Title: Dissecting parameters contributing to the underprediction of aldehyde oxidase-mediated metabolic clearance of drugs

Authors: Sandhya Subash¹, Dilip K. Singh¹, Deepak S. Ahire¹, S. Cyrus Khojasteh², Bernard P. Murray³, Michael A. Zientek⁴, Robert S. Jones², Priyanka Kulkarni⁵, Bill J. Smith³,⁶, Scott Heyward⁷, Ciarán N Cronin⁸, Bhagwat Prasad¹

Affiliations:

1. Department of Pharmaceutical Sciences, Washington State University (WSU), Spokane, WA
2. Drug Metabolism and Pharmacokinetics, Genentech Inc., South San Francisco, CA
3. Drug Metabolism, Gilead Sciences, Foster City, CA
4. Drug Metabolism and Pharmacokinetics, Takeda Development Center Americas, San Diego, CA
5. Drug Metabolism and Pharmacokinetics, Takeda Development Center Americas, Cambridge, MA
6. Terminal Phase Consulting LLC, Colorado Springs, CO (current affiliation)
7. BioIVT Inc., Baltimore, MD
8. Structural Biology and Protein Sciences, Pfizer Global Research & Development and Medical, La Jolla, CA
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Corresponding author: Bhagwat Prasad, Ph.D., Department of Pharmaceutical Sciences, Washington State University, Spokane, WA 99202, USA. Phone: +1-509-358-7739. Fax: +1 509-368-6561. Email: bhagwat.prasad@wsu.edu
ABBREVIATIONS

AO: Aldehyde oxidase
NCE: New chemical entity
HLC: Human liver cytosol
HLM: Human liver microsomes
rAO: Recombinant aldehyde oxidase
pnAF: Protein content normalized activity factor
REF: Relative expression factor
PBPK: Physiologically based pharmacokinetic modeling
CL_{int}: Intrinsic clearance
IVIVE: In vitro to in vivo extrapolation
DDIs: Drug-drug interactions
ABSTRACT

We investigated the effect of variability and instability in aldehyde oxidase (AO) content and activity on scaling of in vitro metabolism data. AO content and activity in human liver cytosol (HLC) and five recombinant human AO preparations (rAO) were determined using targeted proteomics and carbazeran oxidation assay, respectively. AO content was highly variable as indicated by the relative expression factor (REF, i.e., HLC to rAO content) ranging from 0.001-1.7 across different in vitro systems. The activity of AO in HLC degrades at a 10-fold higher rate in the presence of the substrate as compared to the activity performed after preincubation without substrate. To scale the metabolic activity from rAO to HLC, a protein-normalized activity factor (pnAF) was proposed wherein the activity was corrected by AO content, which revealed upto 6-fold higher AO activity in HLC versus rAO systems. A similar value of pnAF was observed for another substrate, ripasudil. Physiologically-based pharmacokinetic (PBPK) modeling revealed a significant additional clearance (CL; 66%), which allowed successful prediction of in vivo CL of four other substrates, i.e., O-benzyl guanine, BIBX1382, zaleplon and zoniporide. For carbazeran, the metabolite identification study showed that the direct glucuronidation may be contributing to around 12% elimination. Taken together, this study identified differential protein content, instability of in vitro activity, role of additional AO clearance and unaccounted metabolic pathways as plausible reasons for the underprediction of AO mediated drug metabolism. Consideration of these factors and integration of REF and pnAF in PBPK models will allow better prediction of AO metabolism.
SIGNIFICANCE STATEMENT

We elucidated the plausible reasons for the underprediction of aldehyde oxidase (AO) mediated drug metabolism and provided recommendations to address them. We demonstrated that integrating protein content and activity differences, accounting for the loss of AO activity, as well as consideration of extrahepatic clearance and additional pathways would improve the in vitro to in vivo extrapolation of AO mediated drug metabolism using physiologically-based pharmacokinetic modeling.
INTRODUCTION

Over the past decade, metabolism by aldehyde oxidase (AO) has been gaining importance as drug design has evolved in the direction of making compounds that are more stable to cytochrome P450 (CYP) mediated metabolism (Argikar et al., 2016). AO is a highly promiscuous enzyme and has been implicated in the oxidative, reductive, and hydrolytic metabolism of drugs belonging to various therapeutic categories (Dalvie and Di, 2019). Failure to sufficiently characterize AO mediated metabolism has led to inaccurate predictions of clearance resulting in either toxicity or poor exposure in humans, leading to discontinuation of compounds such as carbazeran, BIBX1382, FK3453, LuAF09535, and RO1 (Akabane et al., 2011; Dittrich et al., 2002; Jensen et al., 2017; Kaye et al., 1984, Zhang et al., 2011). Recent reports such as methotrexate-induced liver toxicity due to inhibition of its metabolism following co-administration of an AO substrate and inhibitor, favipiravir (Demir et al., 2022) also indicate the possible role of AO in clinically significant drug-drug interactions (DDIs). AO shows wide species differences which makes the translation of toxicological data challenging. For example, dogs do not express functional AO and the activity in rats is significantly lower than humans and monkeys (Terao et al., 2016). Despite the increased incidence of AO-mediated metabolism, challenges associated with in vitro to in vivo extrapolation (IVIVE) and species differences continue to pose hurdles in drug discovery. This leads to drug discovery programs preferring to avoid advancement of new chemical entities (NCEs) with perceived AO liabilities. The AO protein content differences in preclinical models and humans can partly explain the species differences in AO mediated metabolism (Basit et al., 2022), however, the reasons for the poor IVIVE of AO metabolism are not fully characterized.

The challenges of characterizing in vitro activity of AO include high inter-lot variability in hepatocytes, human liver cytosol (HLC) or S9 fractions, and loss of activity on long-term storage (Hutzler et al., 2014, Barr et al., 2013). The variability between lots has been attributed to
differences in method of preparation, handling, and decline in activity during storage and in postmortem tissues (Barr et al., 2013). Alterations in inter-individual AO activity have also been reported due to the presence of allelic variants of AOX1 gene, resulting in proteins with increased or decreased activity (Hartmann et al., 2012; Ueda et al., 2022). The in vitro activity of AO for most of its oxidative substrates is nonlinear with time due to concurrent inactivation (Abbasi et al., 2019) and the enzyme is also liable to substrate inhibition (Barr et al., 2013). However, there has been no documentation of the rates of decline in activity in the presence and absence of substrate.

Heterologously expressed recombinant systems have proven to be key tools in in vitro studies of drug metabolizing enzymes such as CYPs and UDP-glucuronosyl transferases (UGTs). Several attempts have been made previously to prepare recombinant AO (rAO) in different expression systems (Hartman et al., 2012, Coelho et al., 2015, Foti et al., 2016). However, the expression and purification of rAO have been proven to be difficult. Although rAO is available commercially in the form of enriched preparations containing the expressed enzyme, such as those from HEK293 cells (Corning and Origene) and E. coli (Cypex/Xenotech, Hypha discovery), there are no commercially available purified rAO systems. AO is a complex homodimer which makes the production of the catalytically active enzyme challenging (Cronin et al., 2021).

The differences between AO specific activity in HLC and E. coli expressed enzymes have been explained by possible incomplete incorporation of the molybdenum cofactor (MoCo) and iron, perturbed dimerization, and lack of expression of MoCo sulfurase, the enzyme responsible for incorporating the terminal sulfido ligand to the molybdenum cofactor (Barr et al., 2013). As the native sulfurases present in E. coli are not capable of sulfating MoCo effectively, chemical sulfuration techniques have been employed. For example, treatment with sodium sulfide under anaerobic conditions to convert MoCo to its active sulfated form (Foti et al., 2016). Human AO dimers with high specific activity have been produced in baculovirus-infected insect cells without
a requirement for post-production \textit{in vitro} sulfuration, or for co-expression with MoCo sulfurase (Cronin et al., 2021).

The goals of this study were to characterize the factors that affect the IVIVE of AO activity from rAO and HLC. Current practice to determine \textit{in vitro} intrinsic clearance of AO-mediated metabolism are based on the normalization by the total protein amount and expressed as $\mu$L/min/mg protein. A caveat of using this approach is that the maximal rate ($V_{\text{max}}$) for a given reaction would differ based on the protein amount used in the incubation. In the present study, we utilized quantitative proteomics approach to determine the content of AO in rAO from four different sources, human liver cytosol (HLC), and hepatocytes to obtain their relative expression factor (REF) values. Activity assays, with carbazeran as substrate, were carried out using a fixed amount of AO (pmol per incubation) across the different systems.

The substrate independence of protein normalized activity factor (pnAF) was evaluated using another AO substrate, ripasudil. To investigate the loss of activity of AO during incubations, this was determined in HLC, and in the rAO preparation with the highest activity, in the presence and absence of the probe substrate, carbazeran. A proteomics-informed PBPK model was then developed to estimate the additional clearance required to address the underprediction of \textit{in vivo} clearance for carbazeran from \textit{in vitro} metabolism data. This model was used to assess IVIVE of other AO substrates, i.e., O$^6$ benzyl guanine, BIBX1382, zaleplon, and zoniporide.

\section*{EXPERIMENTAL SECTION}

\section*{Materials}

Methanol, dimethyl sulfoxide (DMSO), MS grade acetonitrile, potassium di-hydrogen phosphate, dipotassium hydrogen phosphate, and formic acid were procured from Fisher Scientific (Fair Lawn, NJ). Acetone was purchased from Sigma Aldrich (St Louis, MO). Bicinchoninic acid
A BCA kit for total protein quantification was purchased from Pierce Biotechnology (Rockford, IL). Ammonium bicarbonate (ABC), dithiothreitol (DTT), iodoacetamide (IAA), TCEP (tris(2-carboxyethyl) phosphine), and MS grade trypsin were procured from Thermo Fisher Scientific (Rockford, IL). Human serum albumin (HSA) and bovine serum albumin (BSA) were purchased from Calbiochem (Billerica, MA) and Thermo Fisher Scientific (Rockford, IL), respectively. Synthetic unlabeled peptides with amino acid analysis and stable labeled (heavy peptides) were purchased from New England peptides (Boston, MA) and Thermo Fisher Scientific (Rockford, IL), respectively. Carbazeran, ripasudil, and diclofenac (internal standard) were procured from Sigma Aldrich (St Louis, MO), while 4-oxo carbazeran was procured from Toronto Research Chemical (TRC) (ON, Canada). The list of *in vitro* reagents used in this study is presented in Table 1. Hepatocyte thawing media (INVITROGRO HT) and incubation media (INVITROGRO KHB) were gifted by BioIVT (Baltimore, MD).

**LC-MS/MS quantification of AO content and relative expression factor (REF)**

We applied an optimized targeted LC-MS/MS methodology (Ahire et al., 2021) to selectively quantify AO in rAO systems, HLC, and hepatocyte homogenate (Table 1). The cryopreserved adult hepatocytes (n=8) were thawed using the manufacturer protocol, the cells were counted and diluted using INVITROGRO KHB media. One million hepatocytes were mixed with 300 µL of solubilization buffer and incubated for 60 min at 300 rpm (4°C). rAOP2 (Table 1) was used as calibrators and the stable-labeled (heavy) peptides served as internal standards. Briefly, total protein concentration of HLC and rAO systems was quantified using BCA assay kit and ~80 µg protein was digested as described previously (Ahire et al., 2021). The calibration curve was constructed ranging from 1.95 to 125 fmol/µL of AO protein by serially diluting rAOP2 in ABC buffer (100 mM) containing 1 mg/mL HSA. The calibration curve samples (rAOP2), other rAO systems, HLC, and hepatocytes were digested using a previously optimized protocol (Ahire et al., 2021). 80 µL of each of the calibration standards, rAO systems, HLC (1 mg/mL total protein),
or hepatocyte homogenate was mixed with ABC and BSA and proteins were denatured and reduced with DTT. The sample was cooled to room temperature for 10 min, and the denatured proteins were alkylated with IAA. Ice-cold acetone was added to precipitate proteins followed by vortex mixing and centrifugation. The protein pellet was dried and washed with ice-cold methanol followed by centrifugation. The pellet was dried and resuspended in ABC buffer before digestion by trypsin at 37° C for 16 hours. The reaction was quenched by the addition of the peptide internal standard and the sample was centrifuged. The supernatant was transferred into an LC-MS vial for analysis. Two surrogate peptides of AO (LILNEVSLLGSAPGGK and MIQVVSRR) were quantified using optimized LC-MS conditions (Supp. Tables 1-3). LC-MS/MS data were acquired using an M-class Waters UPLC system coupled with Waters Xevo®TQ-XS microflow mass spectrometer connected to standard ESI source using parameters outlined (Supp. Tables 1 and 2). The peptides were separated on Acquity UPLC HSS T3 C18 column (1.8 µm, 1 mm x 100 mm) and (Vanguard pre column, 1.8 µm, 2.1 x 5 mm) using the MRM transitions specified (Supp. Table 3). The content of AO in the rAO systems, HLC, and hepatocyte, was estimated using the standard curve of rAOP2.

**Quantification of AO activity and relative activity factor (RAF)**

AO-mediated metabolite formation activity of carbazeran or ripasudil was determined in rAO_C, rAO_X, rAO_W, and HLC in 0.1 M potassium phosphate buffer pH 7.4, and for rAOP_1, and rAOP_2 in 25-mM Tris-HCl buffer pH 7.4, 250 mM NaCl, 0.25 mM TCEP. The content of AO in all incubations was maintained at 2 pmol, and the total protein content was adjusted to be 174 µg using HSA. All incubations were carried out in triplicate in a water bath shaker at 37° C with incubation volumes of 100 µL each. The final concentration of DMSO in all the incubations was 0.5% v/v which has been reported not to affect AO activity (Behera et al., 2014). All reactions were initiated by addition of the substrate and terminated at the end of 5 min by adding 200 µL of acetonitrile containing diclofenac as internal standard (33.3 nM, final concentration). Samples
were centrifuged at 10,000 g for 5 minutes (4°C) and the supernatants were collected and transferred to an LC vial for analysis. Calibration standards were prepared by adding stock solutions (1-2000 µM in 25% DMSO) of 4-oxo carbazeran in buffer to give final concentrations of 1-2000 nM and were processed in the same manner as the incubation samples. In case of ripasudil, the samples were quenched and processed as described above and prior to analysis, the supernatants were diluted 4-fold using water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid (95:5 v/v). The formation of the hydroxy metabolite of ripasudil was monitored and the peak area ratio of metabolite to internal standard was determined.

**AO stability in presence and absence of substrate**

We investigated the loss of AO activity in the presence and absence of carbazeran as a substrate as described below:

i) Stability in presence of substrate: In the first experiment 4-oxo carbazeran formation was monitored in HLC and rAOP2 following the addition of carbazeran at different time points (5, 15, 30, and 60 min) using the assay protocol described above.

ii) Stability in absence of substrate: In the second experiment, HLC was preincubated without adding the substrate (carbazeran) at 37 °C for 0, 0.5, 1, 3, and 6 h. Carbazeran was added at the end of each of the preincubation time and the formation of 4-oxo carbazeran was monitored for 5 min using the assay protocol described above.

The data for the formation rates of 4-oxo carbazeran from both these experiments were analyzed using one phase decay model using GraphPad prism version 8.4.3 (La Jolla, CA).

**Enzyme kinetics of AO mediated 4-oxo carbazeran formation**

The incubation time of 5 min was selected to avoid significant decay in protein activity, whereas the AO content of 2 pmol was used for the reactions based off the literature (Xie et al., 2019).
The incubation time of 5 min was found to be within the linear range for metabolite formation. Therefore, HLC and rAO (2 pmol) were incubated with a range of concentrations of carbazeran (0.125-128 µM) at 37° C for 5 min. The reaction was stopped, and the samples were processed using the carbazeran formation activity protocol described above. The processed samples were transferred to LC-MS vials for analysis.

**Determination of protein normalized activity factor (pnAF)**

The reported $K_m$ of carbazeran for AO mediated metabolism is 5 µM (Xie et al., 2019). Therefore, the formation rate of 4-oxo carbazeran was evaluated at 1, 5 and 25 µM (5-fold lower to 5-fold higher than $K_m$) of the substrate in rAO, HLC, and suspended hepatocytes. Similarly, ripasudil was incubated at 25 µM (~5 times reported $K_m$) (Isobe et al., 2016) with rAO and HLC. Hepatocytes in suspension (0.1 million cells) were incubated with 25 µM carbazeran in a total incubation volume of 300 µL. The incubation was carried out in a 5% CO2 incubator at 37° C. The incubation time for hepatocyte assay was 10 min as compared to HLC (5 min) to allow equilibration of the substrate in the cell system so that all metabolic pathways of carbazeran can be captured. Samples were quenched with acetonitrile containing IS (diclofenac) and processed as described earlier, and the processed samples were transferred to LC-MS vials for analysis.

**LC-MS/MS analysis of 4-oxo carbazeran and hydroxy metabolite of ripasudil**

The samples were analyzed using an LC-MS/MS system consisting of microflow LC and Xevo-TQ-XS MS systems (Waters, Milford, MA). Acquity UPLC HSS T3 C18 column (1.8 µm, 1 mm x 100 x100 mm) equipped with a guard column (Vanguard pre column, 1.8 µm, 2.1 x 5 mm). 50 µL/min flow rate of mobile phase A (water containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid) was run using the following gradient programs: i) carbazeran: 0-1 min (5% B), 1-3.5 min (5-65% B) 3.5-4 min (65% B), 4-9 min (65-95% B), 9-9.5 min (95-5% B), 9.5-12 min (5% B), and ii) ripasudil: 0-0.5 min (5% B), 0.5-1.5 min (5-20% B), 1.5-4.5 min (20-
60% B), 4.5-9 min (60-95% B), 9-9.5 min (95-5% B), and 9.5-12 min (5% B). The mass spectrometer was operated in multiple reaction monitoring (MRM) and positive ionization (ESI+) mode with cone voltage (CV) of 27 V. The MRM transitions were: 4-oxo carbazeran (m/z 377.3 → 288.1; collision energy (CE), 25 eV), hydroxy metabolite of ripasudil (m/z 340.11 → 99.0; CE, 20 eV), and diclofenac (m/z 296.0 → 214.0; CE, 25 eV).
Data analysis

The relative expression factor (REF) for the rAO was calculated using Eq 1.

\[
REF = \frac{AO \text{ content in HLC or hepatocyte homogenate (per mg protein)}}{AO \text{ content in recombinant systems (per mg protein)}}
\]  
(Eq 1)

The kinetic parameters for 4-oxo carbazeran formation, \(K_m\) and \(V_{\text{max}}\) (pmol/min/pmol AO) were determined by fitting the Michaelis Menten equation (Eq. 3) to the data using GraphPad Prism (ver. 8.4.3) (La Jolla, CA)

\[
Y = \frac{V_{\text{max}}xS}{K_m+S}
\]  
(Eq 2)

Where, \(V_{\text{max}}\) was calculated as:

\[
V_{\text{max}} \text{ (pmol/min/mg protein)} = V_{\text{max}} \text{ (pmol/min/pmol AO)} \times AO \text{ expression (pmol/mg protein)}
\]  
(Eq 3)

\(p\text{nAF}\) for the rAO preparations was calculated with respect to HLC and hepatocytes as:

\[
p\text{nAF} = \frac{AO \text{ activity in HLC or hepatocytes (per pmol AO)}}{AO \text{ activity in recombinant systems (per pmol AO)}}
\]  
(Eq 4)

The intrinsic clearance (\(CL_{\text{int}}\)) across all systems was calculated using Eq 5

\[
CL_{\text{int}} = \frac{V_{\text{max}}}{K_m}
\]  
(Eq 5)

The \(CL_{\text{int}}\) values obtained from Eq (5) were then integrated with their respective REF and \(p\text{nAF}\) values obtained from Eqs 1 and 4.

\[
CL_{\text{int,HLC}} = CL_{\text{int,rAO}} \times REF \times p\text{nAF}
\]  
(Eq 6)

Metabolite identification of carbazeran in human hepatocytes
Carbazeran is considered to be a highly selective AO substrate, but for more accurate IVIVE analysis, a determination of $f_{m,AO}$ value was advisable. A comprehensive untargeted metabolite identification study was performed to identify any non-AO mediated metabolic pathways in human hepatocytes ($n=8$). The two sets of samples analyzed include control (hepatocytes without carbazeran) and treated (hepatocytes incubated with carbazeran). The samples were analyzed by a nano-flow LC coupled to high resolution mass spectrometer (HRMS) (Thermo Q-Exactive-HF). The mass spectrometer was equipped with a standard easy spray ion source and reverse phase column (0.075 x 250 mm). LC conditions were set at 300 nL/min flow rate and 1 µL injection volume using mobile phase A: 0.1% formic acid in water, and B: 0.1% formic acid in 80% acetonitrile. The LC and source parameters are provided (Supp. Tables 4 and 5, respectively) and MS parameters are given (Supp. Table 6). XCMS online platform (https://xcmsonline.scripps.edu) was used to process the MS raw data. The following screening criteria were employed to filter potential metabolites: i) hepatocytes incubated with carbazeran with >10-fold response compared with the control ii) a mass defect filter (milli Dalton range from -50 to 50), iii) a retention time window of 15–60 minutes (considering carbazeran retention time was observed at about 34 minutes), and iv) an MS intensity of $>10^6$ in the carbazeran treated hepatocyte samples and MS intensity of $<10^6$ in the control samples.

**PBPK model development of carbazeran**

A PBPK model of carbazeran was developed using Simcyp software (version 21, Certara, NY), to estimate unaccounted clearance by *in vitro* scaling. The system-dependent parameters (e.g., organ weight, body composition, and blood flow rates) were already incorporated into the software, whereas drug-dependent parameters were added from literature (Supp. Table 8). The simulated trial designs and virtual population were selected to match the reported clinical data (Kaye et al., 1984). The carbazeran intravenous (IV) PBPK
Disposition model was developed and validated using the published clinical data. A minimal PBPK model was built using the \textit{in vitro} $\text{CL}_{\text{int}}$ obtained from the HLC experiment in this study and corrected using the unbound fraction obtained from literature (de Sousa Mendes et al., 2020).

Initially, a top-down fitting of the PBPK model was done using the reported systemic CL of 157.9 mL/min/kg and $V_{ss}$ value of 0.8 L/kg. By keeping the value of systemic CL constant, $V_{ss}$ values were optimized by visual inspection of simulated plasma concentration-time curve fitting with the literature reported pharmacokinetic (PK) profile. This generated an optimized value of 0.4 L/kg. The $CL_{\text{int}}$ obtained from HLC data was then scaled using well stirred model to get the hepatic clearance.

Optimized additional CL versus adjusted additional CL: The scaled CL (from HLC) was around 66\% lower (53.4 L/h) than the reported CL, even after considering the renal and UGT-mediated CL, suggesting an unaccounted additional CL. A local sensitivity analysis was carried out using a range of 60-120 L/h to estimate the optimized value of the unaccounted additional CL. The effects of the additional systemic CL and $V_{ss}$, on carbazeran PK profile were evaluated using a range of values with multiple virtual trials. For each rAO system, the additional CL values were further adjusted by dividing their respective $CL_{\text{int}}$ with that of HLC (Table 3) and termed as “adjusted additional CL”. The \textit{in vitro} $CL_{\text{int}}$ values from HLC and the rAO systems were then integrated with the optimized value for additional CL and $V_{ss}$.

Contribution of AO mediated metabolism towards total hepatic CL ($CL_H$) for four AO substrates (O-benzylguanine, BIBX1382, zaleplon and zoniporide) was estimated using the well-stirred model, by scaling the \textit{in vitro} $CL_{\text{int}}$ data (de Sousa Mendes et al., 2020). To estimate total AO mediated $CL_H$ for these four substrates, a ratio of the optimized additional CL to the $CL_{AO,\text{hepatic}}$ of carbazeran was considered as shown in Supp. Table 9. Finally, the
reported fraction metabolized by AO ($f_{mAO}$) values were used to estimate the total \textit{in vivo} CL, which was compared with the reported data (de Sousa Mendes et al., 2020).
RESULTS

Aldehyde oxidase (AO) content in recombinant systems, human liver cytosol, and hepatocytes

The contents of AO in recombinant systems, HLC, and hepatocytes were quantified using a calibration curve constructed using rAOP2 ranging from 1.95 to 125 fmol/µL (Fig. 1a). The mean content of AO (Fig. 1b) in rAOC (38.5 pmol/mg) and rAOX (18.4 pmol/mg), were within ~2-fold, whereas rAOW was ~5 fold higher (146.1 ± 24.98 pmol/mg) as compared to HLC (30.9 pmol/mg protein). The contents of AO were highest in rAO P1 (4368 ± 1078 pmol/mg) and rAOP2 (3143 ± 438 pmol/mg). The content of AO in hepatocytes (n=8) ranged from 4.3-7.6 pmol/mg (mean ± SD; 6.07 ± 1.67 pmol/mg hepatocyte homogenate).

Based on AO content, the REF values (Fig. 1c) for rAOC and rAOX were 0.2-1.6 with respect to HLC and hepatocytes, whereas, as expected, the REF values for rAOW, rAOP1 and rAOP2 ranged between 0.001-0.05.

Stability of AO in the presence an absence of carbazeran

The rates of formation of 4-oxo carbazeran in rAOP2 and HLC in the presence of carbazeran declined over time in a similar manner (Figs. 2a and 2b), as indicated by the values of the rate constant (k), a constant relating the rate of the reaction to the substrate concentrations (Table 2), and the residual plots (Supp. Fig. 1). The rate of 4-oxo carbazeran formation was highest at 5 min, hence all subsequent activity assays were carried out using a 5 min incubation time.

The initial value of CLint at 0 min was estimated using the equation of one phase decay model, i.e., \[Y = (Y_0 - \text{Plateau}) \cdot \exp(-K\cdotX) + \text{Plateau}\], which performed better than constraining the plateau to 0. The stability of AO in HLC in the absence of carbazeran (Fig. 2c, Table 2) showed a 10-fold higher decay (k value 0.4 vs 0.047).
Enzyme kinetic parameters for 4-oxo carbazeran formation

A Michaelis Menten plot was fitted to the formation rate of 4-oxo carbazeran in rAO<sub>p2</sub> and HLC (Fig. 3a and 3b). The K<sub>m</sub> value of 4-oxo carbazeran formation in rAO<sub>p2</sub> (15.26 ± 5.9 µM) was two-fold higher as compared to HLC (7.76 ± 2.8 µM), however, the V<sub>max</sub> and the resultant CL<sub>int</sub> values were not statistically different (V<sub>max</sub> = 25.41 ± 2.8 versus 22.4 ± 2 and CL<sub>int</sub> = 1.67 versus 2.88 mL/min/pmol AO, respectively).

Determination of protein normalized activity factor (pnAF) and clearance calculations

The AO content normalized activity in HLC using carbazeran was ~6 fold higher as compared to that in rAO<sub>c</sub> and rAO<sub>x</sub>, at 1, 5 and 25 µM and around ~4-fold higher compared to the rAO<sub>w</sub>, whereas the activities in the rAO<sub>p1</sub> and rAO<sub>p2</sub> were within 2-fold of HLC values (Fig. 4a) with rAO<sub>p2</sub> showing the highest activity comparable to that seen in HLC. The pnAF values for the rAO ranged from 1-6.57 (Fig. 4b). The pnAF values were substrate-independent as both carbazeran and ripasudil showed similar values (Fig. 4c).

Table 3 shows the scaling of in vitro CL<sub>int</sub> of carbazeran to CL<sub>int</sub> from HLC and rAO. Integration of respective REF and pnAF values, resulted in improved scaling with CL<sub>int</sub> values comparable to that obtained in HLC.

Metabolite identification studies with carbazeran

Differences in metabolomic profiles in hepatocytes with and without carbazeran were evaluated using XCMS software. The output Excel file was first processed to shortlist the potential metabolites using the criteria described above. The shortlisted features were compared against the theoretical metabolite list of carbazeran to identify drug metabolites. The comparison confirmed the presence of 4-oxo carbazeran (m/z 377), carbazeran glucuronide (m/z 537), O-desmethyl carbazeran (m/z 347), N-desethyl carbazeran (m/z 333), decarbamylated carbazeran (m/z 290), and decarbamylated 4-oxo carbazeran (m/z 306). These metabolites were further
confirmed by their unique MS$^2$ fragmentation patterns (Supp. Table 7). Based on the MS intensity, 4-oxo carbazeran was found to be the major metabolite (~84%). The direct glucuronide of carbazeran was detected to be around 12%, demethylated carbazeran was 4%, while the other three metabolites were less than 1% of the sum of all metabolites.

**Estimated additional CL of carbazeran and its translation to other AO substrates**

The estimated values for additional CL and $V_{ss}$ using sensitivity analysis were 91.6 L/h and 0.42 L, respectively (Supp. Fig 2a). The observed plasma concentration versus time profiles fell within 95% confidence intervals of the predicted profiles obtained from the *in vitro* $\text{CL}_{\text{int}}$ in HLC (Supp. Fig 2b) and rAO systems (Supp. Fig 3) integrated with the optimized additional CL. However, when the *in vitro* $\text{CL}_{\text{int}}$ from rAO systems were integrated with their respective adjusted additional CL, the observed concentration time profiles did not fall within the 95% confidence intervals of the predicted profiles in case of rAO$_C$, rAO$_X$, and rAO$_W$ (Supp. Fig 4). The plots in Supp. Fig. 4 represent the simulated profiles for scaled $\text{CL}_{\text{int}}$ from rAO systems without integration of pnAF, while those in Supp. Fig. 3 represent the simulated profiles with correction of pnAF. A linear correlation was obtained between the AUC fold ratio (predicted AUC in rAO/AUC *in vivo*) and the pnAF values (Fig. 5).

Using the optimized value of additional CL from the carbazeran model, IVIVE predictions for the four AO substrates (O-benzyl guanine, BIBX1382, zaleplon and zoniporide) as illustrated in Supp Table 9, showed an improvement from several fold underprediction (Fig. 6a) to within 2-fold (Fig. 6b).
DISCUSSION

In vivo CL of compounds cleared by the CYP family of enzymes is reasonably well predicted using both human liver microsomes (HLM) and hepatocytes. However, IVIVE for AO mediated metabolism using HLC, liver S9 fractions, and hepatocytes shows an underprediction of in vivo clearances (Table 4). For example, an underprediction of 13 to 15-fold for IVIVE of AO substrates was reported (Akabane et al., 2012; Zientek et al., 2010) and a scaling factor of 6.5 was recommended to improve predictions within 2-fold (de Sousa Mendes et al., 2020). The reasons postulated for the underprediction include instability of AO enzyme, nonlinearity of AO mediated reactions in vitro and contribution of extrahepatic AO towards clearance. Moreover, rAO systems have not been utilized for the IVIVE of drug metabolism. Given this background, we aimed at better understanding the reasons for the observed underprediction of AO mediated drug clearance from HLC and hepatocyte data and explore the utility of rAO systems for the IVIVE of AO metabolism. We observed five important reasons that should be considered in the IVIVE of AO metabolism, which include, i) differential AO content between rAO systems and HLC, ii) compromised stability of in vitro reagents, iii) faster decay of AO activity during incubation, iv) role of additional AO-mediated (extrahepatic) clearance, and v) substrate-dependent unaccounted metabolic pathways (fm,AO < 1).

rAO from different sources have been isolated and evaluated for determining in vitro intrinsic clearance and fraction metabolized (fm) of an NCE, though most of these preparations have shown to exhibit lower activity than HLC (Barr et al., 2013). Recently, by comparison with known AO substrates, an approximation of NCE turnover using rAO in a plate-based MS assay has been described (Cronin et al., 2021).

In the present study, up to 100-fold difference was seen in AO content between HLC and rAO systems (i.e, REF). Carbazeran is extensively metabolized by AO to 4-oxo carbazeran and this oxidation has been reported to be an AO selective catalytic marker (Xie et al., 2011), and hence
was used as a probe for the activity assays. The metabolite identification study of carbazeran also confirmed that 4-oxo carbazeran formation was the major metabolic pathway, consistent with reported literature (Kaye et al., 1984). The activity assays carried out using the normalized content of AO across rAO from different sources and HLC revealed that the AO protein content differences alone did not account for the observed activity differences. Since AO activity involves protein-protein (MoCo sulfurase) and protein-cofactor interactions (MoCo, Fe, and FAD), the disconnect between the activity and content across rAO systems and HLC could partly be due to variability in these constituents of the reaction affecting the overall stability of the complex. We propose that protein abundance-normalized activity factor (pnAF) should be used for comparing protein activity of a recombinant human enzyme system (e.g., rAO) versus human tissue derived reagents (e.g., HLC). Therefore, both REF and pnAF should be included in scaling AO mediated CL<sub>int</sub> from rAO systems. Collectively, these two factors for non CYP enzymes are sometimes referred to as inter-system extrapolation factor (ISEF). However, ISEF cannot tease out if the difference in overall activity is due to the differences in expression or intrinsic activity (per pmol). Our results show that the pnAF values for two AO substrates, carbazeran and ripasudil, were similar. This substrate-independence suggests that while evaluating AO mediated metabolism for an NCE, laboratories can establish pnAF for AO probe substrates which then can be applied to scaling in vitro clearances of any other AO substrate. This method is particularly important in estimating fractional contribution of AO towards metabolism of NCEs (f<sub>m,AO</sub>) using recombinant systems and for delineating f<sub>m,CYPs</sub> versus f<sub>m,AO</sub>, as shown for other enzymes, (Parvez et al., 2021), which is otherwise not possible using conventional assays with HLC, HLM, or hepatocytes.

The loss of AO activity during the processes of tissue isolation, homogenization and storage has been frequently discussed as one of the probable reasons for poor IVIVE predictions (Kozminsiki et al., 2021; Zientek et al., 2010). Our study shows for the first time that AO enzyme degrades
~10 times faster in the presence of the substrate (carbazeran) which could be possibly triggered by certain catalytic events and an inability of the enzyme to regenerate its active state, as suggested elsewhere (Abbasi et al., 2019). During each catalytic cycle, the molybdenum in MoCo is reoxidized to regenerate fully oxidized enzyme by electron transfer, producing hydrogen peroxide and superoxide anions (Beedham, 2020). These anions are hypothesized to cause loss of AO activity (Abbasi et al., 2019). Degradation of AO activity may be substrate dependent, with slow turnover substrates showing less degradation of AO activity. The in vivo activity of AO would likely depend on the dynamic changes in substrate concentration over time and degradation of activity. These estimates are unknown, but it can be presumed that regeneration of activity is more efficient than in in vitro systems. Nevertheless, we recommend carrying out time course experiments for each substrate of interest and back calculate the activity to obtain the initial rate of reaction at 0 min. Thus, the conventional substrate depletion approach to scale in vitro intrinsic clearances for IVIVE, using longer incubation time, for example >10 min for carbazeran (Zientek et al., 2010, Hutzler et al., 2014, de Sousa Mendes et al., 2020) may not be ideal for AO substrates. A modified activity model which accounts for the loss of activity with time, is also recommended by others for estimating CLint for AO substrates (Abbasi et al., 2019, Kozminski et al., 2021).

Another plausible reason for underprediction of AO mediated metabolism was the unaccounted additional CL. AO is ubiquitously expressed in various extrahepatic tissues in humans, including the endocrine tissues, adrenal gland, reproductive tissues, adipose tissues, skin, etc. (de Sousa Mendes et al., 2020). Though the content of AO in the extrahepatic tissues may be low, the content may not necessarily correlate with the activity and the combined contribution of these tissues towards CL of an AO substrate may be significant. However, for IVIVE predictions, the scaled in vitro CLint of carbazeran integrating data from kidney, lung, vasculature, and intestinal S9 fractions along with hepatic CLint did not show significant role of extrahepatic tissues in in
In vivo clearance (Kozminski et al., 2021). This discrepancy can be explained by the fact that the stability of AO in S9 fractions may be compromised leading to underprediction of AO mediated metabolism. Indeed, the stability of AO enzymes has been reported to be affected during the processes of isolation and handling (Barr et al., 2013). A substantial decline in AO activity has been observed within 24 h of isolation of hepatocytes (Hutzler et al., 2014). Thus, the altered activity of AO in isolated hepatocytes and liver fractions may be a confounding factor in the extrapolation of in vitro data. This conclusion was also supported by another study done in PXB mice, which showed that the CL_int for 4 AO substrates scaled from hepatocytes isolated from chimeric mice showed an underprediction of CL while a good correlation was observed between human CL_int,in vivo and that of PXB mice in vivo (Sanoh et al., 2012). These results suggest that loss of AO activity during isolation has an impact on the prediction of in vivo clearance. In a more recent report, in vivo assessments and single species scaling for AO specific substrates were done in chimeric PXB mice, non-human primate and rat to predict steady state clearance in humans (Miyamoto et al., 2020). The highest correlation was seen with PXB mice (r^2, 0.84), followed by NHP (r^2, 0.7) and rat (r^2, 0.4). This report indicates that the role of extrahepatic AO in the metabolic clearance is limited and the compromised activity of AO in in vitro systems is a likely reason for the underprediction.

The underprediction of in vivo CL could be related to substrate-dependent factors. For example, in the clinical study of carbazeran, two aglycones were detected in the urine samples, following treatment with glucuronidase enzyme (Kaye et al., 1984). The structures of these aglycones were elucidated based on the m/z values and were found to be hydrolysis products of 1) a direct glucuronide of carbazeran, and 2) glucuronide derived from the 4-oxo metabolite. This suggests that a direct glucuronidation is a competing metabolic pathway for carbazeran, however the mass balance study suggests that direct glucuronidation plays a minor role in vivo (~5% of dose). In contrast, in a study by Sharma et al., 2012, carbazeran glucuronide was detected as a
major metabolite (~2 fold more intense UV absorbance than the 4-oxo metabolite) in human hepatocytes. Our investigations in understanding the contribution of non-AO mediated hepatic clearance of carbazeran indicated around 12% (based on MS intensity) contribution of glucuronidation pathway towards its elimination in the liver, which was added to further refine the PBPK model for carbazeran. Therefore, compound specific factors, especially $f_{mAO}$ should be estimated for accurate IVIVE. Indeed, incorporation of additional CL from the present study and consideration of $f_m$ value successfully predicted (within 2-fold) the in vivo CL of O-benzyl guanine, BIBX1382, zaleplon and zoniporide.

In conclusion, we recommend integrating differences in AO content (REF) and activity (pnAF) for extrapolating CL$_{int}$ data from rAO systems to HLC. We demonstrate that accounting for the loss of AO activity and the incorporation of additional clearance would result in improved predictions of IVIVE for AO substrates. PBPK modeling of AO substrates should consider these factors for improved PK predictions.
Supplementary information

Supplementary data are available.

Chromatographic conditions and instrument parameters for quantitation of AO peptides, metabolite identification study, PBPK input parameters for carbazeran, and IVIVE calculations for AO mediated substrates using additional clearance. Residual plots for stability of AO in HLC in the presence and absence of substrate and predicted versus observed concentration time profiles for carbazeran are also provided.
ACKNOWLEDGMENTS

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Data Availability statement:

The authors declare that all the data supporting the findings of this study are contained within the paper.
AUTHOR CONTRIBUTIONS


Conducted experiments: S.S, D.S.A, and D.K.S

Contributed reagents: S.H and C.N.C

Performed data analysis: S.S and B.P

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Cronin CN, Liu JH, Grable N, Strelevitz TJ, Obach RS, and Carlo A (2021) Production of active recombinant human aldehyde oxidase (AOX) in the baculovirus expression vector system (BEVS) and deployment in a pre-clinical fraction-of-control AOX compound exposure assay. Protein Expr Purif 177,105749.


FOOTNOTE

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Conflicts of Interest: BP is cofounder of Precision Quantomics Inc. and recipient of research funding from Bristol Myers Squibb, Genentech, Gilead, Merck, Novartis, Takeda, and Generation Bio. All other authors declared no competing interests for this work.
LEGENDS FOR FIGURES

**Fig. 1:** Calibration curve generated using purified recombinant human AO protein (rAOP2; a), Protein content of AO in HLC (pool of n = 50 donors), hepatocyte homogenate (n=8), and recombinant systems from four sources (b), and relative expression factor (REF) across different recombinant systems (c). The recombinant human systems are the cytosolic extracts of AO-overexpressing HEK293 cells (rAO_C) and E coli (rAO_X). The purified AO proteins were those purified from ecoAO paste (rAO_W) and baculovirus expression vector systems; rAOP_1 and rAOP_2. The data are presented as mean and SD of triplicate analysis, ****p< 0.0001, one- way ANOVA.

**Fig. 2:** Time-dependent formation of 4-oxo carbazeran in purified rAOP2 (a) and HLC (b) Stability of HLC in the absence of carbazeran (c).

**Fig. 3:** Michaelis-Menten plot of 4-oxo carbazeran formation kinetics in rAOP2 (a) and HLC (b).

**Fig. 4:** AO activity (4-oxo carbazeran formation rate) in HLC and the recombinant human systems using three carbazeran concentrations (1, 5, and 25 µM). The data are presented as mean and SD of triplicate analysis. *, **, and *** indicate significant difference with p values < 0.05, <0.01, and ****p< 0.0001, respectively, utilizing, one- way ANOVA (a), Protein normalized activity factor (pnAF) for the recombinant systems with respect to HLC (1, 5, and 25 µM substrate concentration) and hepatocyte homogenate (25 µM substrate concentration) (b), and Comparison of protein normalized activity factor (pnAF) values for carbazeran and ripasudil in three different recombinant systems, i.e., rAO_W, rAOP_1, and rAOP_2 (c).

**Fig. 5:** Correlation of ratio of predicted AUC in rAO versus in vivo AUC and pnAF values.

**Fig. 6:** Predicted CL_{iv} compared with mean observed CL_{iv} before (a) and after scaling with additional clearance from carbazeran PBPK model (b). Dotted lines indicate 2- fold bias across the line of unity.
Table 1: *In vitro* reagents used for investigating AO content and activity

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Abbreviation</th>
<th>Reagent type</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant AO-overexpressing HEK293</td>
<td>rAOc</td>
<td>Cytosol</td>
<td>Corning (Tewksbusy, MA)</td>
</tr>
<tr>
<td>Recombinant AO-overexpressing E coli</td>
<td>rAOx</td>
<td>Cytosol</td>
<td>Xenotech, (Kansas, MO)</td>
</tr>
<tr>
<td>Recombinant AO-overexpressing E coli</td>
<td>rAOw</td>
<td>Cell paste</td>
<td>Dr. Jeff Jones lab, WSU (Pullman, WA)</td>
</tr>
<tr>
<td>Purified recombinant AO (preparation 1)</td>
<td>rAOp₁</td>
<td>Purified protein</td>
<td>Pfizer (San Diego, CA)</td>
</tr>
<tr>
<td>Purified recombinant AO (preparation 2)</td>
<td>rAOp₂</td>
<td>Purified protein</td>
<td>Pfizer (San Diego, CA)</td>
</tr>
<tr>
<td>Pooled human liver cytosol, mixed gender (n=50)</td>
<td>HLC</td>
<td>Cytosol</td>
<td>Xenotech (Kansas, MO)</td>
</tr>
<tr>
<td>Cryopreserved adult hepatocytes, mixed gender (n=8)</td>
<td>HH</td>
<td>Cell</td>
<td>BioIVT (Baltimore, MD)</td>
</tr>
</tbody>
</table>
Table 2: Parameters (mean and 95% CI in parenthesis) for curve fitting of time course of 4-oxo carbazeran formation in rAO$_{P2}$ and in HLC in the presence and absence of carbazeran

<table>
<thead>
<tr>
<th>Parameter</th>
<th>rAO$_{P2}$</th>
<th>HLC</th>
<th>HLC in the absence of carbazeran</th>
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</thead>
<tbody>
<tr>
<td>Y0</td>
<td>36.0 (34.5 to 37.7)</td>
<td>32.4 (27.4 to 39.2)</td>
<td>35.36 (30.9 to 40.2)</td>
</tr>
<tr>
<td>Plateau</td>
<td>3.9 (2.3 to 5.3)</td>
<td>7.3 (-3.1 to 11.1)</td>
<td>10.5 (-62.5 to 17.0)</td>
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<tr>
<td>K (min$^{-1}$)</td>
<td>0.047 (0.04 to 0.05)</td>
<td>0.048 (0.02 to 0.09)</td>
<td>0.43 (0.04 to 0.97)</td>
</tr>
<tr>
<td>Tau</td>
<td>21.2 (18.3 to 25)</td>
<td>20.6 (10.9 to 53.2)</td>
<td>2.3 (1.02 to 22.4)</td>
</tr>
</tbody>
</table>
Table 3: Clearance calculations and IVIVE predictions for carbazeran

<table>
<thead>
<tr>
<th></th>
<th>rAO&lt;sub&gt;C&lt;/sub&gt;</th>
<th>rAO&lt;sub&gt;X&lt;/sub&gt;</th>
<th>rAO&lt;sub&gt;W&lt;/sub&gt;</th>
<th>rAO&lt;sub&gt;P1&lt;/sub&gt;</th>
<th>rAO&lt;sub&gt;P2&lt;/sub&gt;</th>
<th>HLC</th>
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<tbody>
<tr>
<td><strong>CL&lt;sub&gt;int&lt;/sub&gt; µL/min/mg protein</strong></td>
<td>32.45</td>
<td>16.82</td>
<td>19.71</td>
<td>46.89</td>
<td>100.8</td>
<td>89.05</td>
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<tr>
<td></td>
<td>39.31</td>
<td>20.38</td>
<td>23.88</td>
<td>56.80</td>
<td>122.11</td>
<td>107.88</td>
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<tr>
<td><strong>CL&lt;sub&gt;int&lt;/sub&gt; µL/min/mg protein scaled to 0 min</strong></td>
<td>32.45</td>
<td>16.82</td>
<td>19.71</td>
<td>46.89</td>
<td>100.8</td>
<td>89.05</td>
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<td></td>
<td>39.31</td>
<td>20.38</td>
<td>23.88</td>
<td>56.80</td>
<td>122.11</td>
<td>107.88</td>
</tr>
<tr>
<td><strong>REF</strong></td>
<td>0.803</td>
<td>1.679</td>
<td>0.211</td>
<td>0.0071</td>
<td>0.0098</td>
<td>1.000</td>
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<tr>
<td><strong>pnAF</strong></td>
<td>2.74</td>
<td>5.29</td>
<td>4.52</td>
<td>1.90</td>
<td>0.88</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>CL&lt;sub&gt;H&lt;/sub&gt; (well-stirred model)</strong></td>
<td>22.34</td>
<td>13.15</td>
<td>15.04</td>
<td>29.07</td>
<td>45.57</td>
<td>42.78</td>
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<tr>
<td><strong>L/h</strong></td>
<td>33.37</td>
<td>17.30</td>
<td>20.27</td>
<td>48.22</td>
<td>103.65</td>
<td></td>
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</table>

Additional CL for simCYP

- 91.58 (optimized value)
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<tr>
<th>Drugs chosen</th>
<th>Matrix used</th>
<th>In vitro</th>
<th>In vivo/PBPK</th>
<th>Observation</th>
<th>Recommendations</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>MTX, XK-469, RS-8359, zaleplon, 6-deoxypenciclovir, zoniporide, O-BG, DACA, carbazeran, PF-421703, PF-945863</td>
<td>Pooled HLC and HLS9</td>
<td>CL\textsubscript{int} calculated using K\textsubscript{el} and scaled using well stirred model corrected with plasma f\textsubscript{u} and B/P ratios.</td>
<td>Free intrinsic clearance (CL\textsubscript{int,AO}) back calculated from total CL from oral and i.v. data.</td>
<td>Underprediction; 13-fold using (5-32) using HLC and 15-fold (3-52) using HLS9.</td>
<td>Yardstick approach, rank ordering.</td>
<td>Zeintek et.al., 2010 (DMD 38: 1332-1327)</td>
</tr>
<tr>
<td>O-BG, BIBX1382, Carbazeran, Zaleplon, Ziparasidone, Zoniporide (repted i.v CL and f\textsubscript{mAO} &gt; 5%)</td>
<td>Pooled HLC and HLS9</td>
<td>CL\textsubscript{int} calculated using K\textsubscript{el} from parent depletion profiles, corrected by free fractions.</td>
<td>PBPK models developed using in vitro metabolism data, fu\textsubscript{p}, B/P, and fu\textsubscript{mic}. Contribution of microsomal metabolism, renal and biliary</td>
<td>1. Comparison of predicted Cl\textsubscript{iv} and observed Cl\textsubscript{iv}. Underprediction of 3.8 using HLC, 5.8 using HLS9, improvement in prediction by including extra</td>
<td>Every lab develop its own scaling factor using a set of probe substrates using own assay conditions. If CL\textsubscript{int,u} not available, use a factor of 4.6 for HLC.</td>
<td>Mendes et.al. (DMD 48:1231-1238)</td>
</tr>
<tr>
<td>Compound</td>
<td>Type</td>
<td>CL&lt;sub&gt;int&lt;/sub&gt; Calculated</td>
<td>In vivo CL&lt;sub&gt;int&lt;/sub&gt; Calculated</td>
<td>Under Estimation</td>
<td>Use Empirical Scaling Factor as Geometric Average Ratio</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------------</td>
<td>-------------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>FK-3453, O-BG,</td>
<td>Individual and pooled hepatocytes</td>
<td>CL&lt;sub&gt;int&lt;/sub&gt; calculated using K&lt;sub&gt;el&lt;/sub&gt;, scaled using well-stirred model corrected by f&lt;sub&gt;uhepa&lt;/sub&gt;.</td>
<td>In vivo CL&lt;sub&gt;int&lt;/sub&gt; calculated from total CL using dispersion model.</td>
<td>Under estimation of 10-fold (7.2-14.9-fold), similar to Zeintek et al.</td>
<td>Use empirical scaling factor as geometric average ratio of CL&lt;sub&gt;int, in vivo&lt;/sub&gt;/CL&lt;sub&gt;int&lt;/sub&gt;, in vitro from several reference drugs.</td>
<td></td>
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<td>Zaleplon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Akabane et al., 2012 Xenobiotica 42(9):863-871</td>
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<tr>
<td>Compound</td>
<td>Cell Type</td>
<td>CL\textsubscript{int}</td>
<td>Clearance</td>
<td>Correlation</td>
<td>Characterization</td>
<td></td>
</tr>
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<td>-------------</td>
<td>-------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td>BIBX1382, carbazeran, O-BG, zaleplon, XK-469</td>
<td>Pooled mixed gender cryopreserved human hepatocytes</td>
<td>CL\textsubscript{int} calculated using K\textsubscript{el}, scaled using well stirred model.</td>
<td>Total clearance from literature reported data.</td>
<td>Reasonably good correlation between <em>in vitro</em> predicted and observed <em>in vivo</em> clearances.</td>
<td>Characterize all lots of vendor hepatocytes with control AO substrates.</td>
<td>Hutzler et al., 2012 (DMD 40:267-275)</td>
</tr>
</tbody>
</table>
Fig. 1

(a) Peptide area ratio (light/heavy) vs Absolute AO concentration (fmol/μL) with a correlation coefficient $R^2 = 0.9962$

(b) AO content (fmol/mg protein) for different samples

(c) REF concentrations for different peptides

Legend:
- HLC
- Hepatocyte
Fig. 2

(a) 4-Oxo carbazan formation rate (pmol/min/pmol AD) vs. time (min).

(b) 4-Oxo carbazan formation rate (pmol/min/pmol AD) vs. time (min).

(c) 4-Oxo carbazan formation rate (pmol/min/pmol AD) vs. time (h).
Fig. 3

**Diagram a:**
- Y-axis: 4-Oxo carbazepine formation rate (pmol/min/pmol AO)
- X-axis: Carbazepine concentration (µM)

**Diagram b:**
- Y-axis: 4-Oxo carbazepine formation rate (pmol/min/pmol AO)
- X-axis: Carbazepine concentration (µM)
Fig. 4
Fig. 5

A linear regression analysis of the relationship between AUC fold ratio and pNAF shows a high R² value of 0.892. The data points are plotted on a linear scale with AUC fold ratio on the x-axis and pNAF on the y-axis.
Fig. 6

(a) Predicted $\text{Cl}_v$ (L/h) vs. Observed $\text{Cl}_v$ (L/h) for various compounds.

(b) Predicted $\text{Cl}_v$ (L/h) vs. Observed $\text{Cl}_v$ (L/h) with additional details:
- Diamond: Zaleplon
- Circle: O6-Benzyl guanine
- Triangle: Zoniperide
- Square: BIBX1382
- Solid line: Unity
- Dotted line: 2-fold increase

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