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Successful and Unsuccessful Prediction of Human Hepatic Clearance for Lead Optimization

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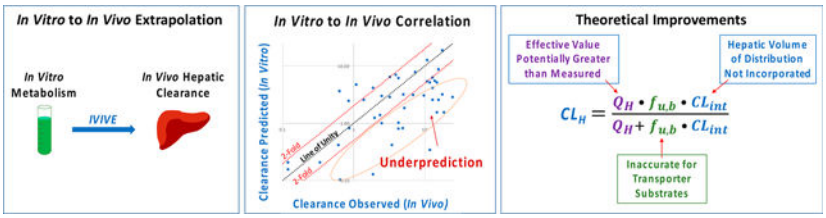
Abstract

Development of new chemical entities is costly, time-consuming, and has a low success rate. Accurate prediction of pharmacokinetic properties is critical to progress compounds with favorable drug-like characteristics in lead optimization. Of particular importance is the prediction of hepatic clearance, which determines drug exposure and contributes to projection of dose, half-life, and bioavailability. The most commonly employed methodology to predict hepatic clearance is termed *in vitro* to *in vivo* extrapolation (IVIVE) that involves measuring drug metabolism *in vitro*, scaling-up this *in vitro* intrinsic clearance to a prediction of *in vivo* intrinsic clearance by reconciling the enzymatic content between the incubation and an average human liver, and applying a model of hepatic disposition to account for limitations of protein binding and blood flow to predict *in vivo* clearance. This manuscript reviews common *in vitro* techniques used to predict hepatic clearance as well as current challenges and recent theoretical advancements in IVIVE.

Graphical Abstract



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1. INTRODUCTION

Accurate prediction of human pharmacokinetic properties of new chemical entities (NCEs) is essential in the drug discovery process. Due to the time-consuming and costly nature of developing a drug,[1] and because very few can be examined directly in humans, it is of interest early on in the drug discovery process to exclude compounds that may display unfavorable pharmacokinetic or ADME (absorption, distribution, metabolism, excretion) properties. Of particular importance is the prediction of human hepatic clearance, which largely determines the exposure of drug in the body, influencing both the efficacy and safety of an NCE. Hepatic clearance also contributes to projection of dose, half-life, and bioavailability and greatly aids in prioritization of compounds with desired drug-like properties for *in vivo* studies, such as decreased systemic clearance, adequate oral bioavailability, and half-life to permit once-a-day oral dosing. To predict the *in vivo* hepatic clearance of NCEs, *in vitro* metabolic stability studies are routinely performed, and if resulting data can be accurately extrapolated, significant benefit can be gained in the development of a new candidate drug. Thus, drug metabolism is considered the leading issue to address in lead optimization efforts and often finds itself as a tier 1 screen for newly synthesized compounds. Here, we discuss common *in vitro* techniques used to predict hepatic clearance of NCEs as well as review recent advancements and current challenges in the *in vitro* to *in vivo* extrapolation (IVIVE) of hepatic clearance.

2. *IN VITRO* TO *IN VIVO* EXTRAPOLATION

The universally accepted and most utilized method of predicting *in vivo* hepatic clearance (CL_H) from *in vitro* measures of drug metabolism is a process known as IVIVE (Figure 1). The three steps of IVIVE are (1) experimentally measuring an *in vitro* intrinsic clearance (CL_{int}), (2) calculating an *in vivo* CL_{int}, and (3) applying a model of hepatic disposition to predict CL_H. In the first step, drug metabolism measurements are conducted in human liver tissue, isolated cells (hepatocytes), or subcellular fractions such as microsomes, and what is being measured *in vitro* is the CL_{int}, or the intrinsic ability of the liver to remove drug in the absence of the limitations of organ blood flow and protein binding. To achieve this, the rate of unbound drug elimination is measured ($k_{inc,u}$), and with consideration of the volume of the incubation (V_{inc}), an *in vitro* CL_{int} can be determined.[2] Step two involves reconciling enzymatic or cell content differences between the *in vitro* incubation and the *in vivo* average human liver, resulting in a prediction of *in vivo* CL_{int}. Finally, CL_H is predicted by applying a model of hepatic disposition, such as the well-stirred model,[3,4] which accounts for liver blood flow, the free fraction of drug in the blood, as well as the predicted *in vivo* CL_{int}.

2.1. Experimental Tools to Study Drug Metabolism.

There are a number of different model systems that can be used to study drug metabolism, as outlined in Figure 2. An *in vivo* pharmacokinetic study (for example, in a preclinical species) most resembles the true *in vivo* scenario, and the complexity of experimental systems decreases as alternate techniques are utilized, such as isolated perfused liver studies, stability assays (in liver slices, hepatocytes, cell fractions, or recombinant enzymes), or an *in silico* prediction of drug metabolism. However, tremendous benefit can be gained

by utilizing less-complex systems, such as decreased cost, increased utility, increased throughput, and using more ethically acceptable methodologies. Table 1 further details useful information for the *in vitro* systems (i.e., liver slices, hepatocytes, microsomes, cytosol, and recombinant enzymes) with respect to which enzymes are contained in each system, cofactors required, the presence of transporters, storage and throughput considerations, advantages, disadvantages, as well as the range of ADME assays that could be performed with each system.

The most commonly used experimental tools in lead optimization efforts are hepatocyte and microsomal stability assays, and Figure 3 outlines how each are isolated from liver tissue. Hepatocytes (liver cells) can be isolated from an intact liver via a two-step collagenase digestion of liver tissue.[5–8] At that point, hepatocytes can be immediately utilized in a suspension assay or plated as primary cell cultures. Often metabolic stability assays are conducted using hepatocyte suspensions, whereas enzyme induction studies require culturing of plated hepatocytes. Alternatively, the isolated hepatocytes can be cryopreserved[7,8] for future use in ADME or toxicity assays. Microsomes are prepared by homogenization of the liver and a process of consecutive centrifugation steps commonly termed “differential centrifugation”.[9,10] First, the liver homogenate is centrifuged at a low speed (9000*g*) to separate the pellet (cell debris) from the supernatant (commonly referred to as the S9 fraction), which contains all the soluble and membrane-bound hepatic proteins. A high-speed centrifugation step (100 000*g*) can then be performed using the S9 fraction to isolate the supernatant, which contains the soluble cytosolic proteins, from the pellet, which contains the endoplasmic reticulum membrane-bound proteins such as the cytochrome P450s (CYPs). Thus, microsomes are artificial vesicles of hepatic endoplasmic reticulum that contain the CYP enzymes that form as a result of this differential centrifugation process. Microsomes can be stored at −80 °C long-term and withstand multiple freeze–thaw cycles while still retaining enzymatic activity[11] and can be thawed at the convenience of the investigator for use in a range of ADME assays (Table 1).

Based on the robustness, ease-of-use, and low cost, microsomal incubations are most often utilized as tier 1 screens in lead optimization efforts. Microsomes contain the membrane-bound phase I enzymes such as the CYPs and flavin-containing monooxygenase (FMO), both of which are primarily oxidative and require the addition of the cofactor NADPH (nicotinamide adenine dinucleotide phosphate).[12] Microsomes also contain the membrane-bound UDP-glucuronosyltransferases (UGTs), which catalyze the phase II conjugation of glucuronic acid to xenobiotics, typically at hydroxyl, carboxyl, carbonyl, or amino functional groups.[13] The reaction requires addition of the cofactor uridine diphosphate glucuronic acid (UDPGA) as well as a pore-forming agent such as alamethicin, as the catalytic active site of UGTs is located within the lumen of the endoplasmic reticulum.[14] It has also been reported by a number of investigators that cytosolic contamination of microsomal fractions can occur as a result of the preparation process, resulting in appreciable nonmicrosomal metabolism by enzymes such as aldehyde oxidase.[15–17] In fact, many investigators choose to perform tier 1 stability assays using the S9 fraction (supplemented with NADPH) to capture both the microsomal Phase I CYP-mediated metabolism as well as contributions of Phase I and II cytosolic enzymes such as esterases, aldehyde oxidase, xanthine oxidase, glutathione S-transferase, and sulfotransferase.[18]

Microsomes are versatile, as they can be used for a range of ADME assays such as metabolic profiling or reaction phenotyping,[19,20] drug–drug interaction studies,[21,22] estimation of drug metabolism and clearance predictions,[2,23,24] and detection of reactive metabolites.[25] Due to the ease-of-use of microsomes, many of these ADME assays are amenable to high-throughput formats, allowing for weekly screens of hundreds of compounds.[21–23] Pooled microsomes are readily available with up to 150 donors to overcome issues related to interindividual differences in activity or expression of metabolic enzymes. Additionally, microsomes can be prepared from any organ (i.e., liver, intestine, lung, kidney, heart, etc.) to allow for evaluation of potential extrahepatic metabolism.[26,27] However, there are some key assumptions when utilizing human liver microsomes for IVIVE of CL_H, most notably that CYPs are primarily responsible for clearance with no involvement of xenobiotic transporters. It is also assumed that the liver is the major site of metabolism with minor contribution of extrahepatic metabolism, and that the *in vitro* compound concentration is representative of the *in vivo* concentrations (i.e., that enzyme saturation is not occurring).

Hepatocytes are considered the gold standard in lead optimization efforts, as they are intact liver cells containing all the major Phase I and II enzymes with cofactors at physiologically relevant concentrations. In addition, membrane transport mechanisms (including xenobiotic transporters) and intracellular compartments are maintained; thus, it is possible to measure the permeability, transport, and metabolism of a test compound in a hepatocyte stability incubation. Hepatocytes can also be specially cultured between two layers of gelled collagen to capture hepatobiliary elimination.[28,29] Thus, hepatocytes are a versatile *in vitro* system that can be utilized to conduct a range of ADME and toxicity assays, including clearance prediction,[30,31] transporter-mediated uptake,[31,32] drug–drug interaction potential,[33] enzyme and xenobiotic transporter induction,[34–36] biliary clearance or toxicity,[28,29,37] and hepatotoxicity.[38–40] Cryopreserved hepatocytes are readily available from multiple vendors and can be procured as pooled lots of up to 100 donors. In comparison to microsomes, hepatocytes are more expensive and typically considered moderate-throughput as additional care is needed in the thawing of hepatocytes and throughout the assay to ensure adequate cell viability. This results in a more labor-intensive assay that poses additional challenges to successfully automate and often finds itself as a tier 2 screen in many lead optimization paradigms.

2.2. IVIVE Step 1: Measure *In Vitro* Intrinsic Clearance.

With firm understanding of the experimental tools available to study drug metabolism, we now highlight each individual step of IVIVE in further detail to cover the theory, process, and limitations of this approach. The first step of IVIVE involves measurement of *in vitro* CL_{int}, typically in hepatocyte or microsomal incubations. A chemical reaction can be considered analogous to drug elimination when metabolism is the major route of elimination, however, intrinsic clearance is not a parameter typically used in chemistry. The rate of a chemical reaction (v) is typically characterized by the Michaelis–Menten relationship:

$$v = \frac{V_{\max}[S]}{K_m + [S]} \quad (1)$$

where $[S]$ indicates the drug concentration, V_{max} is the maximum rate of decrease in drug concentration (units of concentration/time), and K_m is the drug concentration corresponding to half of V_{max} (units of concentration), which results in a reaction rate or velocity in the units of inverse time. To determine these kinetic parameters, a wide range of substrate concentrations is required (to capture both above and below the K_m), metabolite formation should be determined under linear conditions with respect to time and protein concentration (thus requiring a metabolite standard for analytical quantification), and one must ensure less than 20% of parent drug depletion has occurred in each incubation.[41] Figure 4A displays a typical plot of Michaelis–Menten kinetics, visually highlighting the labor-intensive nature of such determinations.

Under conditions where drug concentrations are much less than the K_m value (i.e., $[S] \ll K_m$), the Michaelis–Menten relationship can be simplified and the rate of drug loss (k_{inc}) can be determined under such linear conditions.[2] Thus, this "substrate-depletion" or "*in vitro* half-life" approach is most often utilized in lead optimization efforts (Figure 4B). Depletion of drug is measured at low concentrations, typically 0.1 or 1 μM, under the assumption that this is much less than the K_m value. Measurement of rate of depletion is conducted using the log–linear portion of the curve, resulting in a rate constant with units of inverse time.

To calculate an intrinsic clearance (with units volume/time) from the resulting measured rate of elimination (units of inverse time) from either of the above-discussed methodologies, it is necessary to introduce a volume term into the relationship. This is achieved by multiplying the unbound rate constant of elimination by the *in vitro* incubational volume (V_{inc}), which occurs when normalizing for enzymatic content:[2,42,43]

$$\mathrm{CL}_{\mathrm{int,invitro}} = k_{\mathrm{inc}}\, f_{\mathrm{u,inc}} \frac{V_{\mathrm{inc}}}{\text{amount enzymes or cells} - \textit{in vitro} \quad \text{incubation}} \quad (2)$$

where $f_{u,inc}$ is the fraction unbound in the incubation. Although this aspect was acknowledged in the original publication by Obach et al. that introduced the "*in vitro* half-life" approach,[2] the implications of utilizing a fixed-volume in clearance predictions has not been widely recognized by the field, and this aspect will be discussed in further detail in a subsequent section.

The substrate-depletion approach is commonly utilized in high-throughput screens and some experimental considerations related to the measurement of very low turnover or very high turnover compounds poses additional challenges. Low clearance compounds are becoming increasingly common in drug discovery efforts, due to effective design strategies to overcome metabolic liability in combination with increased assay throughput that can facilitate the rapid establishment of data-rich structure–stability relationships.[44,45] For very low turnover compounds, the loss of compound in the assay should be sufficient to confidently measure k_{inc} to reliably distinguish substrate depletion from bioanalytical or assay variability (Figure 4C). Inadequate attempted solutions include prolonging the

incubation time, as significant decreases in enzymatic activity can occur,[46] or increasing the incubational enzymatic content, as issues related to incubational binding can be introduced[47] because $f_{u,inc}$ is rarely measured for NCEs in tier 1 assays. It should be noted that multiple groups have attempted to elucidate and assess predictive relationships between physicochemical properties and $f_{u,inc}$ for both microsomal and hepatocyte incubations,[48–54] and we emphasize the importance of considering incubational binding in all CL_{int} determinations. Instead, "very low" clearance cutoffs should be employed rather than reporting clearance predictions from such results. For example, it has been recommended that when less than 10–20% turnover occurs by the end of an incubation, a clearance cutoff should be reported rather than an exact value.[46,55,56] This issue can potentially be overcome by alternatively measuring metabolite formation. However, in the lead optimization stage, there is a lack of authentic metabolite standards, and it is not practical in most workflows to anticipate and detect major metabolites for newly synthesized molecules. Of late, a number of *in vitro* systems and methodologies have been developed to more accurately measure low turnover compounds, which will be discussed in a subsequent section together with other notable attempts to improve IVIVE by the field. Extensive loss of drug in the *in vitro* assays also poses challenges, as only the log–linear portion of the curve should be utilized for k_{inc} measurement. As depicted in Figure 4D, inclusion of all time points may result in an underprediction of rate of drug loss, thereby potentially resulting in underprediction of *in vivo* clearance. This aspect is often overlooked in high-throughput screens and is of particular concern in determining rate of drug loss by utilizing assays that only sample a single end-of-incubation time point plus the initial time zero.

2.3. IVIVE Step 2: Calculate *In Vivo* Intrinsic Clearance.

The second step of IVIVE involves estimating *in vivo* CL_{int} from measurements of *in vitro* CL_{int}. This is achieved by reconciling the enzymatic or cell content difference between the incubation and an average whole liver with use of physiologically-based scaling factors:

$$CL_{int,invivo} = \frac{\text{amount enzymes or cells} - \text{whole liver}}{\text{amount enzymes or cells} - \textit{in vitro} \quad \text{incubation}} \times CL_{int,invitro} \qquad (3)$$

These scaling factors first consider microsomal protein or hepatocellularity per gram of liver and then account for liver weight per kg of body weight. Typically used values for human microsomal protein content range from 32 to 48.8 mg microsomal protein per gram of liver[57–60] and values for human hepatocellularity range from 99 to 139 million hepatocytes per gram liver.[57,59,61] The typically utilized value of human liver weight per kg body weight ranges from 21.4 to 25.7 g liver/kg body weight.[62,63] Thus, an *in vitro* CL_{int} can be scaled up to a prediction of *in vivo* CL_{int}.

2.4. IVIVE Step 3: Apply a Hepatic Disposition Model to Predict Hepatic Clearance.

To predict total hepatic clearance, the physiologic limitations of hepatic blood flow (Q_H) and fraction of unbound drug in the blood ($f_{u,B}$) must be considered by utilizing a hepatic disposition model. To describe hepatic drug elimination without being able to measure intraorgan drug concentrations, pharmacokineticists based clearance concepts on chemical engineering reactor models for which only entering and exiting reactant amounts are known

but no measurements within the reactor are possible.[64] Common assumptions of liver models are that (1) only unbound drug can cross membranes and occupy enzyme active sites, (2) no diffusional barriers exist (i.e., passive membrane passage is much larger than metabolic CL_{int}), and (3) hepatic enzymes are homogeneously distributed throughout the liver. Thus, *in vitro* CL_{int} measures, *in vitro* $f_{u,B}$ determinations, and physiologic values of Q_H (20.7 mL/min/kg[62]) can be utilized to predict clearance using a hepatic disposition model.

Figure 5 depicts the most common hepatic disposition models utilized for clearance predictions, including the well-stirred model, the parallel tube model, and the dispersion model, as well as the mathematical relationships that relate entering drug concentration (C_{in}), exiting drug concentration (C_{out}), and Q_H to total clearance CL_H for each model. The simplest model is the well-stirred model:

$$CL_{H,WSM} = \frac{Q_H f_{u,B} CL_{int}}{Q_H + f_{u,B} CL_{int}} \quad (4)$$

The well-stirred model assumes that drug is homogeneously distributed throughout the liver (Figure 5A). Although this well-stirred representation of the liver is far from capturing the complex physiologic aspects of the liver, the simple well-stirred relationship depicted in eq 4 is very useful.

The parallel tube model assumes incremental metabolism where drug concentrations decrease by a first order process throughout the liver. The well-stirred model and the parallel tube model are the two boundary conditions, and there are an infinite number of dispersion models between these two boundary models that are characterized by different dispersion numbers (D_N) that can range from zero (parallel tube mode) to infinity (well-stirred model). A representative dispersion model is depicted in Figure 5C. From examination of each model in Figure 5, one can see that based on the same C_{in} and C_{out} the concentration profile of drug in each model differs significantly, resulting in different hepatic drug exposures (area under the curve) and different average driving force concentrations (C_H) responsible for hepatic drug elimination between the models.

3. IVIVE UNDERPREDICTS *IN VIVO* HEPATIC CLEARANCE

Although measuring drug metabolism in microsomes or hepatocytes is widely used throughout the industry to predict hepatic clearance, *in vitro* measures of drug metabolism significantly and systematically underpredict *in vivo* hepatic clearance.[65–67] It had been reported in 2009 by Chiba et al. that the underprediction of CL_H is approximately 3- to 6-fold in human hepatocytes and approximately 9-fold in human microsomes.[65] More recently, Wood et al.[66] reported that the human hepatocyte underprediction of CL_{int} was 4.2-fold and human microsomes was 2.8-fold, with similar findings in rat hepatocytes (4.7-fold) and rat microsomes (2.3-fold). Bowman and Benet[67] evaluated 11 IVIVE data sets, showing that human hepatocytes underpredicted 1.4- to 21.7-fold and human microsomes underpredicted 1.5- to 7.9-fold, although these reported underpredictions are sometimes associated with CL_H and are sometimes associated with CL_{int} depending on the comparisons

being made in the original publications. More recently, we have pointed out that it is more appropriate to evaluate IVIVE success with respect to total CL_H rather than CL_{int} because potential errors in CL_{int} for high extraction ratio (ER) compounds may not translate to significant error when CL_H is calculated.[42] Further, back-calculating an *in vivo* CL_{int} from total CL_H measurements requires the assumption that the *in vivo* CL_H measurement, the experimentally determined $f_{u,B}$ measurement, and value of Q_H are accurate, and thus any resulting errors in IVIVE are primarily attributed to issues with determining CL_{int}. To date, these assumptions have been considered reasonable by the field; however, we have recently pointed out potential errors in these assumptions.[42] To clarify, we are not implying that accurate determination of CL_{int} is unimportant for high ER drugs, as *in vivo* CL_{int} determines unbound drug exposure for hepatically cleared drugs regardless of ER,[68] we are simply highlighting the additional potential errors that are associated with each parameter that determines total observed CL_H.

The greatest challenge with IVIVE underprediction is that the degree of underprediction can vary greatly from drug-to-drug, and the field does not yet understand why. Attempts to explain this issue by the field have been unsuccessful to date. Explanations of lack of IVIVE have most commonly been attributed to (1) extrinsic factors such as the loss of enzymatic activity due to suboptimal storage or preparation of human liver tissues or due to the presence of metabolic inhibitors present during the isolation process, (2) the inability of *in vitro* incubations to recapitulate hepatic architecture, (3) nonspecific or protein binding that is not fully accounted for in clearance prediction calculations, (4) a neglected contribution of extrahepatic clearance or other clearance mechanisms, or (5) the potential differences between the donors of liver tissue and the young healthy volunteers in which clinical clearance determinations are conducted.[65,69] A number of groups have attempted to simply mitigate the unexplainable underprediction issue by employing a regression-based "fudge" factor to their data,[69–72] and such approaches are common in lead optimization as a practical approach to predict clearance (or rank-order compounds by CL_{int}) despite the unpredictability of IVIVE. Such approaches are commonly referred to as IVIVC, or *in vitro* to *in vivo* correlation. For instance in a simplified example, if it is observed that *in vitro* data underpredicts *in vivo* clearance by 2- to 6-fold for a series of compounds, investigators may choose to apply a 4-fold scaling factor to other compounds in this series to get *in vitro* predictions into the ballpark of *in vivo* values. However, this is a temporary solution that does not address the underlying reasons for underprediction, demonstrating the clear need for a mechanistic understanding of the reasons for underprediction of hepatic clearance.

Throughout the field, many groups both academic and within industry have attempted to understand, explain and mitigate IVIVE underpredictions spanning more than two decades. Many notable efforts to improve IVIVE predictability have addressed issues with nonspecific or protein binding,[24,47,70,73–76] considered differences in drug ionization in extracellular and intracellular liver regions,[77–79] conducted hepatocyte uptake experiments for hepatic or renal transporter substrates,[31,32,80] developed experimental methodologies to account for biliary clearance,[28,29] introduced the Extended Clearance Model that integrates metabolism with membrane passage intrinsic clearances such as hepatic uptake, biliary excretion, and sinusoidal efflux,[81] incorporated the fraction unbound in the liver or liver-to-plasma partition coefficient of unbound drug (Kp_{uu}) for transporter substrates,[82–85]

incorporated intestinal absorption, first-pass elimination and other extrahepatic metabolic contributions,[26,27,86] developed experimental methodologies such as the relay method to extend hepatocyte incubations to 20+ hours and coculture techniques with additional cell types to prolong hepatocyte function in long-term cultures to more accurately measure very low turnover compounds,[87–89] and have investigated the potential for albumin-facilitated uptake by adding serum into hepatocyte incubations.[90–92] We emphasize that each one of these aspects are crucial to consider when attempting to accurately predict *in vivo* clearance, and that the referenced studies are merely representative examples of the extensive efforts by the field to improve IVIVE success in drug discovery.

More recently, our laboratory has thoroughly evaluated the current state of IVIVE and confirmed the interlab variability[93] and clearance-dependent underprediction trends[94] observed by the field.[65,66] We have also found that poor IVIVE for metabolized drugs is not due to transporter involvement.[67] With respect to observations of albumin-facilitated uptake, we have proposed that the albumin effect is due to a protein binding shift due to increased affinity for transporters.[95] Further, we have identified a CYP3A4 underprediction anomaly where microsomes result in markedly higher CL_{int} values and IVIVE success than hepatocytes, but the same trend was not observed for other CYP isoforms,[96] suggesting the potential for enzyme-transporter interplay with the efflux transporter P-glycoprotein present in hepatocyte membranes that may prevent drug access to CYP3A4 as a result of their overlapping substrate specificities,[97] and these results have been further confirmed with a larger number of substrates more recently by the field.[98]

4. ADVANCING IVIVE THEORY

As outlined above, for decades IVIVE underprediction has challenged pharmaceutical scientists both in industry and academia, with extensive efforts directed primarily toward improving experimental outcomes for certain categories of “problem” or hard-to-predict drugs. Although countless mechanistic studies have been conducted representing tremendous efforts across the field, there have only been incremental advances in IVIVE success, and there is still no consensus on the reasons behind the shortcomings of IVIVE for all drugs. Efforts have not yet been able to identify the types of compounds for which IVIVE can be trusted to quantitatively predict clearance. Therefore, in lead optimization IVIVE is regarded as a practical approach to rank-order NCEs based on metabolic stability, but uncertainties remain surrounding the quantitative utilization of IVIVE-based clearance predictions, for instance, in human dose projections. It is surprising that IVIVE is not successful for those drugs that are exclusively eliminated by metabolism (with no involvement of xenobiotic transporters). Why is it that measures of *in vitro* drug metabolism measured in actual liver tissue cannot provide adequate predictions of *in vivo* hepatic elimination? We hypothesize that perhaps the theoretical basis of current IVIVE practices is flawed, and thus significant efforts of our laboratory have been toward advancing IVIVE theory.

4.1. Implicit Well-Stirred Model Assumptions.

We have taken a measured approach to critically evaluate both *in vitro* and *in vivo* assumptions in basic clearance concepts to elucidate the potential reasons for IVIVE underprediction. First, we have recognized by derivation that the extended clearance model,[99] Kp_{uu},[100] and organ ER[101] have all inherently assumed the well-stirred model. Thus, when xenobiotic transporters are involved in drug disposition, there is no advantage in the utilization of alternate models of hepatic disposition (that are considered more physiologic).[99] This is particularly relevant for physiologically based pharmacokinetic (PBPK) modeling approaches, as the dispersion model appears to be universally utilized to model hepatic clearance throughout the literature. Further, the basis of Kp_{uu} on the well-stirred model indicates that nuances of intracellular drug distribution are not considered.[100] Therefore, using Kp_{uu} to improve clearance predictions cannot capture the differences in average drug concentration driving metabolic elimination from the concentrations at the basolateral or apical hepatocyte membranes that drive efflux and biliary elimination, respectively, and thus may provide limited benefits. The recognition that clearance calculations based on ER have inherently assumed the well-stirred model[101] indicates that all clearance calculations are model-dependent when drug concentrations entering and exiting an organ at steady-state are utilized. We have further critically analyzed all such published experimental data that use ER to calculate clearance in isolated perfused rat liver studies, concluding that all *in situ* and *in vitro* data can be described by the well-stirred model.[102]

4.2. The Lower Boundary of IVIVE.

We have also derived IVIVE from first-principles,[42] noting that the lower boundary condition for IVIVE predictions to have the potential to be valid is

$$CL_H \leq f_{u,B} CL_{int} \quad (5)$$

That is, for all drugs regardless of their ER, the product of $f_{u,B}$ and CL_{int} will always be larger than observed CL_H, holds for all models of hepatic disposition, and this relationship is the prerequisite for IVIVE predictions to be accurate. Evaluation of a large IVIVE database[66] and notable IVIVE studies[24,84] revealed that approximately two-thirds of the available published IVIVE data violate the lower boundary condition of the predictive relationship. Until recently, the field has primarily attributed that error to the underprediction of CL_{int}; however, there are a number of assumptions that must also be accurate related to measurements of CL_H and determinations of $f_{u,B}$ for that assessment to be true.

Many investigators believe that the reason *in vitro* rates often fail to predict *in vivo* rates can be due to a variety of assay-centric reasons, such as the ability of enzymes to perform once isolated, the limited architecture of the microsomes and hepatocyte environment, or issues during isolation including the presence of agents that may be inhibitors of metabolic enzymes. This could be true; however, our analysis of the published data suggests that this is not the reason for the observed poor predictability. Obach[24] initially investigated 29 drugs, all based on the same experimental methodology using human hepatic microsomes, and found that 31% of the drugs resulted in accurate clearance predictions within 2-fold.

The compilation of Wood et al.[66] for 83 drugs in human microsomes from many different investigators results in 42% within 2-fold. We find it hard to believe that for 69% of the Obach[24] study drugs there were assay issues, but for 31% there were not because the same procedure was followed for all 29 drugs (and the same point can be made for the Wood et al.[66] IVIVE database). Alternatively, based on our analysis,[42] the drugs exhibiting poor predictability predominantly violated eq 5, while those drugs giving accurate predictability did not violate eq 5, suggesting that poor predictability is drug-specific and not a function of assay-centric reasons.

4.3. Valid Experimental Determinations.

4.3.1. Total Hepatic Clearance.—

Most published IVIVE investigations evaluate error between *in vitro* CL_{int} and *in vivo* CL_{int}. The *in vivo* CL_{int} is back-calculated from total CL_H plasma measurements under the assumptions that each of the individual parameters that determine total clearance are correct. Thus, any resulting errors in IVIVE are primarily attributed to issues with *in vitro* determination of CL_{int} rather than the other factors discussed here. Some of these assumptions may be reasonable, however, because IVIVE has continued to challenge the field, we suggest it is more appropriate to compare total CL_H values and recognize the potential contribution of error in each term. Again, we are not suggesting that accurate CL_{int} determination is not critical, because it is *in vivo* CL_{int} that determines unbound drug exposure (that drives pharmacodynamic outcomes) for all hepatically cleared drugs, regardless of ER.[69] We are just pointing out that there may be additional potential errors associated with each parameter that determines total observed CL_H, thus may introduce additional errors in back-calculations of *in vivo* CL_{int}. We recognize that investigators are aware there may be errors inherent in the experimental determinations of each parameter, for instance due to difficulties in measuring binding parameters for highly bound drugs or due to intrasubject variability. However, here, we further discuss the potential theoretical errors associated with determination of each parameter.

Measurement of *in vivo* clearance values are typically considered to be accurate, however, it must be determined following intravenous dosing or with an accurate estimation of bioavailability (F) following oral dosing. One must also consider interindividual variability, potential for saturation of absorption or metabolism, as well as adequate sampling of the terminal phase (to minimize any errors introduced with extrapolation) and of the absorption phase for high clearance compounds (to accurately capture initial concentrations). Many of these aspects are given due consideration in clinical trial design, however, clearance determinations *in vivo* are typically conducted in plasma. This value is converted to a blood clearance based on a measurement of a blood-to-plasma partitioning ratio $\left(\frac{B}{P}\right)$, and thus it is crucial that such experimental measurements are accurate:

$$CL_{H,blood} = \frac{CL_{H,\,plasma}}{B/P} \quad (6)$$

In the absence of experimental data, investigators often make the assumption that the $\frac{B}{P}$ value is equal to 0.55 for acidic compounds and equal to 1 for basic and neutral compounds.

4.3.2. Fraction Unbound in the Blood.—The $\frac{B}{P}$ ratio is also required to convert measured values of fraction unbound in the plasma ($f_{u,P}$) to $f_{u,B}$:

$$f_{u,B} = \frac{f_{u,P}}{B/P} \quad (7)$$

Plasma is experimentally and analytically advantageous for *in vitro* experimentation; thus, determinations of $f_{u,P}$ are routinely conducted and are converted to $f_{u,B}$ based on a separate experimental determination of $\frac{B}{P}$. But what has not been widely recognized as a potential source of IVIVE error is that in order for the relationship in eq 7 to be true, the free drug theory must hold, which assumes that the free drug concentrations in the blood cell must be equal to that in the plasma. In other words, it is assumed that xenobiotic transporters expressed in the red blood cell are not involved in drug distribution,[42] and this was not an unreasonable assumption at the time this equation was developed, as it was prior to the recognition that transporters were relevant to drug disposition. Xenobiotic transporters have been identified within erythrocyte membranes,[103–105] could potentially have a large impact on the observed unpredictability of IVIVE, and is a fruitful area of future research.

4.3.3. Hepatic Blood Flow Value.—The Q_H value utilized in clearance predictions is based on physiologic determinations of the total blood flow rate entering and exiting the liver. Based on recently published simulations, we have suggested that perhaps blood flow in contact with the metabolic enzymes within the liver may be greater than the actual blood flow into the liver.[42] In all models of hepatic disposition (Figure 5), CL_H cannot exceed Q_H, as drug cannot be eliminated until it is presented to the elimination organ. However, a clearance-dependent underprediction has been observed throughout the field (where the IVIVE underprediction becomes larger with increasing clearance values),[65,66,94] suggesting that such an error could potentially be observed if the commonly used value of Q_H was an underprediction. Simulations revealed that the widely used Q_H value of approximately 20 mL/min/kg underpredicts effective blood flow by about 2.5-fold.[42] At present, this is only a hypothesis that requires experimental validation. However, with recent advancements in hepatic imaging capabilities, it may be possible to improve our understanding of hepatic physiology and potentially revise the relevant Q_H value that should be utilized in clearance predictions.

4.3.4. In Vitro CL_{int} Determinations: Chemistry versus Pharmacokinetics.—We have speculated that a significant source of error in the determination of CL_{int} in basic IVIVE methodologies is that a “chemistry” approach is utilized to predict a “pharmacokinetic” parameter.[42] The term “chemistry” is utilized to describe the *in vitro* scenario in which the incubational volume is fixed, whereas the term “pharmacokinetics” refers to the *in vivo* scenario where volume of distribution can be different for each drug due to each drug’s unique physicochemical properties. The major differences between these

fields with respect to IVIVE are in the definition of V_{max} and the pharmacokinetic volume of distribution that can vary from drug to drug, which is not considered in chemistry where the relevant reaction rates are measured in fixed volumes.

As outlined in detail above, IVIVE is based on principles of Michaelis–Menten kinetics that describe the rate of a chemical (or biochemical) reaction (eq 1) based on reactant concentrations. Under the linear conditions in which the substrate concentration is much less than the K_m of the reaction, the relationship is simplified and the slope of the depletion of parent drug can be used to approximate the eq 1 relationship. The results of such determinations provide the rate of drug loss in units of time^{-1} and are conducted in a fixed incubation volume. But, the desired outcome of IVIVE is to predict a drug clearance in units of volume/time.

In contrast to chemistry, in pharmacokinetics, all derivations are based on mass balance considerations (i.e., amounts rather than concentrations), thus in pharmacokinetics the units of V_{max} are in terms of an amount change in contrast to the chemistry-based V_{max} that has always been expressed as a concentration change. This results in the ratio of V_{max}/K_m in pharmacokinetics as a clearance parameter with the units of volume/time (because V_{max} has the units of amount/time and K_m has the units of amount/volume). However, pharmacokineticists have not derived the classic Michaelis–Menten relationship based on amounts to obtain a V_{max} parameter that has units of amount/time. Rather they just take the chemistry Michaelis–Menten derivation and then change the units of V_{max} for convenience based on no theoretical rationale.

A second potential pharmacokinetic versus chemistry difference relates to volume of distribution. From the incubation, the *in vitro* CL_{in} is implicitly calculated by multiplying the rate constant for elimination (units time^{-1}) by the volume of the incubational fluid (V_{inc}) as outlined in eq 2.[42] This detail (and its implications) have not been widely recognized because the volume term is introduced by dividing the measured $k_{inc,u}$ (determined in IVIVE Step 1) by the concentration of enzymes in the incubation (which is half of the enzyme reconciliation that occurs in IVIVE Step 2). eqs 2 and 3 have been combined here as eqs 8a and 8b to further illustrate how the investigator-selected V_{inc} is incorporated into IVIVE predictions:

$$\mathrm{CL_{int,invivo}} = k_{\mathrm{inc,\,u}} \times \frac{V_{\mathrm{inc}}}{\text{amount enzymes or cells} - \textit{in vitro} \quad \text{incubation}} \times \frac{\text{amount enzymes or cells} - \text{whole liver}}{1} \times \mathrm{CL_{int\,,\,invitro}} \tag{8a}$$

$$\mathrm{CL_{int\,,\,invivo}} = k_{\mathrm{inc,\,u}} \times \frac{V_{\mathrm{inc}}}{1} \times \frac{\text{amount enzymes or cells} - \text{whole liver}}{\text{amount enzymes or cells - } \textit{in vitro} \quad \text{incubation}} \times \mathrm{CL_{int\,,\,invitro}} \tag{8b}$$

where the first two terms on the right-hand side of the equality in eq 8a are how *in vitro* CL_{int} is currently calculated by the field by normalizing $k_{inc,u}$ for *in vitro* enzymatic/cellular

content, and rearrangement of this relationship (eq 8b) highlights how V_{inc} is introduced into the IVIVE relationship.

Pharmacokinetics is a field founded on mass-balance considerations; thus, measurements of systemic drug concentrations are effectively converted to amounts by incorporating a volume of distribution that does not have physiological relevance and can vary by drug. It is a theoretical volume in which a drug must distribute to relate the observed systemic concentrations to the amount of drug present in the body. It is recognized that rate of loss is dependent on both clearance and volume of distribution, and thus changes in either parameter (as a result of drug–drug interactions, disease state, or pharmacogenomic variance of metabolizing enzymes and transporters) can have an impact on observed drug half-life.[106] Current IVIVE approaches are conducted in a fixed-volume incubation and do not account for the pharmacokinetic volume of distribution that can vary for each drug, and drug distribution is not currently recapitulated in traditional metabolic stability incubations.

Figure 6A depicts current IVIVE models that have considered the liver to be a simplified, homogeneous system. Drug enters and exits the liver with Q_H, and the difference between entering and exiting concentrations are attributed to CL_H (and the value of CL_H can be modeled using any of the relationships in Figure 5). However, physiologically the liver is a heterogeneous organ comprised of both aqueous and lipophilic regions into which drugs can distribute. Figure 6B depicts the liver as a two-compartmental model comprised of a hepatocyte water and a lipophilic (nonhepatocyte water) compartment. Drugs primarily cleared by metabolism are typically lipophilic,[107,108] and it is expected that each drug will partition differently into the lipophilic components of the liver (including the hepatocyte membrane) depending on its unique physicochemical properties. Due to the potential for drug distribution within the liver itself, it is highly unlikely that the volume of distribution of drug in the whole liver at steady state ($V_{ss,H}$) is equal to the volume of distribution of drug in the hepatocyte water (V_{hep}) in contact with the drug metabolizing enzymes (Figure 6A–B), and we suggest that the difference of these two volumes of distribution result in the 60–80% of drugs where present IVIVE methods underpredict the *in vivo* measured clearance.[42] We maintain that examination of this potential volume of distribution difference should be a major issue of investigation, as has been recently examined by Riccardi et al.[84]

By inaccurately assuming the liver is a one-compartment homogeneous system, the field has overlooked the potential of drug to distribute out of the hepatocyte water away from the drug metabolizing enzymes. Thus, if one assumes that $V_{ss,H} = V_{hep}$, which is what the field has been unknowingly doing, one is not accurately determining the concentration of drug exposed to drug metabolizing enzymes *in vivo*. Because this difference in volume of distribution is a function of drug distribution within the liver and the physiological characteristics of the liver itself, it is hypothesized that this difference will undoubtedly vary from drug to drug. Therefore, a universal biological scaling factor alone is not appropriate for IVIVE, which many in the field presently believe will succeed (Figure 6C). Theoretical and experimental aspects related to estimating appropriate drug specific correction factors for marketed drugs (to extrapolate to NCEs) and incorporation into IVIVE practices for improved clearance predictions should, in our opinion, be an area of active research in drug metabolism.

5. CONCLUSIONS

In vitro metabolic stability is critically important in lead-optimization for prediction of *in vivo* clearance, and there are a number of experimental systems that could be leveraged for clearance predictions. Microsomal stability is particularly amenable to high-throughput screening for early stages of drug discovery due to the relatively low cost and ease-of-use of microsomal fractions. However, it is critical to anticipate the most likely *in vivo* clearance mechanism to select the appropriate *in vitro* tool for clearance determinations. Although IVIVE approaches are very useful in rank-ordering the metabolic stability of NCEs, IVIVE methods tend to underpredict clearance for reasons that have not yet been fully elucidated, despite significant experimental efforts by the field. Improved methodologies are continuously emerging;[109–111] however, the theoretical basis of the IVIVE process currently employed requires recognition of its inherent assumptions and limitations. There are inherent assumptions with determination of *in vivo* CL_H and $f_{u,B}$, and it is possible that the currently utilized value of Q_H is underpredicted. It is likely that the major limitation of IVIVE is that a chemistry-based determination of rate of drug loss (conducted in a fixed incubation volume) is being utilized to predict an *in vivo* pharmacokinetic clearance parameter in which drug can distribute into hepatic tissues where metabolizing enzymes are not expressed. Thus, it is possible the inexplicable IVIVE underprediction issue challenging the field is due to the fact that current approaches do not account for the pharmacokinetic volume of distribution that can vary for each drug, and drug distribution is not currently recapitulated in traditional metabolic stability incubations nor considered in clearance calculations.

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Biographies

Dr. Jasleen K. Sodhi received her undergraduate degree from the University of California Berkeley and then spent 9 years in the pharmaceutical industry, primarily at Genentech in the Drug Metabolism and Pharmacokinetics department, where she ran the suite of *in vitro* ADME assays and experimentally investigated IVIVE disconnects. More recently, Jasleen received her Ph.D. from the University of California San Francisco under the mentorship of Dr. Leslie Benet, where she also focused on improving the IVIVE of hepatic clearance and understanding complex drug–drug interactions but from a theoretical perspective. Jasleen now leads the Drug Metabolism and Pharmacokinetics efforts at Plexxikon, Inc.

Dr. Leslie Z. Benet, Professor and former Chairman (1978–1998) of Bioengineering and Therapeutic Sciences, University of California San Francisco (UCSF), received his A.B., B.S., and M.S. from the University of Michigan, Ph.D. from UCSF, and has nine honorary doctorates. Dr. Benet was the first President of the American Association of Pharmaceutical Sciences. In 1987, he was elected to membership in the National Academy of Medicine

of the US National Academy of Sciences. He previously served as the Treasurer of the International Society for the Study of Xenobiotics (ISSX), Chair of the Drug Metabolism Gordon Conference, and in 2015 received the ISSX North American Achievement Award. Dr. Benet has published over 600 scientific articles and book chapters, holds 12 patents, and has edited 7 books. His peer reviewed publications have been cited more than 29 000 times.

ABBREVIATIONS USED

$\frac{B}{P}$
blood-to-plasma partitioning ratio

C_H
average hepatic drug concentration

C_{in}
entering drug concentration

CL_H
hepatic clearance

CL_{int}
intrinsic clearance

$CL_{int,invitro}$
in vitro intrinsic clearance

$CL_{int,invivo}$
in vivo intrinsic clearance

C_{out}
exiting drug concentration

CYP
cytochrome P450

D_N
dispersion number

ER
extraction ratio

F
bioavailability

FMO
flavin-containing monooxygenase

$f_{u,B}$

fraction unbound of drug in blood

$f_{u,inc}$
fraction unbound of drug in the *in vitro* incubation

$f_{u,p}$
fraction unbound of drug in plasma

IVIVC
in vitro to *in vivo* correlation

IVIVE
in vitro to *in vivo* extrapolation

k_{inc}
rate of drug elimination in the *in vitro* incubation

$k_{inc,u}$
rate of unbound drug elimination in the *in vitro* incubation

$K_{L \rightarrow W}$
partition coefficient from the lipophilic to aqueous hepatic compartments

K_{m}
Michaelis–Menten dissociation constant

Kp_{uu}
liver-to-plasma partition coefficient

$k_{ss,u}$
steady-state *in vivo* rate of unbound drug loss

$K_{W \rightarrow L}$
partition coefficient from the aqueous to lipophilic hepatic compartments

NCEs
new chemical entities

Q_{H}
hepatic blood flow

SF
physiologically based scaling factors

UGT
UDP-glucuronosyltransferase

V_{d}
volume of distribution

V_{hep}
volume of distribution of drug in the hepatocyte water at steady state

V_{inc}
volume of the *in vitro* incubation

V_{max}
maximal rate of enzymatic reaction velocity

V_{nonhep}
volume of distribution of drug in the nonhepatocyte water (lipophilic regions) at steady state

$V_{ss,H}$
volume of distribution of drug in whole liver at steady state

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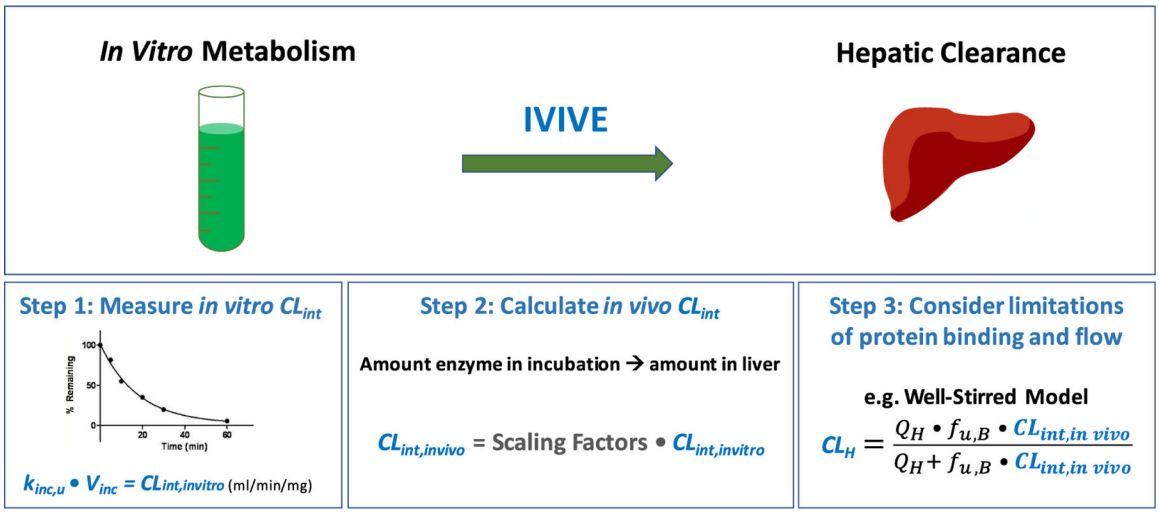

Figure 1.

In vitro to *in vivo* extrapolation (IVIVE). Abbreviations: CL_H, hepatic clearance; CL_{int}, intrinsic clearance; $CL_{int,invitro}$, *in vitro* intrinsic clearance; $CL_{int,invivo}$, *in vivo* intrinsic clearance; $f_{u,B}$, fraction unbound in blood; IVIVE, *in vitro* to *in vivo* extrapolation; $k_{inc,u}$, unbound rate of incubational drug loss; Q_H, hepatic blood flow; V_{inc}, volume of *in vitro* incubation.

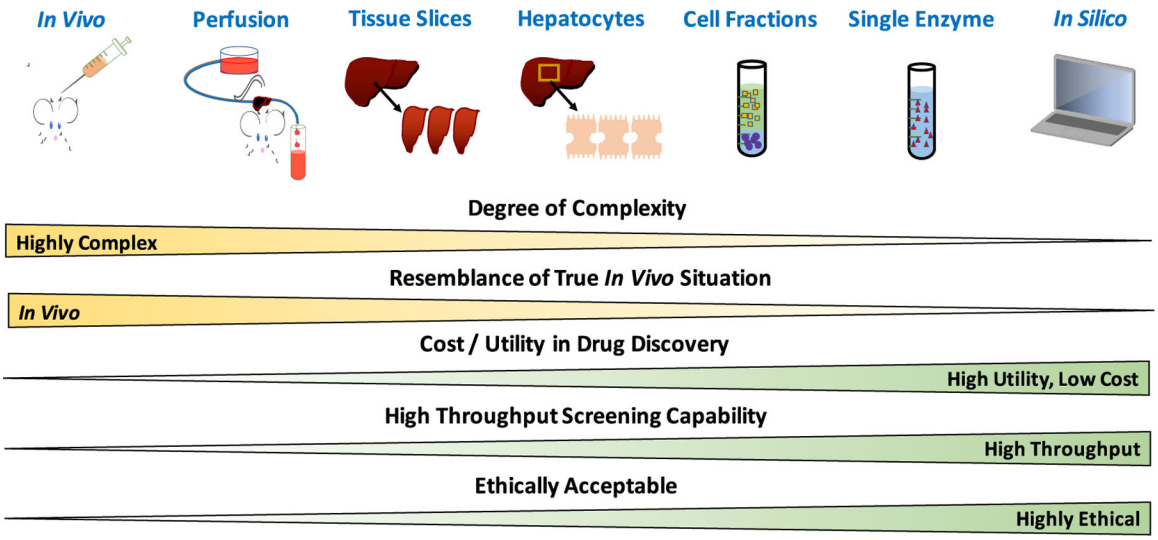

Figure 2.
Experimental methodologies employed to study drug metabolism.

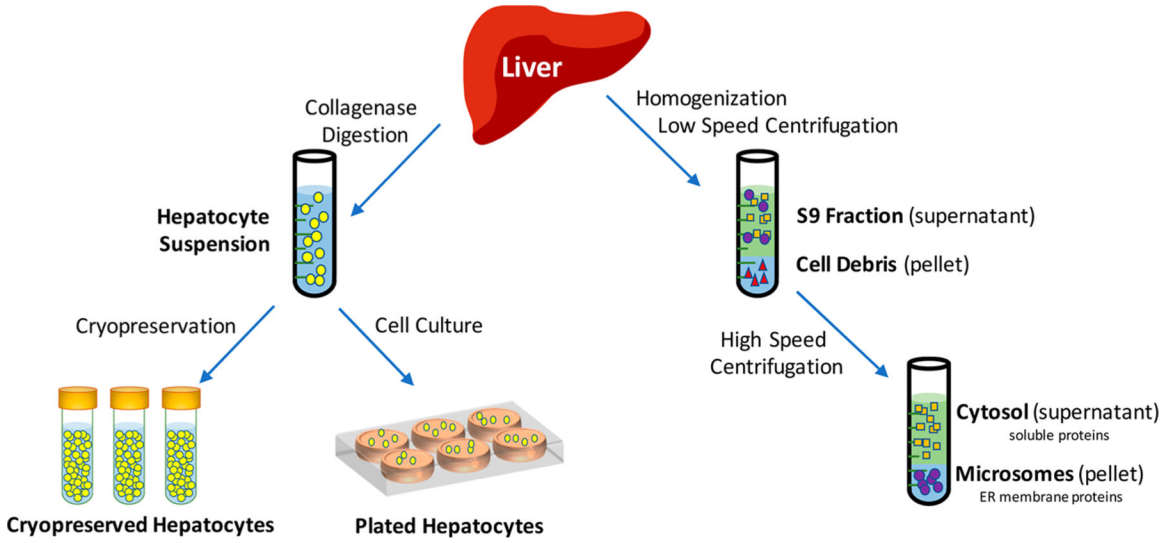

Figure 3.
Isolation of hepatocytes and microsomes from hepatic tissue.

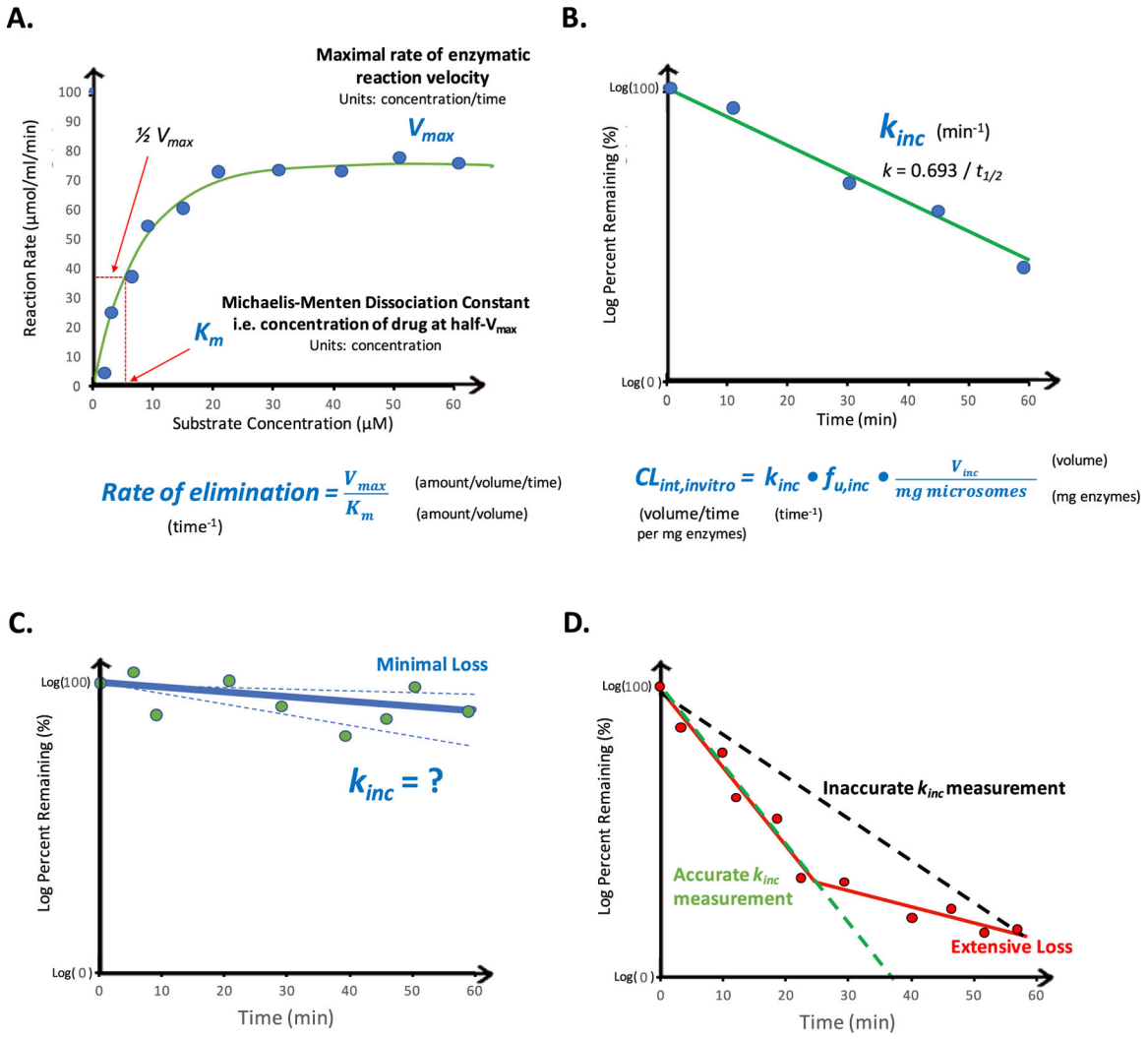

Figure 4.

In vitro determinations of intrinsic clearance and high throughput assay considerations. Abbreviations: $CL_{int,invitro}$, *in vitro* intrinsic clearance; $f_{u,inc}$, fraction unbound in the incubation; k_{inc}, rate of incubational drug loss, K_m, Michaelis–Menten dissociation constant; V_{inc}, volume of *in vitro* incubation; V_{max}, maximal rate of enzymatic reaction velocity.

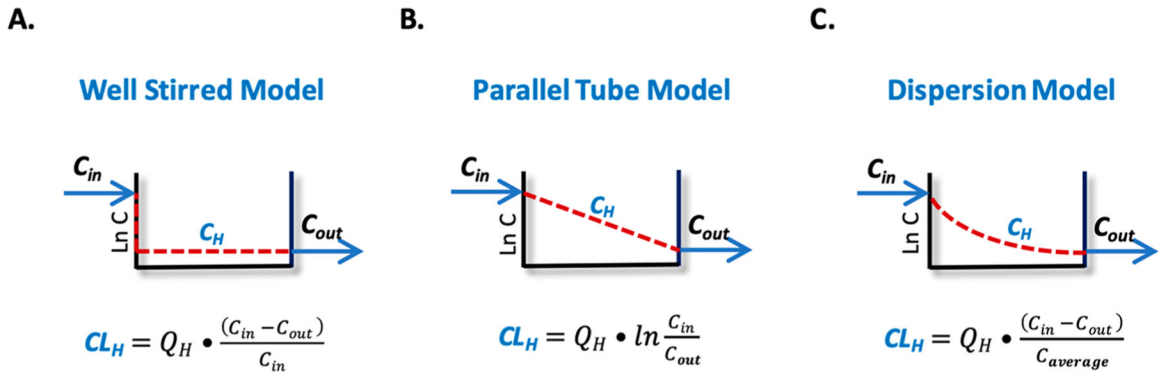

Figure 5.

Hepatic disposition models. Abbreviations: C_H, hepatic drug concentration; C_{in}, entering drug concentration; CL_H, hepatic clearance; C_{out}, exiting drug concentration; Q_H, hepatic blood flow.

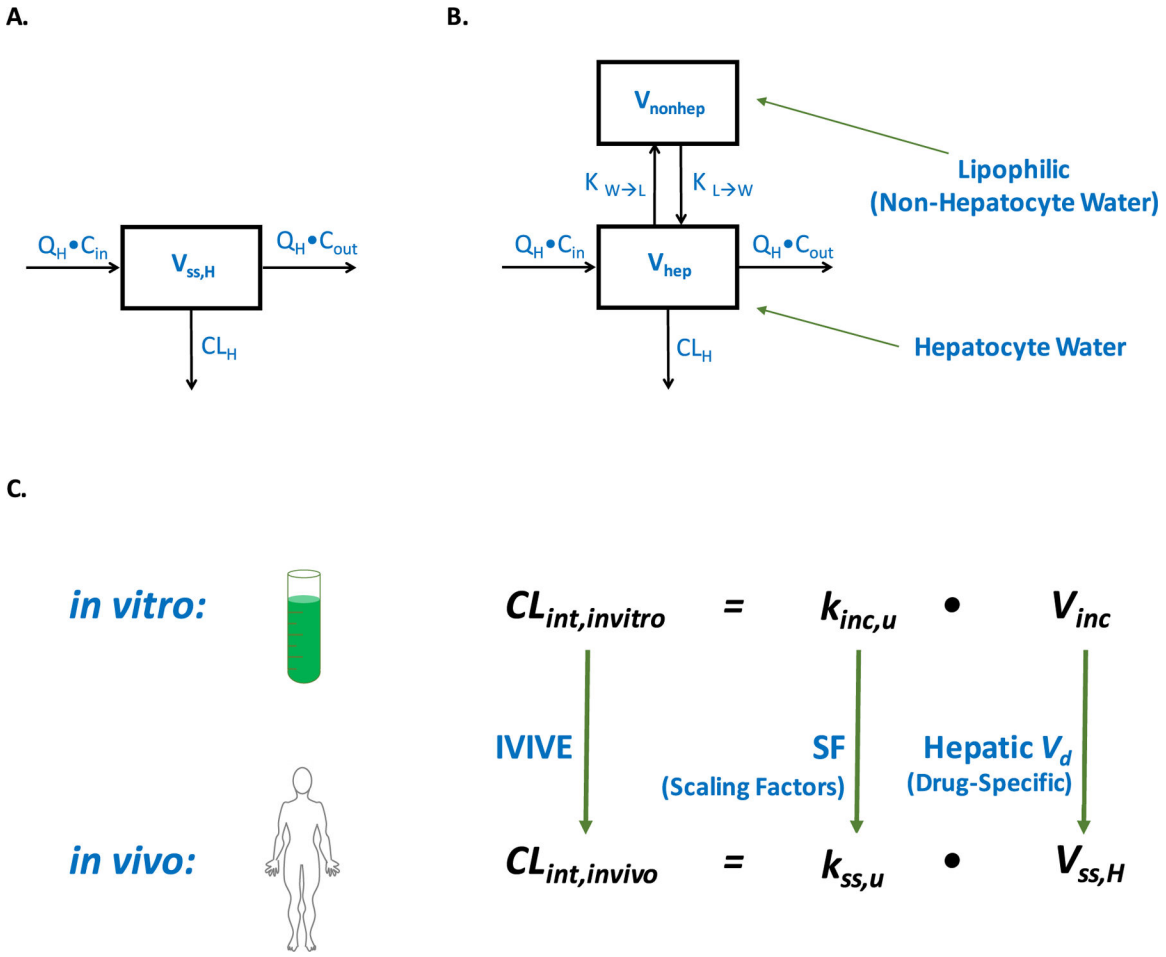

Figure 6.

Hepatic volume of distribution and IVIVE. Abbreviations: CL_H, hepatic clearance; C_{in}, entering drug concentration; $CL_{int,invitro}$, *in vitro* intrinsic clearance; $CL_{int,invivo}$, *in vitro* intrinsic clearance; C_{out}, exiting drug concentration; IVIVE, *in vitro* to *in vivo* extrapolation; $k_{inc,u}$, unbound rate of incubational drug loss; $K_{L \to W}$, partition coefficient from the lipophilic to aqueous hepatic compartments; $k_{ss,u}$, steady-state *in vivo* rate of unbound drug loss; $K_{W \to L}$, partition coefficient from the aqueous to lipophilic hepatic compartments; Q_H, hepatic blood flow; SF, physiologically based scaling factors; V_d, volume of distribution; V_{hep}, volume of distribution of drug in the hepatocyte water at steady state; V_{inc}, volume of *in vitro* incubation; V_{nonhep}, volume of distribution of drug in the nonhepatocyte water (lipophilic regions) at steady state; $V_{ss,H}$, volume of distribution of drug in the whole liver at steady state.

Table 1.

Overview of *In Vitro* Tools Integral in Lead Optimization Efforts[a]

	liver slices	hepatocytes	microsomes	cytosol	recombinant
enzymes	all enzymes	all enzymes	CYPs	NAT	singularly expressed CYP isoform
			FMO	GST	
			UGT	SULT	
			membrane proteins	AO soluble proteins	
cofactors	none	none	NADPH (CYP/FMO)	PAPS (SULT)	NADPH (CYP)
			UDPGA (UGT)	GSH (GST) acetyl-CoA (NAT)	
transporters	all transporters	all transporters	none	none	none
storage	fresh	fresh/cryopreserved	−80 °C freezer	−80 °C freezer	−80 °C freezer
advantages	tissue heterogeneity	transporters/enzymes (+ phase II)	robust, low cost	non-P450 metabolism	specific metabolic pathway
	drug distribution	pooled (5–100 donors)	withstands freeze/thaw pooled (150 donors)	pooled (150 donors)	easy to purchase
disadvantages	difficult to prepare	cell viability	cofactors needed	cofactors needed	difficult to prepare
				difficult to scale-up	difficult to scale-up
throughput	very low	moderate	high	high	high
ADME assays	seldom used	metabolic stability	metabolic stability	metabolic stability	reaction phenotyping
	toxicity	CL prediction	CL prediction	metabolite ID	metabolism of stable compounds
	metabolic profiling	metabolite ID	metabolite ID		
		drug interaction	drug interaction	reaction phenotyping	
		reaction phenotyping sandwich culture	reaction phenotyping		

[a]Abbreviations: AO, aldehyde oxidase; CL, clearance; CYPs, cytochrome P450s; FMO, flavin-containing monooxygenase; GSH, glutathione; GST, glutathione S-transferase; NADPH, nicotinamide adenine dinucleotide phosphate; NAT, *N*-acetyl transferase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; SULT, sulfotransferase; UDPGA, uridine diphosphate glucuronic acid; UGT; UDP-glucuronosyltransferase.