Improving In Vitro-In Vivo Extrapolation (IVIVE) of Clearance Using Rat Liver Microsomes for Highly Plasma Protein Bound Molecules

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**Abbreviations:** AAFE, absolute average fold error; AFE, average fold error; BSA, bovine serum albumin; CL_b, in vivo clearance in blood; CL_h,b, hepatic in vivo clearance in blood; CL_int, intrinsic in vitro clearance; CL_int,u, unbound intrinsic in vitro clearance; CYP, cytochrome P450; DME, drug metabolizing enzyme; EC3S, extended clearance concept classification system; ECCS, extended clearance classification system; fu_b, fraction unbound in blood; fu_inc, fraction unbound in incubation; fu_mic, fraction unbound in liver microsomes; fu_p, fraction unbound in plasma; IVIVC, in vitro in vivo correlation; IVIVE, in vitro in vivo extrapolation; K_m, Michaelis-Menten constant; k_mic, microsomal first order kinetic constant; LC-MS, liquid chromatography-mass spectrometry; PK, pharmacokinetic; PME, protein-mediated-effect; PPB, plasma protein binding; PS_inf, uptake clearance; Q_h, hepatic blood flow; R_bp, blood-to-plasma ratio; RED, rapid equilibrium dialysis; RLM, rat liver
microsomes; RSA, rat serum albumin; sCL\textsubscript{int,u}, scaled unbound intrinsic in vitro clearance; \( t_{1/2} \), half-life; UGT, uridine diphosphate glycosyltransferases; WSM, well-stirred model.
Abstract

It is common practice in drug discovery and development to predict in vivo hepatic clearance from in vitro incubations with liver microsomes or hepatocytes using the well-stirred model (WSM). When applying the WSM to a set of about 3000 Novartis research compounds, 73% of neutral and basic compounds (extended clearance classification system ECCS class 2) were well-predicted within 3-fold. In contrast, only 44% (ECCS class 1A) or 34% (ECCS class 1B) of acids were predicted within 3-fold. To explore the hypothesis whether the higher degree of plasma protein binding for acids contributes to the in vitro in vivo correlation (IVIVC) disconnect, 68 proprietary compounds were incubated with rat liver microsomes (RLM) in the presence and absence of 5% plasma. A minor impact of plasma on clearance IVIVC was found for moderately bound compounds (fu_p ≥ 1%). However, addition of plasma significantly improved the IVIVC for highly bound compounds (fu_p < 1%) as indicated by an increase of the average fold error (AFE) from 0.10 to 0.36. Correlating fu_p with the unbound intrinsic clearance (CL_int,u) ratio in the presence or absence of plasma allowed the establishment of an empirical, non-linear correction equation that depends on fu_p. Taken together, estimation of the metabolic clearance of highly bound compounds was enhanced by the addition of plasma to microsomal incubations. For standard incubations in buffer only, application of an empirical correction provided improved clearance predictions.

**Keywords:** Intrinsic clearance (CL_int), in vitro in vivo correlation (IVIVC), in vitro in vivo extrapolation (IVIVE), liver microsomes, metabolic stability, plasma protein binding (PPB), protein-mediated effect (PME), well-stirred model.
Significance statement: Application of the well-stirred liver model for clearance IVIVE in rat generally underpredicts the clearance of acids. The strong protein binding of acids is suspected to be one responsible factor for the underprediction. 

CL_{int,u} determinations using rat liver microsomes incubations supplemented with 5% plasma resulted in an improved IVIVC. An empirical equation was derived from fu_p and the ratio of CL_{int,u} in the presence and absence of 5% plasma which can be applied to correct CL_{int,u}-values from incubations in buffer.
Introduction

Drug discovery programs generally aim for low to moderate blood clearance (CL\textsubscript{b}) to achieve sufficient target engagement. Historically, the majority of marketed drugs were eliminated by hepatic oxidative metabolism, predominantly mediated by CYP enzymes (78%) (Zanger et al., 2008) and the importance of CYPs for drugs’ elimination was recently confirmed by Cerny (2016) and Saravanakumar et al. (2019). Common tools to study the metabolic stability in vitro are isolated liver microsomes, hepatocytes, liver slices and recombinant drug metabolizing enzymes (DME) (Sodhi and Benet, 2021) and mechanistic physiological models such as the well-stirred-model (WSM) (Rowland et al., 1973) are applied for in vitro to in vivo extrapolation (IVIVE) (Sodhi and Benet, 2021). The common implicit assumptions of these models are (i) only unbound compounds have access to DME (e.g. CYP), (ii) permeability doesn’t limit access and (iii) the DME are distributed homogeneously in the liver (Sodhi and Benet, 2021). Accordingly, measured intrinsic clearance values (CL\textsubscript{int}) are corrected for the free fraction in the incubation (f\textsubscript{Uinc}) and applied to the WSM accounting for the liver blood flow (Q\textsubscript{h}) and the protein binding in blood (f\textsubscript{ub}) to predict the hepatic clearance (CL\textsubscript{h,b}).

However, the widely applied IVIVE approaches typically lead to an underprediction of in vivo clearance (Wood et al. (2017), Bowman and Benet (2016), Chiba et al. (2009), and Hallifax et al. (2010)). Various potential reasons have been proposed: (i) for hepatocytes: the depletion of co-factors, the permeation rate limitation or the unstirred water layer limiting access to hepatocytes (ii) for humans: the inherent variability of the biological material or the mismatch of enzyme activity in donors and healthy volunteers in clinical studies (iii) for liver microsomes: the absence of
additional potentially critical elimination pathways, and (iv) in general: the loss of enzymatic activity or the inappropriate substrate concentration above the $K_m$ of key pathways leading to saturation and lower $CL_{int}$-values with the parent depletion approach. To account for the above-mentioned limitations, empirical scaling factors have been proposed by various groups (Hallifax and Houston (2019), Williamson et al. (2020), Jones et al. (2022) and Tess et al. (2023)). Nevertheless, a considerable imprecision remains (Francis et al., 2021). Moreover, constant scaling factors are not optimal for highly protein bound compounds (Jones et al., 2022) and additional scaling factors have been proposed to account for protein-mediated-effects (PME) leading to increased uptake into hepatocytes (Poulin et al., 2016).

While the PME gained increased attention (Schulz et al., 2023) and the addition of plasma protein to incubations with hepatocytes was shown to be beneficial for IVIVE of metabolically cleared compounds (Francis et al., 2021) and transporter substrates (Li et al., 2020), the impact of plasma protein addition on non-cellular systems, like liver microsomes, has been investigated less. Rowland et al. (2008a), Rowland et al. (2007) and Rowland et al. (2008b) reported a protein effect with liver microsomes by sequestering the inhibitory effect of endogenous (or added) fatty acids in liver microsomes. Reduced $K_m$-values in liver microsomal incubations supplemented with bovine serum albumin (BSA) and fatty acid free human serum albumin (HSA-FAF) led to increased $CL_{int}$-values, improving IVIVC for substrates of UGT2B7 and CYP2C9 ((Rowland et al., 2008a) and (Rowland et al., 2007)). Skaggs et al. (2006) performed incubations in human liver microsomes in the presence of 30-50% plasma to account for plasma protein binding (PPB) and eliminate the need for additional binding experiments. Hepatic metabolic clearance for 7 marketed drugs was more accurately predicted. In contrast, this approach revealed systematic IVIVC
disconnects as pointed out by Berezhkovskiy et al. (2009) emphasizing the importance of measuring PPB (in plasma and incubations), and also for experiments performed in the presence of diluted plasma. Another approach, proposed by Yan et al. (2023), is to measure the dynamic binding (called dynamic free fraction $f_D$) using a reporter enzyme and measure the kinetics in presence and absence of plasma proteins. Improved IVIVE performance was shown for 9 marketed drugs when applying the dynamic free fraction $f_D$ instead of the static measured unbound fraction ($f_u$) to the WSM with $CL_{int}$ from human liver microsomes. However, this approach relies on a kinetic reporter enzyme assay, which is not necessarily CYP-related.

In the present study, we aimed to systematically investigate the impact of plasma on the clearance in liver microsomal incubations and on clearance prediction. Upon conducting a statistical analysis on rat clearance IVIVC for a large data set of internal research compounds, we focused the experimental work on highly protein bound compounds, predominantly lipophilic acids. Finally, we explored whether supplementing RLM with plasma provides improved IVIVE for highly bound drugs.
Materials and Methods

Regents and Chemicals

Novartis proprietary compounds were obtained from the Novartis compound store as 10 mM stock solutions in Dimethyl sulfoxide (DMSO). β-Nicotinamide adenine dinucleotide 2′-phosphate tetrasodium salt (NADPH), uridine diphosphate glucuronic acid (UDPGA) and all other reagents, chemicals and buffer salts were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA), Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany). Pools of male Sprague-Dawley rat liver microsomes from ≥ 3 individual rats (catalog number: M00001, lot number: YLQ) were obtained from BioIVT (Brussels, Belgium).

Selection of Compounds

For an extensive IVIVC analysis using the traditional WSM approach, about 3000 Novartis research compounds were selected based on availability of rat low dose PK (up to 1 mg/kg i.v. dose), permeability, PPB and RLM in vitro clearance.

For RLM incubations in the presence or absence of 5% plasma, 67 compounds were carefully selected from the above-mentioned data set comprising 35 different drug discovery projects representing current Novartis chemistry. The selected compounds also had $P_{app}$ values $> 5 \times 10^{-6}$ cm/s (ECCS class 1/2 compounds) and in vitro $f_{mic}$, $f_{p}$, and $CL_{int}$ values in a measurable range (details in Table S4). Two controls (NVP-010 and NVP-011) with low PPB ($f_p > 10\%$) were added to investigate the impact of plasma on the enzymatic activity of RLM, one of which had in vivo CL information and was included in the IVIVC analyses. Using the traditional WSM approach (Rowland et al., 1973), clearance IVIVC for 37 and 31 compounds was within and outside 3-fold, respectively.
For initial experiments, a subset of 22 compounds was selected fulfilling the following criteria: \( f_{\text{mic}} > 5\% \), in vivo \( \text{CL}_{p} > 10 \text{ mL/min/kg} \), in vitro \( \text{CL}_{\text{int}} > 100 \mu\text{L/min/mg} \) and \( f_{\text{p}} < 5\% \). As high PPB compounds often reach the assay limit (\( f_{\text{p}} < 1\% \)), the incubations were repeated in diluted plasma (5% rat plasma) and extrapolated to 100% plasma using Equation 5 (Table 1).

**Metabolic Stability in Rat Liver Microsomes**

Microsomal incubations were performed in 100 mM phosphate buffer (pH 7.4) in the presence or absence of increasing concentrations of rat plasma (0%, 1%, 3% and 5%). For higher protein concentrations (5%, 15% and 50%), rat serum albumin (RSA) was used as a surrogate since diluted plasma undergoes gelation. Rat plasma from Sprague-Dawley contains 28 g/L (425 µM) RSA (Barber et al., 1990) which corresponds to 100% plasma-equivalent and hence, 50% plasma-equivalent was established with 14 g/L RSA, 15% plasma-equivalent with 4.2 g/L RSA and 5% plasma-equivalent with 1.4 g/L RSA. Incubations were performed in 384-well PCR plates at 37°C on an automated Hamilton STARlet platform (Hamilton, Bonaduz, Switzerland). An acoustic dispenser (ECHO 650T, Beckman Coulter, Nyon Switzerland) was used to dispense 5 nL of a 10 mM solution of the test articles into 25 µL 100 mM phosphate buffer only or supplemented with plasma or RSA containing 2 mM NADPH or 2 mM UDPGA as co-factor. A 13 µL aliquot of this solution was added to 13 µL RLM (1 mg/mL protein in the same matrix as the co-factor solution) containing Alamethicin (50 µg/mg protein) and 40 mM MgCl\(_2\) in case of UDPGA after 10 min pre-incubation at 37°C. At specific time points (0.5, 5, 10, 20, 30, 40, 50, 60 min), the reactions were terminated by the addition of 10 µL acetonitrile/water/formic acid (90:10:0.1) containing the analytical internal standards (1 µM warfarin and 1 µM glyburide). This mixture was transferred to a new 384-well
plate containing 15 µL acetonitrile/water/formic acid (90:10:0.1) and was centrifuged at 5000g for 15 min at 4°C. A 30 µL aliquot of the supernatant was transferred to another 384-well plate pre-filled with 50 µL water/acetonitrile (50:50) and subjected to LC-MS analysis for measuring test article and internal standard. Assay variability was recently demonstrated to be within 2-fold for about 80% of investigations (Rodríguez-Perez et al., 2023).

**Equilibrium Dialysis**

Binding to plasma proteins and RLM was measured in triplicate by equilibrium dialysis using the RED device from ThermoFisher (Rockford, IL, USA). Test articles (5 µM for plasma and 1 µM for liver microsomes) were incubated with plasma (5% and 100%) and RLM (0.5 mg/mL protein). Aliquots of 300 µL were dispensed in the red chamber of the RED device and 500 µL of 100 mM phosphate buffer (pH 7.4) in the white chamber. The RED device was sealed with a gas permeable membrane and was incubated for 4 hours on an orbital shaker at 750 rpm (Kisker model V 2000) in an incubator (HERA cell 150 from Thermo Scientific) at 37°C with 5% CO₂.

At the end of the incubation period, 50 µL aliquots from both compartments were transferred to a 96 deep-well-plate prefilled with 600 µL acetonitrile containing the analytical internal standards (0.2 µM glyburide) and 50 µL buffer or matrix for a matrix match. The samples were centrifuged at 5000g for 20 minutes at 4°C and the supernatant was transferred to a new 384-well plate prefilled with 30 µL water. Test articles and internal standard were subsequently measured by LC-MS.

**Ultracentrifugation**

For compounds with a measured fuₘic < 10%, follow-up incubations were performed using ultracentrifugation to accurately determine the estimate of the binding value to liver microsomes. Test articles (1 µM) were added to 1000 µL phosphate buffer (100
mM, pH 7.4) containing RLM (0.5 mg/mL protein) and incubated for 10 min at 37°C in a glass vial. For the determination of the total concentration, 3 times 50 µL were added to a 96 deep-well-plate prefilled with 300 µL acetonitrile containing the analytical internal standard (0.2 µM glyburide) and 50 µL phosphate buffer. For the free fraction, an aliquot of 700 µL was removed from the glass vial and centrifuged (Beckman UC Optima Max-XP) at 436000g for 3.5 hours at 37°C (centrifugation was stopped without the use of brakes to avoid mixing of the layers). 300 µL of the supernatant was carefully removed, transferred to a new vial and 3 times 50 µL were added to the 96 deep-well-plate prefilled with acetonitrile containing the internal standard and 50 µL blank microsomes (RLM in phosphate buffer) for a matrix match. Test articles and internal standard were subsequently measured by LC-MS.

**Passive Permeability**

Passive permeability of compounds was investigated using the transwell MDCK-V2 assay as described by Huth et al. (2021). In brief, 96-well plate permeable inserts were plated with Madin-Darby Canine Kidney cells and cultured for 3 days. The test compound in DMSO stock solution (10 mM) was added to Hanks’ balanced salt solution (HBSS) resulting in a final concentration of 10 µM. Moreover, the HBSS buffer contained 0.02% bovine serum albumin (BSA) and 10 mM HEPES. The acceptor compartment was HBSS with 5% BSA and 10 mM HEPES. Both compartments contained Bafilomycin A at 10 nM. The assay was run for 120 min, determining the donor concentration at time zero, and the donor and acceptor concentration after 120 min by LC-MS.

**In vivo PK in Rat**

In vivo PK studies were performed according to the Animal Welfare country legislation and regulations and to the global Novartis Animal Welfare Policy and
Standards. Six to four days before first drug administration, male Sprague-Dawley rats (body weight 250-300 g) were anesthetized by inhalation of an oxygen/isoflurane mixture (97/3, v/v; isoflurane, Forene, Abbott AG, Zug, Switzerland), and catheters were surgically implanted into the two jugular veins (one for blood collection and the other for intravenous injection). The catheters were exteriorized at the neck. Animals received analgesic treatment before surgery and subsequently at appropriate times after surgery. Animals were kept individually in standard cages, with free access to food and water throughout the experiment. After complete surgery recovery, the awake cannulated rats were dosed intravenously at a dose of 0.1-1.0 mg/kg of test article solubilized in a mixture of N-1-methylpyrrolidone (NMP) and polyethylene glycol 200 (PEG200) with an administration volume of 0.5 mL/kg. Blood (EDTA, ~10-20 µL) was collected via the catheter implanted into the jugular vein at different time points (0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 24 h). Immediately after collection, these whole blood samples were frozen on dry-ice and then stored at −20°C until LC-MS analysis for test article determination.

For bioanalytical investigation of blood samples, protein precipitation was performed by mixing an aliquot of blood with acetonitrile, followed by centrifugation at 4 °C. The supernatant was transferred into a microtiter plate and an aliquot of each sample was injected into the LC-MS system for analysis.

**LC-MS analysis**

Analysis of samples was performed on a liquid chromatography–mass spectrometry system (LC-MS) consisting of a Shimadzu Nexera LC-system and a Sciex QTrap 6500 MS controlled by Analyst 1.7 from AB Sciex (Darmstadt, Germany). Compound specific parameters (parent ion, fragment and collision energy) were obtained by automatic tuning using DiscoveryQuant 3.0.7. These parameters were stored in a
database to be used for selective quantitation of each test article. Samples (2 µL) were injected onto a Phenomenex Kinetex Polar C18, 2.1 x 30 mm, 2.6 µm column (Brechbühler, Schlieren, Switzerland) and were eluted with a gradient of 0.1 % formic acid in water (mobile phase A) versus 0.1 % formic acid in acetonitrile (mobile phase B) at a flow of 0.8 mL/min at 50°C using the following gradient: 0 min 2 % B; 0.2 min 2 % B; 1 min 60 %B; 1.3 min 100 % B; 1.7 min 100 % B; 1.71 min 2 % B and 1.95 min 2 % B.

Data Analysis

For the calculation of the in vitro metabolic clearance, the percentage of test article remaining relative to time zero (0.5 min) incubation was used to determine the elimination-rate constant ($k_{mic}$) and intercept with an exponential decay model (XLfit 5.5.0.5 model 500). Intrinsic clearance ($CL_{int}$) was calculated by correcting for the used microsomal protein concentration ($C_{protein} = 0.5$ mg/mL).

$$CL_{int} = \frac{k_{mic}}{C_{protein}}$$

Equation 1

The unbound scaled intrinsic clearance ($sCL_{int,u}$) was calculated from the $CL_{int}$ using physiological scaling factors (SF). The SF1 is 61 mg microsomal protein per gram liver (Smith et al., 2008) and SF2 is 40 gram liver per kg body weight (Davies and Morris, 1993) for rat:

$$sCL_{int,u} = CL_{int} * SF_1 * SF_2 / f_{u_{inc}}$$

Equation 2

The free fraction in the incubation $f_{u_{inc}}$ was measured with the RED device using either RLM (0.5 mg/mL) or 5% rat plasma in the donor compartment for incubations with buffer or supplemented with 5% plasma. For compounds with no measured
microsomal binding, \( f_{\text{mic}} \), was calculated based on logP/D using the model from Austin et al. (2002).

\[
f_{\text{inc}} = \frac{(\text{AREA}_{\text{test article}}/\text{AREA}_{\text{internal standard}})_{\text{receiver}}}{(\text{AREA}_{\text{test article}}/\text{AREA}_{\text{internal standard}})_{\text{donor}}}
\]

Equation 3

The predicted hepatic in vivo clearance in blood (\( CL_{h,b} \)) was calculated using the well-stirred model (Rowland et al., 1973), where \( Q_h \) is the hepatic blood flow of 80 mL/min/kg for rat (Musther et al., 2017) and \( R_{bp} \) the blood-to-plasma-ratio. The \( R_{bp} \) was set to 1 for the statistical analysis of 3000 Novartis research compounds. For the IVIVC calculations of 68 proprietary compounds, generic values for \( R_{bp} \) were used depending on the ionization state of the corresponding compounds with 0.67 for acids, 0.81 for neutrals and 1.0 for bases and zwitterions (median values from about 1800 compounds with measured ex vivo \( R_{bp} \), (Figure S1)).

\[
CL_{h,b} = \frac{Q_h \cdot SC_{\text{int,u}} \cdot f_{u_p} / R_{bp}}{Q_h + SC_{\text{int,u}} \cdot f_{u_p} / R_{bp}}
\]

Equation 4

The values for the fraction unbound in (100%) plasma (\( f_{u_p} \)) were extrapolated from the measured values with 5% plasma (\( f_{u_p5\%} \)) using Equation 5 where \( D \) is the dilution factor (\( D = 100\% / 5\% = 20 \)) (Kalvass and Maurer, 2002):

\[
f_{u_p} = \frac{1}{1 + \frac{1}{D}} f_{u_p5\%} - \frac{1}{D}
\]

Equation 5

To compare the accuracy of the predictions for the different incubations, the average fold error (AFE) was used to investigate the bias and the absolute average fold error (AAFE) was calculated to get a measure about the precision using the equations below where \( n \) corresponds to the number of compounds.

\[
AFE = 10^{\frac{1}{n} \sum \log \left( \frac{\text{predicted } CL_{h,b}}{\text{observed } CL_{h,b}} \right)}
\]

Equation 6
$$\text{AAFE} = 10^{-\frac{1}{2} \sum \left| \log \left( \frac{\text{predicted } CL_{b,h}}{\text{observed } CL_{h,b}} \right) \right|} \quad \text{Equation 7}$$

Data analysis of in vivo samples was done using a non-compartmental approach. PK calculations were performed on individual concentration profiles. All calculations were based on the compound’s free form.

Briefly, the apparent terminal slope $\lambda_z$ (rate-constant in h$^{-1}$) of the semilogarithmic concentration-time curve was estimated between the 3 last measured time point concentrations (with a square of correlation coefficient, also named goodness of fit statistic, $R^2 > 0.75$); then the apparent elimination half-life ($t_{1/2,z}$) was calculated as $t_{1/2z} = \ln 2 / \lambda_z$. The areas under the curve (AUC) were calculated by the linear trapezoidal rule and extrapolated to infinity time as $AUC = AUC_{\text{last}} + C_{\text{last}}/\lambda_z$, where $AUC_{\text{last}}$ is the area under the curve between zero and the last measurable time point ($T_{\text{last}}$) and $C_{\text{last}}$ the last measurable blood concentration. The extrapolation of the intravenous AUC from the last time point to infinite (i.e. $C_{\text{last}}/\lambda_z$) did not exceed 25% of the AUC. Systemic plasma clearance ($CL_b$) data were calculated as: $CL_b = \frac{\text{Dose}_{\text{iv}}}{AUC_{\text{iv}}}$. 
Results

Impact of PPB on IVIVC

A systematic analysis of the Novartis research database was performed to investigate the predictive performance of the well-stirred model (WSM). A total of about 3000 compounds with available rat in vivo PK and in vitro metabolic stability data were extracted from the Novartis research database. In vivo CL\textsubscript{h,b} was predicted using the WSM based on CL\textsubscript{int}-values from RLM corrected for microsomal binding (fu\textsubscript{mic}), plasma-protein binding (fu\textsubscript{p}) and setting the blood-to-plasma ratio (R\textsubscript{bp}) to 1. As shown in Figure 1, CL\textsubscript{h,b} predictions were within 3-fold in 73% of the cases for highly permeable (P\textsubscript{app} > 5 \times 10^{-6} \text{ cm/s}) neutral and basic compounds (ECCS class 2) and 62% for low permeable compounds (ECCS class 4). In contrast, for highly permeable acids, prediction performance decreased to 44% (ECCS class 1A) and 34% (ECCS class 1B) and even more for low permeable acids (40% (ECCS class 3A) and 21% (ECCS class 3B), respectively) (Figure 1 panel A).

Transporters might play an important role in the elimination of low permeable compounds, which is rationalized by extended clearance classification systems such as ECCS (Varma et al., 2015) or EC3S (Camenisch, 2016). For further evaluation and the experimental part of this study, only ECCS / EC3S class 1/2 compounds were included, which are expected to be predominantly eliminated by hepatic metabolism. We also include lipophilic acids, which may depend on hepatic uptake but are ultimately metabolized.

Figure 1 panel C shows that one of the success criteria (% within 3-fold) of neutral and basic compounds were similar for compounds with fu\textsubscript{p} > 10% (84% within 3-fold) compared to compounds with fu\textsubscript{p} > 5% (80% within 3-fold). For permeable acids...
(Figure 1 panel B), the % within 3-fold is more sensitive towards fu_p, with only 42% of the CL_h,b predictions within 3-fold for compounds with an fu_p > 2% but up to 84% for compounds with fu_p > 10%, matching the success rate of neutral compounds and bases.

**Addition of plasma led to increased CL_{int,u} values.**

Initial incubations in RLM for a subset of 24 compounds (Table 1) were performed with 0%, 1%, 3% and 5% rat plasma. According to the free-drug hypothesis, a reduction of apparent CL_{int} with increasing plasma concentration is expected. However, for compounds with an IVIVC outside 3-fold (Figure 2 panel A), the decrease in measured CL_{int}-values (blue lines) was less than anticipated with the different plasma concentrations in the RLM incubations. Correcting measured CL_{int} for fu_{inc} resulted in increasing unbound CL_{int,u}-values along with increasing plasma concentration (green lines). Compounds with an IVIVC within 3-fold (Figure 2 panel B) showed no major change of the apparent CL_{int} and were within a factor of 2 compared to incubations in buffer. The unbound clearance CL_{int,u} of NVP-015 and NVP-022 was less affected because of a high microsomal binding which is comparable to binding in diluted plasma (fu_{mic} < 20%). NVP-022, with a fu_{mic} of 1.7% showed even stronger binding compared to diluted plasma leading to a higher unbound CL_{int,u} value in buffer compared to plasma. Other compounds had on average higher fu_p-values and behaved as expected according to the free-drug hypothesis, showing a reduced impact on unbound CL_{int,u} (NVP-013a, NVP-016, NVP-017, NVP-018 and NVP-019). The two control compounds (NVP-010 and NVP-011 with fu_p > 10%) as well as NVP-020 with a high fu_p of 17.3% also showed no reduced CL_{int}-values (and CL_{int,u}-values), indicating the activity of CYP enzymes is not significantly impacted by the added plasma.
Determination of optimal plasma concentrations for CL assays

In order to determine the minimum plasma concentration required to reach the highest \( CL_{\text{int,u}} \) value, incubations were performed with 12 compounds using up to 50% plasma-equivalent. For incubations at higher protein concentrations, matching RSA concentrations were used to overcome gelling issues (see Methods section Metabolic Stability in Rat Liver Microsomes). A comparison of the PPB for the 12 compounds with 5% rat plasma and 1.4 g/L RSA (5% plasma-equivalent) showed no difference in binding with nearly all \( fu_p \)-values within 2-fold (Table S1), an AFE of 0.80 and an AAFE of 1.35. Also, no impact on the \( CL_{\text{int}} \) and \( CL_{\text{int,u}} \) was observed with all \( CL_{\text{int}} \) values being within 1.5-fold (Table S3), an AFE of 1.01 and an AAFE of 1.20.

A plot of \( CL_{\text{int,u}} \) vs plasma concentration indicates that a plateau was reached at about 5% plasma for 10 out of 12 compounds (Figure 3) and \( CL_{\text{int,u}} \)-values did not increase by more than two-fold with further increasing plasma concentrations (Table S3). An equilibrium model was fitted (Figure 3 and Table S2) and the resulting parameters are highly variable. Nevertheless, it indicates that the maximal effect is usually achieved with less than 5% plasma.

For two compounds (NVP-012 and NVP-005), the maximum \( CL_{\text{int,u}} \) increased beyond 5% plasma (estimated plasma concentration reaching half of the maximal effect on \( CL_{\text{int,u}} \) is 26.9% and 6.9%, respectively) (Figure 3 and Table S2). Nevertheless, the effect was considered sufficient and the calculated \( CL_{\text{h,b}} \)-values with 5% plasma were within 2-fold compared to the measured in vivo \( CL_b \) for both compounds (Table S3 and Figure S3). For the control NVP-010 (\( fu_p = 23.3\% \)), there was essentially no effect and all \( CL_{\text{int}} \) values were within 1.5-fold which is within the expected assay variability (two-fold) and results were regarded as similar (Table S3 and Figure S3).
Impact of plasma on the free fraction in RLM

Traditionally, microsomal incubations are performed in protein-free buffer and data are corrected for incubational binding that is predominantly driven by membrane sequestration. To investigate the impact of plasma on the free fraction in microsomal incubations supplemented with plasma, binding experiments were performed with 5% plasma alone and 5% plasma with RLM (0.5 mg/mL protein) (Table 2). The $f_u$ data from both conditions were generally within the same range with ratios close to unity (AFE 0.92) and with a calculated AAFE of 1.53. The difference was greater than 2-fold for only 2 compounds (NVP-003 and NVP-004a). No compound showed stronger binding (more than factor 2) in plasma with RLM and we concluded that there is no significant additional binding for the investigated compounds when adding RLM to 5% plasma.

IVIVC with 5% plasma

Finally, all selected compounds (Table S4) were incubated with RLM in the presence or absence of 5% plasma. Plots for the IVIVC are shown in Figure 4. For compounds with $f_{up} \geq 1\%$ (Figure 4 panel A & C), addition of plasma had limited impact on the IVIVC for $sCL_{int,u}$ and $CL_{h,b}$. With and without plasma, 35 (74%) and 32 (68%) compounds were within a 3-fold range (by $CL_{h,b}$), respectively (Table 3). Also, the statistical parameters AFE and AAFE were comparable (Table 3). In contrast, for compounds with $f_{up} < 1\%$ (Figure 4 panel B & D), the IVIVC improved by the addition of plasma with 14 (67%) of compounds predicted within 3-fold compared to 4 (19%) compounds with buffer only (Table 3). Seven compounds which remained outside of the 3-fold range nevertheless also showed increased $CL_{int,u}$ values (Table S4). Four of these compounds had in vivo $CL_b$-values above the liver blood flow ($Q_h$ of 80 mL/min/kg), indicating extrahepatic elimination. The predicted $CL_{h,b}$-values
were increased for all 7 compounds, in particular for 5 compounds which showed increased $CL_{h,b}$ by more than a factor of 3 (Table S4). The prediction accuracy improved, although still an underprediction, with an AFE moving from 0.10 to 0.36 with 5% plasma (Table 3). When limiting the IVIVC analysis to compounds with a $CL_b < 100$ mL/min/kg, the AFE for highly bound compounds ($fu_p < 1\%$) moved up to 0.47 and the AAFE down to 2.18 which is comparable to values observed for compounds with lower binding (Table 3).

**Protein-mediated effects on $CL_{int,u}$ are dependent on $fu_p$**

Figure 5 shows fold changes in $CL_{int,u}$ with and without plasma plotted against $fu_p$. A strong increase in the ratios was observed for $fu_p$-values below 1%. Above this value, addition of plasma had no relevant impact on $CL_{int,u}$. Recently, Bi et al. (2021) reported a similar correlation for $fu_p$-dependent hepatic uptake, which was described by a non-linear facilitated-dissociation model. Applying the same concept, we derived the following equation to describe the correlation in Figure 5: ratio ($CL_{int,u}$ 5% plasma / $CL_{int,u}$ buffer) = 1 + 1/$fu_p$ * 1.186. In line with Figure 5, compounds with the largest change in $CL_{int,u}$ (highest ratio) showed the largest improvements in IVIVC (shown in green). Two compounds (NVP-022 and NVP-066) with lower ratios showed stronger binding to microsomal proteins ($fu_{mic} < 20\%$, confirmed by ultracentrifugation) compared to 5% plasma. Three compounds (in purple) with $fu_p \geq 1\%$ showed no impact on $CL_{int,u}$ (ratio within 2-fold). Nevertheless, they moved outside the 3-fold range of the IVIVC, likely due to assay variability.

For highly protein bound compounds ($fu_p < 1\%$), there was a clear increase of the derived $CL_{int,u}$ values measured with 5% plasma (up to 100-fold) and IVIVC was improved for 10 compounds (Figure 5). For 6 compounds, $CL_{int,u}$ (and calculated $CL_{h,b}$) increased by more than a factor of 2 but IVIVC was still outside 3-fold (4 have
measured in vivo $\text{CL}_{h,b}$ beyond $Q_h$) indicating the potential for other elimination pathways.
Discussion

Despite many efforts to improve in vitro systems for metabolic stability, it remains challenging to predict clearance across a broad chemical space. There is a clear trend for underprediction of in vivo CL and a need to gain more understanding of the limitations of the WSM approach (Wood et al., 2017). We identified ECCS class 1 and class 3 compounds (acids) as the most challenging sub-groups to predict in vivo clearance. While this can be rationalized by the poor permeability of ECCS class 3 compounds, violating one of the implicit assumptions of not limiting the access to DME (Varma et al., 2015), there is no obvious explanation for ECCS class 1 compounds (permeable acids). In general, lipophilic acids strongly bind to plasma proteins, predominantly to albumin (Urien et al., 2001). This holds true for our data set of about 3000 compounds, where the average fuₚ of acids is substantially lower compared to other ionization classes (Figure S2). When considering compounds with higher fuₚ values only, the IVIVC for acids (ECCS class 1A/B) and bases/neutrals (ECCS class 3) was similar (Figure 1) indicating the PPB essentially causes the IVIVC disconnect. The observed IVIVC disconnect for ECCS class 1A/B compounds and/or highly protein bound molecules is in-line with previous studies using microsomes and/or hepatocytes (e.g. Jones et al. (2022), Francis et al. (2021) and Tess et al. (2023)), which was corrected by empirical scaling factors attenuating the binding correction in the WSM (or parallel-tube model) with decreasing fuₚ-values. This indicates a violation of the free-drug hypothesis which may be explained by a protein mediated update mechanism for cellular incubations (Schulz et al., 2023). However, despite a similar outcome, this hypothesis cannot explain the disconnect in non-cellular incubations like liver microsomes. On the other hand,
there are quite high protein concentrations present in the hepatocyte cytosol (Roll and Willenbring, 2010).

The underestimation of hepatic clearance is highest when the plasma \( f_{up} \) is small, and therefore the binding correction in the well-stirred liver model (Equation 4) is highest. The traditional approach for binding correction implies that only the free drug is available for metabolism. We postulate that this might not be entirely true (Figure 6) and that there may be a residual activity or hand-over of bound molecules that cannot be neglected anymore when \( f_{up} \) becomes very small. We explored this hypothesis by directly measuring the impact of protein on the metabolic stability in rat liver microsomal incubations. The unbound clearance (\( CL_{int,u} \)) was then used to predict the in vivo CL using the WSM (Equation 4). Interestingly, for highly bound compounds, we observed higher metabolic turnover than anticipated from the binding correction based on the free fraction that was measured by independent equilibrium dialysis experiments.

The increased \( CL_{int,u} \) values from these incubations with 5% plasma led to an improved IVIVC for highly bound compounds (\( f_{up} < 1\% \)) and, when limiting the IVIVC to compounds with \( CL_b < 100 \text{ mL/min/kg} \), reached similar levels (% within 3-fold, AFE and AAFE) as compounds with lower binding. Despite a significant increase of \( CL_{int,u} \)-values observed with 5% plasma (up to 100-fold), no \( CL_{h,b} \) overprediction was observed and there was no or little effect of the 5% plasma on \( CL_{int,u} \) for compounds with \( f_{up} \geq 1\% \) (Figure 5). This observation is in line with the hypothesis of a residual reactivity or hand-over of bound molecules which is expected to be relevant for compounds with low \( f_{up} \) (< 1%) only.
An improved IVIVE was also reported by Bi et al. (2021) for OATP substrates by increased in vitro uptake clearance values ($PS_{inf}$) from incubations in the presence of plasma and the ratios of plasma-to-buffer $PS_{inf,u}$ showed correlation with $fu_p$ which was best described by a facilitated-dissociation model. Developed for hepatic uptake but conceptually applicable to enzymatic turnover, we applied a simplified version of this model, and the derived equation enabled the estimation of corrected $CL_{int,u}$ values based on available $fu_p$ and in vitro $CL_{int}$ data determined under standard conditions (i.e., without plasma).

Despite the improved rat clearance IVIVC, the results still indicated a general underprediction in the presence of 5% plasma (AFE 0.47 and 0.62 for $fu_p < 1\%$ and $fu_p \geq 1$, respectively). Potential explanations may be the reduced activity of in vitro systems resulting from the isolation process (Wood et al., 2017) or additional metabolic or extra-hepatic elimination mechanism, which are not covered by liver microsomes. Since many acids were included in this study, we also investigated the contribution of uridine diphosphate glycosyltransferases (UGT) by incubating RLM with UDPGA as a co-factor. No relevant turnover was observed in the absence or presence of 5% plasma (Table S5), suggesting no major involvement of UGTs in the elimination of the tested compound. These experiments also addressed the question whether fatty acids, typically contained in in vitro incubations, may have inhibitory effects on metabolizing enzymes as reported for UGT1A9, UGT2B7 and CYP2C9 by Rowland et al. (2008a), Rowland et al. (2007) and Rowland et al. (2008b). While in these studies, the addition of BSA resulted in enhanced enzyme activity, we did not observe relevant changes in UGT activity. Although it is not known by which CYP isoforms the tested compounds are metabolized, enhanced activity by the rat
CYP2C9 homologue in the presence of plasma may partially contribute to the improved IVIVC.

According to Berezhkovskiy et al. (2009), with a plasma concentration of 100% in the microsomal incubation, the measured CL_{int}-values are dependent on the binding to RLM and the R_{bp} only. When R_{bp} is neglected (set to 1 or using generic values), a plasma concentration of 100% can be theoretically used in the incubations without requiring an independent measurement of PPB as intended by Skaggs et al. (2006). Based on our experience, however, this approach comes with technical hurdles since the reduction of turnover will move many compounds below the assay limit. We therefore propose to use a plasma concentration of 5%, representing a good balance between measurable clearance and achieving a maximum effect on unbound clearance for most compounds.

RLM incubations in buffer are traditionally corrected for f_{inc} raising the question about the f_{inc} in RLM with plasma. In order to assess the free fraction when adding 5% plasma to incubations, we performed equilibrium dialysis experiments containing RLM in the presence or absence of 5% plasma. The data revealed that microsomal binding for the investigated compounds becomes negligible in the presence of plasma (Table 2), indicating that highly bound acids preferably bind to albumin rather than to membrane fractions. Consequently, it is sufficient to correct RLM CL_{int} by the fu measured in 5% plasma. Conducting the plasma protein binding assay in diluted plasma is especially helpful for highly bound compounds (fu_p < 1%) as it enhances the dynamic range with regards to bioanalytical sensitivity. The measured fu-values in 5% plasma can be extrapolated to 100% plasma for the WSM predictions. Saturation of binding sites, which can compromise the back calculation in 100% plasma, is unlikely as most of the investigated compounds are acids and binding is
expected to happen to albumin (Urien et al., 2001) which is present at high concentration in rat plasma (28 g/L (Barber et al., 1990)).

With this study, we have demonstrated that rat CL\textsubscript{h,b} of highly plasma protein-bound compounds (fu\textsubscript{p} < 1%) is generally underpredicted by the WSM using RLM and the common binding corrections. We show that addition of protein (plasma) to rat microsomal incubations improves IVIVC for those compounds and further work will be needed to confirm it in species other than rat. It is plausible to conclude that, besides the free compound, there is also a hand-over of plasma protein-bound compound to the metabolizing enzyme or a residual enzymatic activity to the protein bound compound as depicted in Figure 6, which cannot be neglected anymore when fu\textsubscript{p} is very small. We propose adding 5% plasma to liver microsomal incubations for highly protein bound compounds (fu\textsubscript{p} < 1%) to overcome this residual binding. Alternatively, in vivo clearance in rat can be estimated by applying an empirical correction equation similar to that previously established in hepatocyte studies (Bi et al., (2021) and Francis et al. (2021)).
Acknowledgments

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Data availability statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Information.

Author contributions

Participated in research design: Trunzer, Teigão, Huth, Poller, Rodríguez-Pérez, and Faller

Conducted experiments: Trunzer, and Teigão

Performed data analysis: Trunzer, Teigão, Huth, Poller, Desrayaud, Rodríguez-Pérez, and Faller

Wrote or contributed to the writing of the manuscript: Trunzer, Teigão, Huth, Poller, Desrayaud, Rodríguez-Pérez, and Faller

Footnote

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Competing interests: The authors declare no competing interests.
References


Figure legends:

**Figure 1: Clearance IVIVC for Novartis rat data.** Panel A shows the IVIVC for about 3000 research compounds in rat according to the ECCS class. Data are presented as percentage of rat $CL_{h,b}$ predictions using the WSM within 3-fold. Panels B and C show the corresponding data for ECCS class 1A/B (permeable acids) and class 2 (permeable bases/neutrals), respectively, depending on the free fraction in plasma ($fu_p$).

**Figure 2: $CL_{int}$ and unbound $CL_{int,u}$ in RLM in the presence of plasma.** Measured apparent $CL_{int}$-values (blue lines) and expected $CL_{int}$-values (red lines) in the presence of 0, 1%, 3% and 5% rat plasma. Black lines show the apparent $CL_{int}$ at 0% plasma with grey lines indicating a difference of factor 2 (assay variability). Green lines show the unbound $CL_{int,u}$ corrected for $fu_{inc}$ (measured $fu_{mic}$ for 0% plasma and $fu_p$ of the corresponding plasma concentrations extrapolated by Equation 5). Compounds showing an underprediction of CL (ratio $< 0.3$ and $fu_p < 4\%$) are shown in panel A and compounds with an IVIVC within 3-fold including controls with weak PPB ($fu_p > 10\%$) are shown in panel B.

**Figure 3: $CL_{int,u}$ with increasing plasma concentrations.** Measured unbound $CL_{int,u}$-values in the presence of increasing plasma concentration. Combined data of incubations supplemented with 0%, 1%, 3%, 5% plasma and 1.4, 4.2 and 14 g/L RSA which corresponds to 5%, 15% and 50% plasma-equivalents. Dots with squares were removed from the fit for equilibrium parameter indicated by red lines and given in Table S2.

**Figure 4: IVIVC for 68 Novartis compounds.** Panel A and B show the IVIVC by $CL_{h,b}$ for 47 compounds with $fu_p \geq 1$ (A) and 21 highly bound compounds with $fu_p <$
1% (B). Panel C and D show the IVIVC by sCL_{int} for compounds with f_u \geq 1 (C) and with f_u < 1% (D). Indices in dark blue show the IVIVC based on incubations with 5% plasma and in light blue based on incubations in buffer. Acids are depicted as squares, bases as circles and neutral compounds as diamonds.

**Figure 5: Impact of 5% plasma on CL_{int,u}.** Ratio of CL_{int,u} measured with 5% plasma and CL_{int,u} in phosphate buffer versus PPB (f_u). Compounds shown in light green show an IVIVC within 3-fold with and without 5% plasma. Dots in dark green show improved IVIVC (moved within 3-fold) and red dots show compounds which did not move into the 3-fold range. Dots in purple are compounds which moved outside the 3-fold range with 5% plasma. Triangles indicated compounds with a in vivo CL_{b} greater than hepatic blood flow (80 mL/min/kg). The grey horizontal lines indicate factor 1 (solid) and the 2-fold error (dotted). The blue curves show a fit with the formula ratio \( \frac{\text{CL}_{\text{int,u}} \text{ 5% plasma}}{\text{CL}_{\text{int,u}} \text{ buffer}} = 1 + \frac{1}{f_u} \times 1.186 \) (solid) and the 2-fold error range (dotted).

**Figure 6. Proposed workflow for CL IVIVE.** Standard approach for the application of the WSM based on in vitro CL_{int} and PPB regarding the free drug hypothesis and proposed new approach for highly protein bound compounds based on measured impact of plasma proteins with 5% plasma in microsomal incubations and an empirical correction equation.
Table 1: Parameters of compounds selected for initial incubations with 0, 1%, 3% and 5% plasma.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ionization</th>
<th>In vivo CL&lt;sub&gt;b&lt;/sub&gt; (mL/min/kg)</th>
<th>P&lt;sub&gt;app&lt;/sub&gt; (10&lt;sup&gt;-6&lt;/sup&gt; cm/s)</th>
<th>f&lt;sub&gt;u,p&lt;/sub&gt; (%)</th>
<th>f&lt;sub&gt;u,fin&lt;/sub&gt; (%)</th>
<th>rat CL&lt;sub&gt;int&lt;/sub&gt; (µL/min/mg)</th>
<th>predicted CL&lt;sub&gt;H,b&lt;/sub&gt; (mL/min/kg)</th>
<th>Ratio CL predicted/observed</th>
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<td>NVP-001</td>
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<td>973</td>
<td>8.0</td>
<td>0.02</td>
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<td>31.2</td>
<td>973</td>
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<td>&gt;80</td>
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<td>2.0</td>
<td>0.06</td>
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<tr>
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<td>1.1</td>
<td>&gt;80</td>
<td>442</td>
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<td>NVP-007</td>
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<td>1770</td>
<td>65.5</td>
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<td>8.5</td>
<td>2.5</td>
<td>34.9</td>
<td>601</td>
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<td>7.9</td>
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<td>401</td>
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<td>17.3</td>
<td>79.6</td>
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<td>NVP-021</td>
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<td>NVP-023</td>
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<td>7.1</td>
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<td>52</td>
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<tr>
<td>NVP-010** Neutral</td>
<td>96</td>
<td>1.4</td>
<td>23.3</td>
<td>75.4</td>
<td>705</td>
<td>71.3</td>
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<td>NVP-011** Acid</td>
<td>13.7</td>
<td>&gt;80</td>
<td>298</td>
<td>56.0</td>
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* $f_{u_p}$ (at 100% plasma) was extrapolated from the measured $f_{u_p}$ at 5% plasma

** control compounds with $f_{u_p}>10%$
Table 2: Protein binding of 14 compounds: Binding measured for 0.5 mg/mL RLM (fu\textsubscript{mic}), 5% plasma and 5% plasma with 0.5 mg/mL RLM and the ratio of fu\textsubscript{5%plasma} to fu\textsubscript{5%plasma & RLM}. Mean values of 3 measurements (intra assay) with the standard deviation are shown.

<table>
<thead>
<tr>
<th>Compound</th>
<th>fu\textsubscript{mic} (%)</th>
<th>fu\textsubscript{5%plasma} (%)</th>
<th>fu\textsubscript{5%plasma &amp; RLM} (%)</th>
<th>Ratio</th>
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<tr>
<td>NVP-003</td>
<td>53.8 ± 7.2</td>
<td>3.6 ± 1</td>
<td>9.1 ± 1</td>
<td>0.39</td>
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<td>NVP-004a</td>
<td>26.8 ± 2.2</td>
<td>1.3 ± 0.1</td>
<td>4.6 ± 0.5</td>
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<tr>
<td>NVP-006</td>
<td>&gt;80</td>
<td>18.8 ± 1.6</td>
<td>23.8 ± 0.8</td>
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<tr>
<td>NVP-009a</td>
<td>17.6 ± 1.2</td>
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<td>80.7 ± 0.7</td>
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<td>NVP-031</td>
<td>&gt;80</td>
<td>30.1 ± 0.7</td>
<td>29.8 ± 0.9</td>
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<td>NVP-035*</td>
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<td>26.3 ± 1.6</td>
<td>27.6 ± 4.1</td>
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<td>27 ± 2</td>
<td>15.6 ± 1.3</td>
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<td>NVP-045</td>
<td>60.5 ± 12.8</td>
<td>26.6 ± 1.1</td>
<td>32.5 ± 1.9</td>
<td>0.82</td>
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</tbody>
</table>

* fu\textsubscript{mic} was confirmed by ultracentrifugation
Table 3: Summary of IVIVC for 68 Novartis compounds

<table>
<thead>
<tr>
<th>PPB</th>
<th>within 3-fold</th>
<th>AFE</th>
<th>AAFE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% Plasma (CL_{h,b})</td>
<td>(\text{fu}_p &lt; 1%)</td>
<td>19% (4/21)</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>(\text{fu}_p \geq 1%)</td>
<td>68% (32/47)</td>
<td>0.59</td>
</tr>
<tr>
<td>5% Plasma (CL_{h,b})</td>
<td>(\text{fu}_p &lt; 1%)</td>
<td>67% (14/21)</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>(\text{fu}_p \geq 1%)</td>
<td>74% (35/47)</td>
<td>0.61</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>PPB</th>
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</thead>
<tbody>
<tr>
<td>0% Plasma (CL_{h,b})</td>
<td>(\text{fu}_p &lt; 1%)</td>
<td>29% (4/14)</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>(\text{fu}_p \geq 1%)</td>
<td>70% (32/46)</td>
<td>0.61</td>
</tr>
<tr>
<td>5% Plasma (CL_{h,b})</td>
<td>(\text{fu}_p &lt; 1%)</td>
<td>79% (11/14)</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>(\text{fu}_p \geq 1%)</td>
<td>76% (35/46)</td>
<td>0.62</td>
</tr>
</tbody>
</table>
Figure 1

A. RLM CL<sub>WSM</sub> Performance

<table>
<thead>
<tr>
<th></th>
<th>High-perm acids</th>
<th>Low-perm acids</th>
<th>Neutrals/bases</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW&lt;400</td>
<td>44</td>
<td>34</td>
<td>21</td>
</tr>
<tr>
<td>MW&gt;400</td>
<td>40</td>
<td>62</td>
<td>73</td>
</tr>
</tbody>
</table>

B. Permeable acids (n=495)

<table>
<thead>
<tr>
<th></th>
<th>≤5% fup</th>
<th>&gt;5% fup</th>
<th>&gt;10% fup</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>73</td>
<td>80</td>
<td>84</td>
</tr>
</tbody>
</table>

C. Permeable bases/neutrals (n=1506)

<table>
<thead>
<tr>
<th></th>
<th>≤5% fup</th>
<th>&gt;5% fup</th>
<th>&gt;10% fup</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>73</td>
<td>80</td>
<td>84</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3

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Figure 5
Figure 6

**Standard approach**

- Metabolic Stability $\text{CL}_{\text{int}}$
  - Liver microsomes in buffer
  - $\text{CL}_{\text{int}} = \text{CL}_{\text{int}} / f_u \text{blood}$

- Microsomal Binding $f_u \text{mic}$
  - RLM (0.5 mg/mL)

- Protein Binding $f_u \text{protein}$
  - with 100% plasma

- $\text{CL}_{\text{int}} = \text{CL}_{\text{int}} / f_u \text{mic}$
- $\sigma \text{CL}_{\text{int}} = \sigma \text{CL}_{\text{int}} \times SF_1 \times SF_2$
- $\text{CL}_{\text{int}} = Q_h \times \sigma \text{CL}_{\text{int}} \times f_u \text{blood}$

- $\text{CL}_{\text{int}} = \text{CL}_{\text{int}} / f_u \text{mic}$

**Proposed approach for highly protein bound compounds ($f_u \text{protein} < 1\%$)**

- Metabolic Stability $\text{CL}_{\text{int}}$
  - Liver microsomes in 5% plasma

- $\text{CL}_{\text{int}} = \text{CL}_{\text{int}} / f_u \text{protein}$

- Microsomal Binding $f_u \text{mic}$
  - RLM (0.5 mg/mL)

- Protein Binding $f_u \text{protein}$
  - with 5% plasma

- $\text{CL}_{\text{int}} = \text{CL}_{\text{int}} / f_u \text{protein}$
- $\sigma \text{CL}_{\text{int}} = \sigma \text{CL}_{\text{int}} \times SF_1 \times SF_2$
- $\text{CL}_{\text{int}} = Q_h \times \sigma \text{CL}_{\text{int}} \times f_u \text{protein}$

- $\text{CL}_{\text{int}} = \text{CL}_{\text{int}} / f_u \text{protein}$

**Empirical Correction**

- $\text{CL}_{\text{int}} = \text{correction} \times SF_1 \times SF_2$
- $\text{CL}_{\text{int}} = (1 + 1/f_u \text{protein} \times 1.186) \times \text{CL}_{\text{int}}$

**Well Stirred Model**

- $f_u \text{blood} = f_u \text{protein} / R_{bp}$
- $f_u \text{protein} = f_u \text{blood} / R_{bp}$

$\text{CL}_{\text{int}}$ and $\text{CL}_{\text{int}}$ refer to intrinsic clearance, $f_u$ to free fraction of drug, and $SF_1$ and $SF_2$ to physiological scaling factors. $R_{bp}$ is the receptor-binding protein.