography (PET) imaging, are widely used to assess biodistribution and have been applied to the measurement of hepatobiliary excretion.[45,46] These imaging modalities offer an advantage over tissue homogenization in terms of increased resolution to suborgan-level compartments and the ability to serially sample the same subject. However, they rely on measurements of radiolabeled drug divided by a theoretical volume that includes the combination of intravascular, extracellular, and intracellular compartments. Radiochemical modification of the test article with a particle-emitting probe (e.g., ^{18}F, ^{11}C, ^{89}Zr, and ^{124}I) could theoretically alter the distribution properties or transporter affinity of the molecule. In addition, differentiation between parent and metabolite usually is accomplished only through pharmacokinetic modeling.

Microdialysis. Microdialysis is limited in application and provides data from extracellular fluids to indirectly estimate intracellular drug concentrations. This primarily preclinical technique is typically used to measure unbound drug concentrations in the brain, but it also has been applied for measurement of unbound drug in the bile.[47] Assumptions regarding equilibrium dynamics, permeability, and the role of active vs. passive processes govern the utility of this technique and limit its applicability to calculations of intracellular drug concentrations. An interesting approach that provides a better estimate of intracellular drug concentrations is PET imaging combined with microdialysis.[48] In this approach, a microdialysis probe is placed in the tissue/region of interest during a dynamic PET scan, thus enabling measurement of unbound drug concentrations in the extracellular space corresponding to the PET data.

Microautoradiography. Microautoradiography tracks the distribution of radiolabeled molecules in tissues and cells in culture.[49] The technique has traditionally been used in conjunction with quantitative whole-body autoradiography for research on absorption, distribution, metabolism, and excretion during drug development. Resolution in microautoradiography is generally of multicellular scale, but the technique has been shown to be useful for the study of single-cell microorganisms and can be applied to hepatic drug quantification.

Fluorescence imaging. Traditionally, quantitative chemical imaging methods have been used to study the distribution of fluorescent or pigmented dye molecules in cells or tissue samples. By combining optical (transmitted light or fluorescence) imaging microscopy with quantitative computational analysis techniques, spatial variations in the absorbance of a tissue sample, or in the fluorescence excitation and emission of a tissue sample, can be converted to relative differences in the spatial distribution of an optical probe. Recently, additional deep-tissue-imaging techniques have been developed to facilitate the study of fluorescent probes in cells and live animals. However, only a handful of drugs fluoresce in the visible wavelengths or are pigmented sufficiently to allow detection above the endogenous background of tissue samples. Therefore, the application of fluorescence or absorbance imaging techniques holds very limited promise for pharmacokinetic analysis of subcellular drug disposition in the liver.

Direct methods

Currently, there are no established methods that can be used to directly measure subcellular drug concentrations in the liver. In hepatocytes, measuring drug concentrations in different subcellular compartments by direct chemical analysis is complicated by the small volumes and the dynamic nature of transport and retention phenomena that can be perturbed by invasive measurement schemes. As an alternative, noninvasive *in vitro*, *in vivo*, and *ex vivo* quantitative, microscopic–chemical imaging approaches have been developed. These methods could potentially be used to measure drug concentrations in hepatocytes (**Table 3**). The following techniques seem most promising and relevant.

Capillary electrophoresis. To our knowledge, capillary electrophoresis (CE; **Table 3**) is the only direct subcellular analysis method that can be used to monitor the subcellular distribution of chemical agents in the hepatocytes of live animals.[50] In CE, whole cells and subcellular organelles are drawn into a capillary tube through electrokinetic or siphoning methods. Although CE requires cell disruption before measurement, the nature of this technique could theoretically provide analytical results within seconds after disruption, minimizing the effect of drug diffusion during the process. Chemical agents in the capillary are then measured with any one of a variety of highly sensitive detection methods, including laser-induced fluorescence, absorbance, and electrochemical and mass spectrometry.[51,52] Nevertheless, although CE can be used to measure the amount of drug associated with a specific organelle fraction, drug concentration is also dependent on organelle volume.

Microscopic optical imaging. To visualize the subcellular distribution of unlabeled small-molecule drugs, Raman confocal microscopy is perhaps the most promising and broadly applicable technique (**Table 3**). Although this optical imaging technique can be used to monitor the relative intracellular mass distribution of small molecules, it could potentially be combined with independent calibration and volume measurements using three-dimensional reconstructions of confocal sections through a cell or tissue slice to calculate the concentrations of these molecules in individual subcellular compartments.

Microscopic mass spectrometry imaging. Secondary ion mass spectrometry (SIMS) measurements are performed by directing a primary, micron-, or nanometer-diameter ion beam of a few kiloelectron volts of energy onto the surface of a solid tissue section to form secondary ions reflecting the atomic composition of the surface (**Table 3**). These secondary ions are then

Table 2 ***In vitro*, *in situ*, and *in vivo* models to estimate the intracellular concentrations of drugs and metabolites in the liver**

Model system	Advantages	Disadvantages	Examples of parameters estimated[a]
Membrane vesicles	• Specific cell membrane or transporter protein can be studied in isolation • Substrate can have direct access to transporter-binding site	• Direct correlation between transport kinetics and intracellular concentrations has not been extensively studied • Variations between batches • Not possible to directly measure intracellular hepatic concentrations	• Canalicular efflux of pravastatin through Mrp2 was quantified using rat canalicular membrane vesicles: *in vitro* biliary clearance (*in vitro* CL_{bile}) was estimated[2]
Recombinant proteins	• Simple experimental design and rapid generation of test system • Identification of vectorial transport of substrates in polarized system • Kinetic parameters can be generated for PBPK models to estimate intracellular hepatic concentrations	• Not possible to directly measure intracellular hepatic concentrations • For polarized systems, permeation across second membrane can be rate limiting, leading to the requirement for complex kinetic models	• Intracellular accumulation of ezetimibe and metabolites measured in cell lines expressing OATP1B1, UGT1A1, and MRP2 using both radiolabel and LC–MS/MS[82]
Hepatocytes	• Direct measurement of initial uptake rate and intracellular concentrations • Many parameters can be determined from a single liver because a large number of cells will be available	• Loss of polarity in suspension	• Intracellular concentrations of glutathione determined using capillary electrophoresis–laser-induced fluorescence[83] • *In vitro* studies to assess the impact of hepatic uptake transporters compared with passive diffusion into rat hepatocytes. K_{puu} and CL_{diff}, along with other parameters, were estimated[2] • Parameters describing hepatic uptake (both active and passive) and intracellular binding were estimated[44,63]
Sandwich-cultured hepatocytes	• Polarity of hepatocytes is restored. Bile canalicular structures are formed, thus allowing vectorial transport across the hepatocytes • Direct measurement of intracellular *in vitro* hepatic concentrations possible	• Expression of transporters and drug-metabolizing enzymes can increase/decrease during culture	• Estimation of kinetic parameters to describe disposition of parent compound and metabolites[84] • Parameters describing hepatic uptake (both active and passive) and biliary efflux were estimated for several OATP substrates[61]
Perfused liver	• Direct measurement of intracellular hepatic concentrations possible • Architecture of the liver remains intact • Extrahepatic processes cannot influence outcomes	• Perfusion of human livers is not feasible • Viability of liver will decline throughout the duration of experiment • The experiments are labor intensive	• Estimation of $f_{u,liver}$ and other kinetic parameters to describe hepatic disposition of parent compound and metabolites of both pafuramidine and CPD-0868[84] • The impact of Oatp and Mrp2 on the intrahepatic concentrations of benzyloxypropionic tetraacetate were measured by γ scintillation probe, with kinetic parameters estimated for uptake and biliary efflux[85] • Impact of hepatic uptake and efflux on the exposure of digoxin to hepatic metabolism (Cyp3a). Liver-to-perfusate ratios were measured[37]
Animal models	• Direct measurement of hepatic concentration possible • Architecture of liver remains intact	• The effect of individual transporters can be determined from mutant strains of animals; however, data may be confounded by alterations in compensatory pathways • Species differences in expression levels and substrate recognition	• Exposure of liver to methotrexate by microdialysis[86]

Table 2 Continued on next page

Table 2 Continued

Model system	Advantages	Disadvantages	Examples of parameters estimated[a]
Knockout animal models	• Complete system where the impact of the abolition of one or more pathways can be explored	• Impact of one pathway can be determined; however, up- or downregulation of other pathways may occur	• Differences in exposure of digoxin in liver and other tissues were determined by bulk tissue analysis via radiometric endpoints[87] • Hepatic exposure of metformin in Oct1$^{-/-}$ relative to wild-type mice[18,19] • Effects of Mrp2 and/or Mrp3 on the hepatic exposure of methotrexate and its 7–OH metabolite; liver-to-plasma ratios (K_p) were estimated[26]
Human *in vivo* studies	• Complex system; however, potential to determine intracellular concentrations is a possibility that may be realized	• Direct measurement of unbound intracellular drug concentrations is challenging	• PET imaging of hepatobiliary processes[46] • Imaging and pharmacokinetic modeling of hepatic levels of ^{99m}Tc-mebrofenin[22] • ^{99m}Tc-mebrofenin–ritonavir hepatic DDI evaluated in humans based on imaging data, *in vitro* IC_{50} values, and intracellular unbound ritonavir concentrations[72]

DDI, drug–drug interaction; $f_{u,liver}$, fraction unbound in the liver; IC_{50}, half-maximal inhibitor concentration; K_{puu}, hepatocyte-to-medium partition coefficient for unbound drug concentration; LC–MS/MS, liquid chromatography–tandem mass spectrometry; OATP, organic anion–transporting polypeptide; PET, positron emission tomography; PBPK, physiologically based pharmacokinetic.

[a]Refs. 76–98 are listed in **Supplementary Data** online.

analyzed by mass spectrometry. Although SIMS can be used to monitor the relative spatial distribution of drug molecules with nanometer resolution, sample preparation involving fixation or freezing could lead to artifacts affecting drug distribution. Matrix-assisted laser desorption ionization, which utilizes soft ionization of sample surfaces using ultraviolet or infrared laser coupled with time-of-flight mass spectrometry, currently lacks the sensitivity to yield subcellular resolution.

Particle-induced photon emission. Similar to SIMS, micro-PIXE (particle-induced X-ray emission) and micro-PIGE (particle-induced gamma ray emission)[53,54] are two additional microscopic chemical imaging techniques that could be used to detect the subcellular distribution of drugs in hepatocytes based on quantifying the emission of photons that are produced upon interaction of an ion beam with a tissue surface. As in SIMS, sample preparation involving fixation or freezing could be problematic and may lead to artifacts.

As described above, direct methods to measure intracellular drug concentrations are not routinely available. Instead, there are established, indirect methods for measuring drug concentrations in the liver that provide parameters for mathematically modeling drug concentrations in the hepatocytes. These modeling techniques are discussed in the following section.

ESTIMATION OF INTRACELLULAR DRUG CONCENTRATIONS BY MODELING AND SIMULATION

Modeling and simulation are a useful approach to estimating unbound intracellular drug concentrations in different tissues. Currently, reported modeling and simulation methods use physicochemical properties of drugs, *in vitro* cell-based data such as passive permeability, cellular uptake/efflux, or *in vivo* animal and human pharmacokinetic data applied for estimation/refinement of certain parameters using PBPK modeling approaches. Models that can reasonably delineate the impact of transporters/enzymes on cellular or *in vivo* disposition provide useful estimates of intracellular concentrations, but all involve a number of assumptions. Although integration into models of tissue concentration data obtained by advanced experimental methods as highlighted above is currently limited, it represents a key step for further refinement of the existing models and their validation.

Estimation of unbound intracellular drug concentration based on physicochemical properties

When permeation of drugs into cells is driven primarily by passive diffusion, steady-state partitioning into a cell can be reasonably predicted using $logD_{7.4}$ for acidic compounds and logP for basic and neutral molecules. These empirical models have been used to predict the unbound fraction in both microsomes and hepatocytes (**Table 4**, methods 1 and 2). More recently, an empirical relationship was defined between $f_{u,cell}$ values obtained by mechanistic modeling and $logD_{7.4}$ using uptake data from either suspended or plated hepatocytes (**Table 4**, methods 3 and 4, respectively). This empirical relationship was established for a series of acidic and neutral drugs, many of which are substrates for hepatic uptake transporters.[10,44] Extending these results, another recent study reported a multivariate structure-binding relationship based on $f_{u,cell}$ measurements in cultured cells.[13] Although empirical, these approaches can provide an initial estimate of the $f_{u,cell}$ for a transporter substrate before further mechanistic modeling.

The physicochemical characteristics of drugs can determine their unique intracellular distribution/accumulation. For example, basic and cationic drugs can differentially distribute into lysosomes and mitochondria, respectively.[11,13,14] Consideration of lysosomal partitioning in hepatocytes has been reported for lipophilic basic drugs (e.g., fluoxetine),[12] but a more detailed analysis has been provided for brain tissue,[11] for which inclusion of pH partitioning into the cell and lysosomes with a

Table 3 Direct and indirect methodologies for the estimation of intracellular drug concentrations

Direct measurement method[a]	Analyte/matrix	Detection method	Utility/limitations/assumptions
Capillary electrophoresis[51,52,88]	"Bioparticles", whole cells, organelles	Laser-induced fluorescence, UV, electrochemical, LC–MS	Nano- to femtoliter sample volumes required for analysis
			Technically challenging and limited accessibility
			Can isolate individual cells or organelles for analysis
			Multiple techniques for different culture/cell types
MSI: Nano-SIMS, MIMS[89–91]	Individual cells, potentially subcellular fractions, "bioparticles"	Secondary ion mass spectrometry, with mass analyzer, multi-isotope imaging mass spectrometry	Nano-SIMS ^{14}C resolution potentially <0.1 μm
			Nano-SIMS sensitivity could achieve 1,000 times that of ^{14}C autoradiography
Raman microscopy[92–94]	Analysis of cells and tissues, material surface	Light scatter through change in polarization potential, rotation, or vibration energy	Probes' vibrational states within chemical bonds
			Applicable to biological systems with lower energy excitation for sample preservation
			Recent, advanced detection systems have shortened data collection times for increased imaging throughput
Nuclear microscopy (microbeam PIXE/PIGE)[53,54,95]	Single cell; platinum and endogenous metals	Ion microbeam with particle-induced X-ray/γ-ray emission	Achieves ≤1-μm diameter resolution
			Not a widely accessible technology
			Limited to metal-containing drugs/compounds (e.g., platinum drugs)
Microautoradiography[49,96]	Radiolabeled sample in cryosection	Exposure of radiolabel, FISH, IHC, confocal microscopy	Grain density evaluation can be combined with micro-FISH and confocal microscopy for structure–function analyses
			Resolution generally limited to multicellular level
PET/SPECT imaging[45,46,97]	Positron/γ particle–emitting total drug or metabolite(s) in imaged tissue or organs of interest	PET/SPECT image with PK samples/LC–MS/LSC	Residualizing vs. nonresidualizing isotopes allow for derivation of internalization rate and concentration
			Receptor occupancy measurements possible
			Expensive, technically challenging, limited by resolution to mathematically deriving concentrations in tissues
PET imaging with simultaneous microdialysis[48]	Same as PET plus microdialysate from volume of interest corresponding to PET scan	PET/in-line HPLC radioligand detector, γ-counter	Requires kinetic modeling to parameterize analyte flux and derive intracellular concentrations
			Similar limitations as PET, yields small sample volumes
			Requires physicochemical characterization of the test article to draw meaningful conclusions
Bulk analysis[11,40]	Total drug or metabolite(s) in tissue homogenate or section	HPLC–UV, LC–MS, radioactivity, MALDI	Often fails to describe suborgan distribution
			Pharmacokinetic model-based approach often used to derive intracellular concentrations from resultant data
			Low-technology method and easily accessible

FISH, fluorescence *in situ* hybridization; HPLC, high-performance liquid chromatography; IHC, immunohistochemistry; LC–MS, liquid chromatography–mass spectrometry; LSC, liquid scintillation counting; MALDI, matrix-assisted laser desorption ionization; MIMS, multi-isotope imaging mass spectrometry; MSI, mass spectrometry imaging; PET/SPECT, positron emission tomography/single-photon emission computed tomography; PIGE, particle-induced γ-ray emission; PIXE, particle-induced X-ray emission; PK, pharmacokinetic; SIMS, secondary ion mass spectrometry; UV, ultraviolet.

[a]Refs. 76–98 are listed in **Supplementary Data** online.

three-compartment model accounted for the difference in the f_u between brain slices and brain homogenates. In tumor cells, more than 90% of cellular MKT-077, a lipophilic cation, was estimated to reside in mitochondria;[14] modeling of the intracellular concentration of MKT-077 was performed using the equations detailed in Method 7, Table 4. Further investigation of such mechanistic cellular models and their applicability to hepatocytes is required.

Monolayer cell–based permeability models

A common method to measure cellular permeability and transport is to determine the flux across a cell monolayer. Monolayer systems have been used to identify efflux transporter substrates and to determine the kinetic constants for transport. Although hepatocytes do not have the required tight junctions needed for monolayer permeability experiments, both hepatocytes and monolayer models include passive permeability and active transport, and the interactions between these processes may be similar for both cell systems. Several reports have used three-compartment models to represent Caco-2 or Madin–Darby canine kidney cell monolayers,[55–59] for which the three compartments represent the apical, cellular, and basolateral compartments. Three-compartment models also have been applied to simulate the combinations of passive permeability, active uptake, and efflux transport, as well as to study metabolism in Caco-2 cells.[58]

Such mechanistic modeling of drug permeability has revealed the importance of local concentrations in the design and interpretation of drug transport experiments for efflux transporters such as P-gp and BCRP. Using elementary rate constants to model permeability across monolayer model systems, the actual K_m (Michaelis–Menten constant) parameters for transporter-mediated flux were shown to differ greatly[55] from the apparent K_m obtained by fitting a Michaelis–Menten equation to the observed transport data ($K_{m,app}$). Another report showed that kinetic parameters calculated from concentration–efflux data overestimate the half-maximal inhibitor concentration (IC_{50}) and K_m values.[56] Moreover, using verapamil, quinidine, and vinblastine as substrates, K_m values determined based on intracellular concentrations calculated from a three-compartment model were consistent for different cell systems, whereas $K_{m,app}$ varied greatly with transporter expression.[59] These reports suggest that the differences in observed K_m and K_i (inhibition constant) values are due to changes in the intracellular concentration of substrates and inhibitors caused by the action of transporters. Use of calculated intracellular concentrations (instead of apical or basolateral concentrations) resulted in more consistent kinetic parameters.

Because the substrate-binding site of P-gp is accessed from within the inner leaflet of the apical membrane,[60] the three-compartment model described above was extended to include explicit basolateral and apical membrane compartments.[57] This model showed significant differences in predicted intracellular concentrations for apical vs. basolateral addition of drug. Apical efflux by P-gp resulted in very low intracellular concentrations following apical addition, whereas intracellular concentrations were lower by twofold or less after basolateral addition. These observed differences are consistent with the impact of efflux transporters on *in vivo* drug concentrations in the brain (apical exposure) and liver (basolateral exposure). Although the intracellular concentrations in the above studies have not been confirmed experimentally by direct measurement, correlations among predicted intracellular concentrations, K_m values, and tissue concentrations are encouraging.

Hepatocyte models to estimate unbound fraction

Studies that have attempted to assess the extent of intracellular binding of transporter substrates in conjunction with multiple ongoing processes within hepatocytes are summarized in **Table 4**. One method to estimate the $f_{u,cell}$ value is indirectly from the K_p (hepatocyte-to-medium total drug concentration ratio) and K_{puu} (hepatocyte-to-medium partition coefficient for unbound drug concentration) data.[9,10] The parameter K_p reflects the extent of both intracellular binding and active transport processes, whereas K_{puu} reflects only active transport. Certain studies incorrectly assume that K_p reflects an increase in cellular unbound drug concentration, which is subsequently available to metabolic enzymes. A study using suspended rat hepatocytes showed that no direct correlation could be established between K_{puu} and $f_{u,cell}$, highlighting the fact that the measurement of only one of these processes in isolation is insufficient to characterize drug distribution in hepatocytes.[10] The importance of the K_{puu} value for statins, which are taken up into hepatocytes by OATPs, is exemplified in interpreting the difference in the IC_{50} of statins for the inhibition of 3-hydroxy-3-methyl-glutaryl–coenzyme A reductase activity between the hepatocyte and the microsomal systems. Theoretically, the ratio of the IC_{50} values (microsome/hepatocyte) of statins should be the same as their K_{puu} values because only unbound drug will be responsible for the enzyme inhibition. The reported data seem to support this hypothesis.[9]

Recently, mechanistic two- or three-compartment models including media, cellular, and bile compartments have been applied.[44,61–64] These models allow dynamic evaluation of multiple processes occurring in hepatocytes, including active transport, passive permeability, efflux, and intra/extracellular binding. Unlike static models (e.g., the conventional two-step approach in which passive diffusion is measured separately and subtracted from the transport observed in a complex system), mechanistic compartmental models allow simultaneous fitting of all concentration–time points during uptake at 37°C and estimate the extent of intracellular binding as an output model parameter.[44,63] Intracellular binding is estimated in conjunction with other processes, including metabolism, if the cellular concentrations of both parent and metabolite(s) are measured. The $f_{u,cell}$ in the mechanistic *in vitro* models can be obtained/predicted in some instances by other methods and implemented as a constant in the model, as shown recently in the analysis of sandwich-cultured hepatocyte uptake data.[61] One of the assumptions in the current mechanistic compartmental *in vitro* models is that the intracellular binding is not saturated at the substrate concentrations studied, which may lead to overestimation of the $f_{u,cell}$ value if saturation were to occur. These mechanistic models provide more dynamic and physiologically relevant characterization of cellular processes, but the main limitation is the requirement for a large number of data points/cells for appropriate parameter definition. Subsequently, estimated $f_{u,cell}$ values are applied as input parameters in the PBPK models to allow a mechanistic description of unbound drug concentrations in the liver.

PBPK models

Whole-body PBPK models integrate drug-related kinetic parameters and physiological parameters to predict drug disposition *in vivo*. PBPK models are useful to simulate not only blood (plasma) concentrations but also tissue

Table 4 Summary of methodologies available to estimate intracellular fraction of unbound drug in cells

Method[a]	Predictive equations developed for $f_{u,cell}$	Comments
1. Empirical prediction of f_u for nontransporter substrates[42]	$\frac{\log(1-f_u)}{f_u} = 0.40\log D(P) - 1.38$	
2. Empirical prediction of $f_{u,cell}$ for nontransporter substrates[98]	$f_{u,hep} = \frac{1}{1+(125\times VR\times 10^{0.072\log P(D)^2 + 0.067\log P(D) - 1.126})}$	Thirty-nine acidic, neutral, and basic drugs
3. Indirect estimation from K_{puu} and K_p data for transporter substrates[10]	$K_{puu} = \frac{CL_{act,uptake} + CL_{diff}}{CL_{diff}}$, $f_{u,cell} = \frac{K_{puu}}{K_p}$	Empirical model for prediction of $f_{u,cell}$ developed using acidic and neutral drugs: Log $f_{u,cell}$ = −0.9161–0.2567 $\log D_{7.4}$ (suspended hepatocytes)
4. Mechanistic compartmental uptake model[44,63]	$\frac{dC_{cell}}{dt} = \frac{\frac{V_{max}\times C_{med,u}}{K_{m,u}+C_{med,u}} + CL_{diff}\times C_{med,u} - CL_{diff}\times C_{cell}\times f_{u,cell}}{V_{cell}}$	$f_{u,cell}$ is the output parameter of the mechanistic model
		Extended incubation times (45–90 min) required for precise $f_{u,cell}$ estimation
		Empirical model for prediction of $f_{u,cell}$ developed using acidic and neutral drugs: Log $f_{u,cell}$ = −0.4379–0.4129 $\log D_{7.4}$ (plated hepatocytes)
		$f_{u,cell}$ Obtained using extensive rat hepatocyte studies can be applied for the analysis of human hepatocyte uptake data
5. Mechanistic compartmental uptake and metabolism model[44,63]	$\frac{dC_{cell}}{dt} = \frac{\frac{V_{max}\times C_{med,u}}{K_{m,u}+C_{med,u}} + CL_{diff}\times C_{med,u} - C_{cell}\times f_{u,cell}\times(CL_{diff}+CL_{met,u})}{V_{cell}}$	
6. pH partitioning[11]	$K_{puu,cell} = V_{ISF} + K_{puu,cyto}(V_{ISF} + V_{lyso}K_{puu,lyso})$	$K_{puu,cyto}$ and $K_{puu,lyso}$ are determined by pH partition theory
7. Model based on plasma membrane potential and mitochondrial uptake[14]	$C = (1+\beta)V_{cell}e^{\gamma}C_{0,med}(1-e^{-\kappa t})$	Cellular concentration of unbound fraction ($C_{cell,u}$) estimated using total concentration and cytosol/mitochondria equilibrium constant (β), which is determined from the C(t) profile
	$\kappa = k\gamma/(1+\beta)(1-e^{\gamma})$	
	$C_{cell,u} = C/(1+\beta)$	

$C_{0,med}$, initial drug concentration in medium; C_{cell}, total cellular concentration; $CL_{act,uptake}$, intrinsic active uptake clearance; C_{diff}, passive diffusion clearance; $C_{med,u}$, unbound drug concentration in medium; $CL_{met,u}$, unbound metabolic clearance; dC/dt, the differential form describing the rate of change in concentration with respect to time; f_u, fraction of unbound drug; $f_{u,cell}$, fraction of unbound drug in cell; $f_{u,hep}$, fraction of unbound drug in hepatocyte incubations; k, mass transfer coefficient across the cell membrane; $K_{m,u}$, unbound drug affinity constant; K_p, the membrane partition coefficient, which can be measured as the hepatocyte-to-medium total drug concentration ratio; K_{puu}, hepatocyte-to-medium partition coefficient for unbound drug concentration; $K_{puu,cyto}$, ratio of concentration of the unbound fraction in the cytosol to that in brain interstitial fluid (ISF); $K_{puu,lyso}$, ratio of concentration of the unbound lysosomal fraction to that of the unbound cytosolic fraction; V_{cell}, intracellular volume; V_{ISF}, volume of the brain interstitial fluid; V_{lyso}, volume of the lysosome; V_{max}, maximum transport or metabolic rate; γ, constant associated with plasma membrane potential.

[a]Refs. 76–98 are listed in **Supplementary Data** online.

concentration–time profiles. This allows assessment of (i) potential drug-related efficacy and toxicity (e.g., statin profiles in the liver and muscle, respectively)[2] and (ii) changes in systemic and liver exposure due to DDIs or genetic polymorphisms of transporter/enzyme proteins.

Most PBPK models consider tissues as well-stirred, perfusion rate–limited compartments, i.e., the concentration of the unbound drug in the tissue is in equilibrium with the unbound concentration in the emergent blood.[65,66] Although acceptable for many highly permeable drugs, a permeability rate–limited tissue model is often required to describe distribution of low-permeability molecules. For these drugs, additional compartments are included to account for vascular or cellular diffusional barriers.[2,61,64–67] **Figure 2b** represents a generic permeability-limited liver model that can be expanded to account for additional processes, if needed (e.g., basolateral efflux, organelle sequestration, or saturable binding (as in the case of cyclosporine)).[68] However, the model expansion and complexity depend on the availability of adequate tissue data to allow a mechanistic description of these processes and precise estimation of the PBPK model parameters.[68]

Drug distribution within the liver is a complex process, and determining the steps that modulate hepatic exposure of drugs is important, as illustrated in **Figure 3**. For drugs with high passive diffusion and substantial metabolic elimination (e.g., saquinavir), profiles such as those simulated in **Figure 3a–c** are expected. However, for many drugs, active uptake from the blood into hepatocytes is the dominant process, in comparison with passive permeability, metabolism, or active efflux,[10,63,69] resulting in unbound liver/blood concentration ratios greater than unity ($K_{puu,liver}$ >>1) (**Figure 3f**). In such cases, reduced activity in basolateral uptake transporters (due to either inhibition or polymorphism) leads to increased drug concentrations in blood and lower $K_{puu,liver}$. However, the effect on liver exposure (expressed as AUC_{liver}) will depend on whether alternative elimination routes exist: if hepatic elimination is the predominant route, AUC_{liver} will be determined primarily by biliary excretion and metabolic clearance. In that case, reduced activity of the basolateral uptake

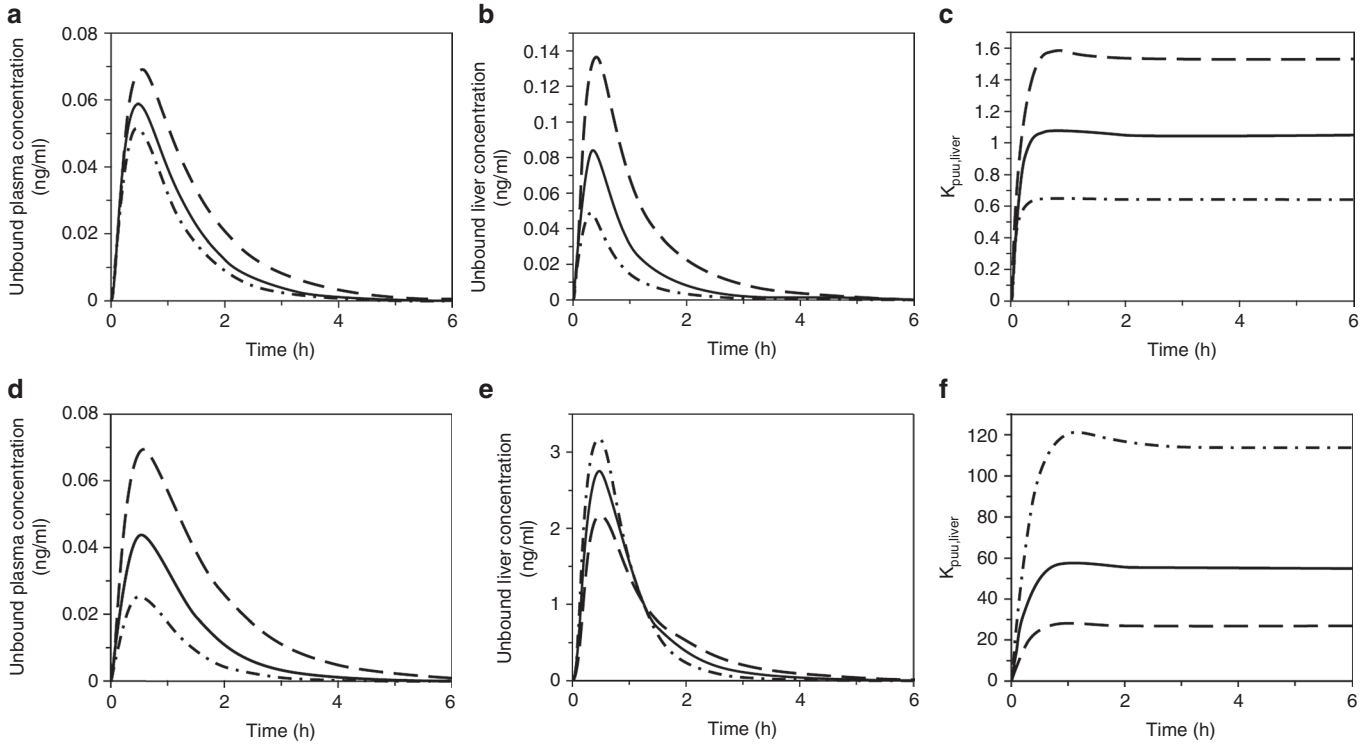

Figure 3 PBPK simulations of the unbound plasma and liver concentration–time profiles and the ratio of unbound liver tissue to liver sinusoidal concentrations ($K_{puu,liver}$). A permeability-limited liver model was used for simulations to account for active transport processes. (**a**–**c**) Drugs with a large contribution of passive diffusion to total uptake and substantial metabolic elimination/biliary excretion. Profiles represent the impact of variation in hepatic elimination resulting in differential liver exposures, where the solid line represents CL_{met} (or CL_{bile}); dashed line, 0.5× CL_{met} (or CL_{bile}); and dashed-dotted line, 2× CL_{met} (or CL_{bile}). (**d**–**f**) Active uptake is the major contributor to the total uptake ($CL_{act,uptake} > CL_{diff} > CL_{met}$ or CL_{bile}). Variation in uptake transporter activity (solid line represents $CL_{act,uptake}$; dashed line, 0.5× $CL_{act,uptake}$; and dashed-dotted line, 2× $CL_{act,uptake}$) results in differential blood exposure (**d**) with no effect on liver AUC (**e**) for drugs primarily cleared by liver, either via biliary excretion (e.g., pravastatin) or CYP450-mediated metabolism (e.g., repaglinide). (**a**–**f**) The drug is assumed to have a high extent of intracellular binding ($f_{u,cell} < 0.1$) and minimal contribution of renal clearance to overall elimination. Changing the $f_{u,cell}$ in the PBPK model will affect the total liver concentration, but the overall trends will remain the same. AUC, area under the concentration–time curve; $CL_{act,uptake}$, intrinsic active uptake clearance; CL_{bile}, intrinsic biliary excretion clearance; CL_{diff}, passive diffusion clearance; CL_{met}, intrinsic metabolic clearance; $f_{u,cell}$, unbound fraction of drug in cell; PBPK, physiologically based pharmacokinetic.

transporter will lead to a decrease in $C_{max,liver}$ and a prolonged terminal half-life, but the AUC_{liver} value will remain unchanged (Figure 3e). In contrast, inhibition of either biliary transporters or metabolism could have a pronounced effect on liver exposure (e.g., pravastatin).[2] If renal clearance is a significant contributor to the total drug clearance, liver and plasma exposure will be affected by multiple mechanisms, including not only hepatic basolateral uptake, metabolism, and/or biliary excretion but also renal elimination (as in the case of methotrexate,[70] Table 1). A recent clinical microdose study performed in the presence of either OATP1B or CYP3A4 inhibitors[71] supported the principles stated above for atorvastatin, but clinical data delineating the relative importance of OATPs and metabolism are lacking for most other drugs. The interplay between these processes is very complex, and direct measurement of exposure in liver and other human tissues is crucial to further validate this model because systemic exposure data, in many cases, are not an adequate surrogate for tissue concentration–time profiles. Clinical studies using PET/single-photon emission computed tomography imaging and other advanced experimental methods in conjunction with mechanistic modeling may help refine our understanding of tissue distribution of a drug and its implications.[45,72]

For a PBPK model to accurately predict drug disposition (including intracellular concentrations), mechanistic input parameters from *in vitro* cellular systems (e.g., active uptake/efflux, passive permeability, intracellular binding, and metabolism) need to be integrated with systems parameters (e.g., abundance of transporters and enzymes).[2,64,67] In certain instances, the kinetic parameters related to hepatic clearance (active uptake or biliary excretion) can be estimated *in vivo* in rats.[2] Despite species differences in transporter expression and activity,[73] initial model optimization in rats can be useful for understanding the underlying mechanisms (e.g., enterohepatic recirculation of valsartan) and application as empirical scalars for human prediction.[2,74] Further validation/refinement of the human PBPK models should be carried out when relevant clinical data become available. Use of PBPK model–simulated local tissue concentrations at the relevant sites of action (instead of plasma exposure as a surrogate) allows prediction of transporter- and transporter-metabolism-based DDIs in a more mechanistic manner. Examples include use of the unbound hepatic inlet concentrations for the assessment of the interaction potential on basolateral hepatic uptake transporters, liver intracellular concentrations for efflux transporters/enzymes, and enterocyte

concentrations for intestinal efflux transporters/enzymes, as conducted recently for the prediction of cyclosporine DDIs.[67]

CURRENT CHALLENGES IN MEASURING AND PREDICTING INTRACELLULAR CONCENTRATIONS OF DRUGS AND METABOLITES

Many challenges and knowledge gaps currently exist in determining the relevant hepatocellular concentrations of drugs and metabolites to accurately predict drug efficacy, toxicity, and clearance (metabolic or biliary) difficult. In general, our understanding of intracellular drug disposition in the liver is rudimentary. Although mathematical models commonly assume that the liver is a "well-stirred" compartment, subcellular distribution (e.g., lysosomal trapping and mitochondrial accumulation) may affect the intracellular concentration of some drugs, thereby influencing specific target binding, metabolism, and toxicity. How these processes affect intracellular drug concentrations is not well understood, especially at the kinetic level. Furthermore, the involvement of transporters in determining local drug concentrations in subcellular domains and organelles remains generally unexplored. In fact, the fundamental definition of "intracellular concentration" is debatable. The unbound liver concentration, calculated as the sum of unbound drug mass in all hepatocyte compartments divided by the total cytosolic volume, may not accurately represent the relevant concentrations of most drugs at the sites of action (efficacy), toxicity, and elimination.

Currently available *in vitro* and *in vivo* models have limited capability to quantitatively predict the impact of transporters on intracellular drug concentrations. In many cases, empirical scaling factors need to be applied when extrapolating uptake/efflux transporter activity from *in vitro* to *in vivo* models in humans. For compounds that undergo hepatic metabolism, prediction of enzyme–transporter interplay adds another level of complexity. *In vitro* models retaining the activity of hepatic basolateral uptake/efflux and canalicular transporters, in addition to drug-metabolizing enzymes, may be required to accurately predict $K_{puu,liver}$ in humans. Recently, some three-dimensional cultured-hepatocyte systems/microfluidic devices have been developed to mimic the *in vivo* architecture. Without direct measurement of *in vivo* hepatic pharmacokinetics, it is difficult to reconcile how these sophisticated models will provide better predictions of hepatocellular concentrations than traditional two-dimensional sandwich-cultured hepatocytes. Transporter-knockout animal models provide a mechanistic understanding of the *in vivo* roles of transporters in determining both systemic and tissue exposure to drugs. However, these models are limited by species differences in substrate specificity, tissue distribution, relative abundance of transporters, and potential compensatory mechanisms.[75] The utility of animal models expressing human transporters and/or enzymes in predicting intracellular drug concentrations remains to be determined.

Currently, there are no standardized, accepted methods to directly measure unbound intracellular drug concentrations. Noninvasive approaches to measure unbound drug concentrations in human tissues are limited. As a result, total drug concentrations in tissues and cell-based models are used as surrogate measurements and often are not correlated with unbound intracellular drug concentrations. Therefore, using total drug concentrations to predict efficacy, toxicity, and DDIs can be misleading. Although some novel imaging techniques appear promising, in most cases these techniques have not been applied to quantification of drug molecules in the liver microenvironment. In the future, techniques such as confocal Raman microscopy, nano-SIMS, and CE may be used to spatially quantify the hepatocellular pharmacokinetics of drug molecules that do not contain a fluorophore. However, these techniques may not be readily applicable to humans.

Significant progress has been made in using modeling approaches to predict the effect of drug-metabolizing enzymes and transporters on the systemic exposure of drugs in preclinical species and humans. However, the ability of these models to predict intracellular drug concentrations for transporter substrates is unknown. These models require certain assumptions, and the lack of methods to directly measure tissue concentrations of the unbound drug in humans makes it difficult to validate these assumptions and refine the predictions. Therefore, establishing a publicly available, peer-reviewed database of liver-to-plasma "steady-state" partition coefficients based on unbound drug concentrations in preclinical species and humans will be helpful. Furthermore, complicated mechanistic models require large *in vitro* and/or *in vivo* data sets to predict and simulate intracellular drug concentrations. The accuracy of models also relies on the quality of input parameters, which are extrapolated from *in vitro* and/or *in vivo* models; inaccurate conclusions may be drawn when too many unknown parameter values are estimated on the basis of too few measurements. Because of the lack of experience and high level of uncertainty, these complicated models are not currently applied routinely to new drug candidates.

SUMMARY

The intracellular concentration of the unbound form of a drug is an important parameter for predicting drug efficacy, toxicity, and DDIs. Although we have made great progress over the past two decades in elucidating the roles of transport proteins in drug disposition, many fundamental questions remain unanswered. In particular, understanding the impact of transporters on the modulation of intracellular drug concentrations is still a challenging area, representing an important direction for research in this field. This white paper highlights the importance of intracellular concentrations in drug development and provides an update on current progress, issues, and critical scientific gaps in quantitatively assessing intracellular drug concentrations by direct measurement or modeling/simulation. The unbound drug concentration in the liver is an important parameter used to predict (i) DDIs at the level of metabolic enzymes and transporters and (ii) drug-induced liver toxicity. Due to current limitations in existing experimental model systems and, more importantly, the lack of preclinical and clinical data to establish *in vitro*–to–*in vivo* correlations, it is premature to propose the use of predicted maximal intracellular unbound drug concentrations to avoid false-negative DDI predictions. Currently, uncertainties in intrahepatic drug concentrations, including those due to transporter activity, are addressed by incorporating safety

factors or using total (instead of unbound) plasma concentrations. Incorporation of K_{puu} values would be expected to decrease these safety factors and improve the prediction of DDIs. Specific recommendations regarding best practices to measure intracellular concentrations of new drug candidates include (i) direct measurement of total and unbound drug concentrations in whole liver tissue; (ii) determination of K_{puu} in polarized hepatocytes, which have functional basolateral uptake/efflux transporters, metabolizing enzymes, and biliary efflux transporters; (iii) application of scaling approaches from hepatocytes to whole body based on these data; and (iv) use of PBPK modeling to assist in quantitative analyses and to make predictions. However, as detailed in this white paper, many of the technologies and approaches discussed above have limitations and/or are still under development. Continued technological and methodological innovation is necessary to expand our knowledge and to drive this field forward. Improved experimental models, development, and refinement of cutting-edge technologies (e.g., tissue imaging), as well as application of mechanistic modeling approaches to estimate intracellular drug concentrations, will lead to more accurate predictions of drug efficacy, pharmacokinetics, DDIs, and toxicities, ultimately enhancing the quality and use of drugs in the clinic.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/cpt

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CONFLICT OF INTEREST

J.W.P. is an employee of GSK and owns stock in the company. Y.L. is an employee of Bristol-Myers-Squibb and has no conflict of interest. K.F. is an employee of AstraZeneca. X.C. is an employee of Merck Sharp & Dohme Corp. and potentially owns stock and/or holds stock options in the Company. R.E. is an employee of, and owns shares in, AstraZeneca. K.L.R.B. is chair of the Scientific Advisory Board for Qualyst Transporter Solutions, which has obtained an exclusive license for the sandwich-cultured hepatocyte technology used for quantification of biliary excretion (B-CLEAR).

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