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Title page

Challenges and opportunities for in vitro-in vivo extrapolation of aldehyde oxidase-mediated clearance: Towards a roadmap for quantitative translation

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Running title page

Running title: IVIVE of aldehyde oxidase-mediated clearance

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Abbreviations

AO: aldehyde oxidase, AUC: area under the curve, B/P: blood-to-plasma ratio, CL: clearance, $CL_{int,\textit{in vitro}}$: *in vitro* intrinsic clearance, $CL_{int,u,}$: unbound intrinsic clearance, $CL_{int,u,AO}$: unbound intrinsic clearance by aldehyde oxidase, CPPGL: cytosolic protein per gram liver, ESF: empirical scaling factor, fm_{AO}: fraction metabolized by aldehyde oxidase, fu_{cyt}: unbound fraction in incubations of human liver cytosols, fu_{hep}: unbound fraction in incubations of human hepatocytes, fu_{inc}: unbound fraction in incubation medium, fu_{mic}: unbound fraction in incubations of human liver microsomes, fu_{p}: unbound fraction in human plasma, fu_{S9}: unbound fraction in incubations of human liver S9, gmfe: geometric mean fold error, HPGL: hepatocytes per gram liver, IVIVE: *in vitro-in vivo* extrapolation, L-O-O: leave-one-out, rmse: root mean squared error, PBPK: physiologically-based pharmacokinetic, S9PPGL: S9 protein per gram liver

Abstract

Underestimation of AO-mediated clearance by current *in vitro* assays leads to uncertainty in human dose projections, thereby reducing the likelihood of success in drug development. In the present study we first evaluated the current drug development practices for AO substrates. Next, the overall predictive performance of *in vitro-in vivo* extrapolation (IVIVE) of unbound hepatic intrinsic clearance ($CL_{int,u}$) and unbound hepatic intrinsic clearance by AO ($CL_{int,u,AO}$) was assessed using a comprehensive literature database of *in vitro* (human cytosol/ S9/ hepatocytes) and *in vivo* (iv/oral) data collated for 22 AO substrates (total of 100 datapoints from multiple studies). Correction for unbound fraction in the incubation (fu_{inc}) was done by experimental data or *in silico* predictions. The fraction metabolized by AO (fm_{AO}) determined via *in vitro/in vivo* approaches was found to be highly variable. The geometric mean fold errors (gmfe) for scaled $CL_{int,u}$ (mL/min/kg) were 10.4 for human hepatocytes, 5.6 for human liver cytosols, and 5.0 for human liver S9, respectively. Application of these gmfe's as empirical scaling factors improved predictions (45-57% within 2-fold of observed) compared with no correction (11-27% within 2-fold), with the scaling factors qualified by leave-one-out cross-validation. A road map for quantitative translation was then proposed following a critical evaluation on the *in vitro* and clinical methodology to estimate *in vivo* fm_{AO}. In conclusion, the study provides the most robust system-specific empirical scaling factors to-date as a pragmatic approach for the prediction of *in vivo* $CL_{int,u,AO}$ in the early stages of drug development.

Keywords

Aldehyde oxidases, hepatic elimination, in vitro-in vivo prediction (IVIVE)

Significance Statement

Confidence remains low when predicting *in vivo* clearance of aldehyde oxidase (AO) substrates using *in vitro* systems, leading to de-prioritisation of AO substrates from the drug development pipeline to mitigate risk of unexpected and costly *in vivo* impact. The current study establishes a set of empirical scaling factors as a pragmatic tool to improve predictability of *in vivo* AO clearance. Developing clinical pharmacology strategies for AO substrates by utilizing mass balance/clinical DDI data will help build confidence in fm_{AO}.

Introduction

Aldehyde oxidase (AO) is a molybdenum cofactor (MoCo)-containing cytosolic enzyme that catalyzes the oxidation of aldehydes and nitrogen-containing aromatic heterocycles, the reduction of N- and S-oxides, and the hydrolysis of amides (Beedham, 2001). AO has taken a more important role in drug metabolism in part because of efforts to mitigate single cytochrome P450 (CYP) isoform mediated drug metabolism, as well as the predisposition of chemical structures in some therapeutic areas to be AO substrates during the drug discovery process. Overlapping substrate specificity between AO and CYP has led to increasing need of an understanding of AO-mediated drug metabolism and its contribution to drug clearance (Manevski et al., 2019). Initial screening in human liver microsomes and hepatocytes, followed by incubation of compounds in human liver cytosols or S9 with and without an AO inhibitor, is generally used for identification of an AO substrate (Pryde et al., 2010). Despite recent research efforts, confidence in the prediction of *in vivo* AO mediated clearance using *in vitro* systems or preclinical species remains low, with many defining characteristics appearing to be substrate-dependent (Table 1).

Inter-species differences is a key challenge for pharmacokinetic and toxicological translational of AO-mediated metabolism (Sahi et al., 2008). While humans and monkeys have one active isoform of AO (AOX1), mice and rats express four structurally conserved genes of AO (Aox1, Aox3, Aox3l1, and Aox4), yet dogs and cats do not express any AO isoforms (Beedham et al., 1987; Garattini et al., 2009). To overcome these species-differences during preclinical research, humanized mouse models have been developed, which have >80% of native hepatocytes replaced with human donor hepatocytes (Katoh et al., 2004; Katoh et al., 2005). Humanized mice have been shown to replicate human specific metabolite generation by AO and other enzymes (Jensen et al., 2017; Uehara et al., 2020). For six AO substrates, total clearance (CL_{total}) for chimeric mice with humanized livers (PXB mice; PhoenixBioCO; Ltd., Hiroshima, Japan) correlated strongly with human CL_{total} (R^2 of 0.84), outperforming non-human primate (0.7) and rat (0.4). Interestingly, the allometrically scaled CL_{total} was overpredicted using PXB mice, but underpredicted using non-human primate and rat (Miyamoto et al., 2017).

High variability of *in vitro* activity and inhibition measurements for AO has been linked to inconsistencies in *in vitro* matrix preparation, enzyme instability/dimerization, and genetic variability of donors. Concerns about enzyme instability have been linked to inadequate cofactor composition (e.g., MoCo, FAD), peroxide formation in catalytic cycles (Manevski et al., 2019) and inactivation of AO by small sulfhydryl-containing reducing agents (e.g. dithiothreitol) (Esmaeeli et al., 2023). Like many enzymes, appropriate experimental design is needed to capture initial product formation rates for AO-mediated reactions. For AO substrates, deviation from the initial linear phase can happen rapidly leading to underestimation of reaction rates. A modulated activity model was therefore proposed to estimate the early fast rate of the enzyme reaction from extended time-course data. While for some compounds the conventional analysis using the Michaelis-Menten model was sufficient, for other compounds the modulated activity model estimated rate constants of up to 42-fold higher than those obtained from the Michaelis-Menten model (Abbasi et al., 2019) (Table 1). Conversely, such nonlinearity is not evident in the more commonly applied substrate depletion assay format. Thus, great care must be taken in experimental design and data analysis when evaluating AO kinetics.

In vitro to *in vivo* extrapolation (IVIVE) of *in vitro* clearance data using physiological scaling factors generally underpredicts human metabolic clearance (Wood et al., 2017); this trend is evident for AO substrates (Zientek et al., 2010; Toselli et al., 2022). To overcome the limitations of quantitative IVIVE, a yardstick approach has been proposed as a qualitative tool to predict if a new drug candidate has high, medium, and low clearance. The yardstick approach compares a new drug's intrinsic clearance to that of known AO substrates in liver cytosol and S9 fraction (Zientek et al., 2010), with co-incubation with hydralazine as AO inhibitor when using hepatocytes to isolate the AO-specific metabolism (Strelevitz et al., 2012; Toselli et al., 2022). In addition, empirical scaling factors have been explored as a pragmatic approach to bridge the gap between predicted intrinsic clearance from *in vitro* data and *in vivo* values obtained either using static (Akabane et al., 2012a; Hutzler et al., 2016) or physiologically-based pharmacokinetic (PBPK) models (De Sousa Mendes et al., 2020).

The present study aimed to (i) investigate the current industry practices on developing AO substrates, and (ii) perform a comprehensive analysis on the overall predictive performance of IVIVE of unbound

hepatic intrinsic clearance ($CL_{int,u}$) and unbound hepatic intrinsic clearance by AO ($CL_{int,u,AO}$) from *in vitro* studies in human hepatocytes, human liver cytosols and human liver S9 based on physiological scaling to derive and evaluate empirical scaling factors (ESFs). Additionally, to support translational modeling, methods for estimating fraction metabolized by AO (fm_{AO}) were critically evaluated.

Materials and Methods

Survey Analysis

In this survey we aimed to evaluate the extent and coverage of current *in vitro* assays used in drug development for measurement and translational modeling of drug metabolism by AO or mixed AO- and CYP-substrates. A 9-question, self-completion questionnaire was developed, and administered within the AO-focus group of the Centre for Applied Pharmacokinetic Research (https://www.capkr.manchester.ac.uk/), a consortium of academic researchers at The University of Manchester and nine industrial pharmaceutical companies. Participants were from industry and experts in drug metabolism, pharmacokinetics and/or clinical pharmacology; participants were asked to report on their experience developing AO substrates, the *in vitro* techniques they use for measuring rates of AO-mediated metabolism, and translational methods used for predicting *in vivo* clearance and fm_{AO} / $fm_{parallel\ pathways}$. Open-ended questions asked about preferred *in vitro* system(s) for companies based on the relative performance for prediction of *in vivo* AO clearance, and whether AO instability associated with storage of human tissue samples is a concern. In addition, consideration of hepatic or extrahepatic AO abundance/activity, and utilization of PBPK models for AO/mixed AO-CYP substrates across companies were investigated. Survey responses were returned between October 2021 and December 2022. Collected data were anonymized prior to analysis.

Database of *in vitro/in vivo* intrinsic clearance of aldehyde oxidase substrates

Systematic Literature Search: *In vitro* intrinsic clearance ($CL_{int,in\ vitro}$) data measured with substrate depletion or enzyme kinetics assays using cryopreserved human hepatocytes, human liver cytosols and human liver S9 were retrieved from the PubMed electronic database (https://pubmed.ncbi.nlm.nih.gov)) and Clinical Pharmacology and Biopharmaceutics Reviews uploaded on the Drugs@FDA database (https://www.accessdata.fda.gov/scripts/cder/daf/)) between October 2021 and October 2022. PubMed analysis was carried out with search terms “aldehyde oxidase” (AND) “human hepatocytes” (OR) “human liver cytosols” (OR) “human liver S9”. Other related data including fraction unbound in incubation (fu_{inc}), *in vivo* clearance in human, the octanol–water partition coefficient (logP), fraction unbound in plasma (fu_p), blood-to-plasma ratio (B/P), and fraction metabolized by aldehyde oxidase

(fm_{AO}) were obtained from PubMed, Drugs@FDA, and DrugBank database (Wishart et al., 2018). Data were excluded if reported below the quantitation limit due to sensitivity in substrate depletion assay or analytical sensitivity. In cases where predicted hepatic clearance was reported after physiological scaling, the data were included if back-calculation of $CL_{int,in\ vitro}$ (e.g. µL/min/million hepatocytes) from the original study was possible. For fu_p and B/P, means across reported values were used, assuming B/P is not concentration-dependent. No exclusion criteria were applied with respect to assay format or incubation time for fu_p measurements. fm_{AO} from human quantitative excretion data, clinical CYP-mediated drug-drug interaction (DDI) data, *in vitro* inhibition studies, model predictions and literature reports were collated. Compounds with only *in vitro* fm_{AO} values reported below the lower limit of quantification (or equal to zero) were excluded.

Reported $CL_{int,in\ vivo}$ and renal/biliary clearance data were collated from human pharmacokinetic studies after oral or intravenous (iv) dosing. Data were excluded if data from different doses suggested a non-linear dose-AUC relationship. Weighted mean (by number of subjects) of plasma and apparent oral clearance (CL/F) was calculated when more than one clinical study or studies at multiple dose levels were available for a specific compound.

If clinical data for a specific compound were unavailable for healthy subjects, studies in patient groups were included. Only one study has reported abundance of AO differences between tissue from healthy subjects and a diseased population (colorectal cancer liver metastasis patients) (Vasilogianni et al., 2022). Thus, due to lack of sufficient data in the literature, where pharmacokinetics data were reported only for diseased patients, observed $CL_{int,u}$ and $CL_{int,u,AO}$ were back-calculated directly assuming there was no difference in AO activity and abundance between healthy and diseased populations.

Inclusion of in-house data: Unpublished in-house data including $CL_{int,in\ vitro}$ from cryopreserved human hepatocytes, fu_p and B/P for 9 AO substrates (BIBX1382, carbazeran, DACA, idelalisib, O6-benzylguanine, XK469, zaleplon, ziprasidone and zoniporide) were used to enrich the IVIVE dataset (Suppl. Table S1). Methodology of substrate depletion assay using human hepatocytes (BioreclamationIVT Lot number: XCK [Pool of 10; mixed gender], Baltimore, MD, USA) at 0.3 and 1

µM substrate concentrations (Study 1), and at 0.1 µM (Study 2); equilibrium dialysis (HTDialysis™, Gales Ferry, CT) (Banker et al., 2003) assay for plasma binding; and blood partitioning assay (Yu et al., 2005) are described in Supplement 1. The human biological samples (BioreclamationIVT, Baltimore, MD, USA) were sourced ethically, and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol.

IVIVE of unbound aldehyde oxidase intrinsic clearance

Correction of *in vitro* data for nonspecific binding: $CL_{int,in\ vitro}$ data from literature and in-house measurements were corrected for the nonspecific binding in the incubation, as outlined in Suppl. Fig S1. Briefly, where experimental unbound fraction data were available for the compound and *in vitro* system of interest, the unbound fraction in human hepatocytes (fu_{hep}), human liver microsomes (fu_{mic}), human liver cytosols (fu_{cyt}) or human liver S9 (fu_{S9}) incubations were used after correction for specific protein or cell concentrations used for $CL_{int,in\ vitro}$ measurement. These corrections were based on relative protein concentration and cell concentrations used in each study assuming that cell volume to incubation volume ratio was 0.005 at the cell concentration of 10^6 cells/mL (Brown et al., 2007). If experimental fu_{hep} was not available, then it was predicted from experimental fu_{mic} as reported previously (Kilford et al., 2008).

Previous studies on a limited number of compounds (n=3) indicated that non-specific protein binding may differ between human liver cytosols and human liver microsomes at the same protein concentration (Cubitt et al., 2009; Cubitt et al., 2011). Because of insufficient data in the literature, when experimental fu_{cyt} data were not available, fu_{mic} data were used as a surrogate for fu_{cyt} accounting also for the protein concentration used in the *in vitro* studies. To evaluate the impact of this assumption, re-analysis of the data without correction of $CL_{int,in\ vitro}$ in human liver cytosols for non-specific binding (fu_{cyt}=1) was explored as an alternative approach. If no experimental fu_{inc} data were available for a particular compound in any system, fu_{inc} were predicted from logP data (experimental or *in silico* predicted) using the Hallifax and Houston algorithm (Hallifax and Houston, 2006; Kilford et al., 2008). In studies where

fu_{inc} was not reported alongside $CL_{int,u,in\ vitro}$, corrections were applied with standardized fu_{inc} in the current study.

IVIVE of unbound aldehyde oxidase intrinsic clearance using standard physiological scaling factors: The collated unbound hepatic intrinsic clearance ($CL_{int,u,in\ vitro}$) obtained in human hepatocytes, human liver cytosols and human liver S9 were scaled following a standardized scaling approach. Physiological scaling factors were 120 x 10^6 hepatocytes per gram of liver (HPGL) (Naritomi et al., 2003), 80.7 mg cytosolic protein per gram of liver (CPPGL) (Houston and Galetin, 2008), and 120.7 mg S9 protein per gram of liver (S9PPGL) as total of microsomal (Barter et al., 2007) and cytosolic protein per gram of liver, respectively. Standard liver weight of 21.4 g per kg body weight (Ito and Houston, 2005) was used for further scaling to predict $CL_{int,u}$ in milliliters per minutes per kilogram using eq.1. Predicted $CL_{int,u}$ was considered to be equal to predicted $CL_{int,u,AO}$ in human liver cytosols and human liver S9 in the absence of CYP cofactor NADPH, assuming the contribution of non-AO enzymes in these incubation conditions was negligible. Following every possible effort to standardize the *in vitro* data and minimize the uncertainty associated with the use of combined datasets from different laboratories and biological matrices, the mean of predicted $CL_{int,u}$ or $CL_{int,u,AO}$ was used for further bias and precision calculations.

$$Predicted\ CL_{int,u}\ (mL/min/kg\ ) = \frac{CL_{int,in\ vitro}}{fu_{inc}} \times HPGL\ (or\ CPPGL\ or\ S9PPGL) \times \frac{Liver\ weight\ (g)}{Body\ weight\ (kg)} \quad (1)$$

Back-calculation of observed *in vivo* intrinsic clearance: Collated *in vivo* plasma clearance after iv or oral administration, fu_p and B/P data were used for the back-calculation of observed hepatic $CL_{int,u}$. Unless specified in the clinical studies, 1.7 m^2 and 70 kg were assumed as body surface area and body weight, respectively to calculate plasma clearance in milliliters per minute per kilogram. The well-stirred liver model was utilized to back-calculate $CL_{int,u}$ from iv data using eq. 2 (Pang and Rowland, 1977), whereas eq. 3 was applied for compounds with oral pharmacokinetics data. Extrahepatic AO metabolic clearance was considered negligible based on low/ unquantifiable AO abundance reported in human kidney, intestine, heart, and lung S9 (Basit et al., 2020).

$$Observed\ CL_{int,u}(mL/min/kg) = \frac{CL_H}{fu_b \times \left(1-\frac{CL_H}{Q_H}\right)} \quad (2)$$

$$Observed\ CL_{int,u}(mL/min/kg) = \frac{Dose}{AUC \times fu_b} \times F_G \times f_a \quad (3)$$

where CL_H and Dose/AUC represent the hepatic blood clearance obtained from mean plasma data after correcting for renal clearance (where applicable) and B/P. The fu_b represents the fraction unbound in blood, Q_H represents the average hepatic blood flow of 20.7 ml/min/kg (Kato et al., 2003), Dose represents the oral drug dose (mg/kg), AUC represents the area under the drug concentration-time curve (mg.min/mL), F_G represents the fraction escaping intestinal metabolism and f_a represents the fraction absorbed.

For compounds with reported hepatic blood clearance that exceeded the average hepatic blood flow, the hepatic blood clearance was set at 90% of hepatic blood flow (i.e., 18.63 mL/min/kg) (Cubitt et al., 2009; Kilford et al., 2009). F_G was assumed to be equal to 1 due to lack of available data across all compounds. For compounds predominantly metabolized by AO, this assumption was supported by low AO abundance in the intestine (Basit et al., 2020). However, for mixed AO and other enzyme (e.g. CYPs) substrates administered orally (e.g. capmatinib, idelalisib, lenvatinib), this assumption may cause over-prediction of observed $CL_{int,u}$. If mass balance data after oral administration were available, f_a was calculated using eq. 4, otherwise complete absorption was assumed.

$$f_a = 1 - \frac{\%\ unchanged\ drug\ in\ feces}{\%\ dose\ recovered} \quad (4)$$

Observed $CL_{int,u}$ values were multiplied by fm_{AO} values to calculate *in vivo* AO unbound intrinsic clearance ($CL_{int,u,AO}$) to compare with predicted $CL_{int,u,AO}$ from human liver cytosols and human liver S9. Various fm_{AO} values from human mass balance studies, *in vitro* assays and literature were collated and critically evaluated.

Bias and precision calculations: Bias and precision for $CL_{int,u}$ or $CL_{int,u,AO}$ predictions were calculated as geometric mean fold error (gmfe) in eq. 5 and root mean squared error (rmse) in eq. 6 (Sheiner and Beal, 1981; Gertz et al., 2010), respectively. The gmfe indicates the absolute deviation from the line of

unity as it does not allow over- and underpredictions to cancel each other out. Correlation between predicted and observed parameters (or between predicted values from different *in vitro* systems) was evaluated using the squared Pearson correlation coefficient (R^2).

$$gmfe = 10^{\frac{1}{n}\sum\left|log\left(\frac{predicted}{observed}\right)\right|} \quad (5)$$

$$rmse\ (mL/min/kg) = \sqrt{\frac{1}{n}\sum(predicted - observed)^2} \quad (6)$$

Empirical scaling factors: Database gmfe values for each *in vitro* system (hepatocytes, cytosols and S9) were used as empirical scaling factors (ESF) to further scale $CL_{int,u}$ as a pragmatic approach to improve IVIVE accuracy of AO clearance prediction according to eq. 7 (Matsunaga et al., 2019). Bias and precision were re-analyzed for predictions of $CL_{int,u}$ and $CL_{int,u,AO}$ using these newly developed ESFs.

$$Predicted\ CL_{int,u}\ (mL/min/kg) = Physiologically\ scaled\ predicted\ CL_{int,u} \times \text{system specific ESF} \quad (7)$$

where physiologically scaled predicted $CL_{int,u}$ was calculated using eq. 1.

The leave-one-out (L-O-O) cross-validation was adapted to assess bias and variance in ESFs (Hastie et al., 2009). In this approach, for all n compounds, a L-O-O ESF was generated for each (n-1) dataset (leaving out the i^{th} compound). The variability of these n L-O-O ESFs was assessed to identify outlier compounds in datasets. These L-O-O ESFs were then applied to predict the $CL_{int,u}$ or $CL_{int,u,AO}$ of the i^{th} left-out compound. The overall bias (gmfe) of the L-O-O $CL_{int,u}$ predictions were calculated using eq. 5.

Results

Survey analysis

The survey was limited to nine pharmaceutical companies within the AO Focus group of the Centre for Applied Pharmacokinetic Research. To minimize potential bias due to inter-company communications, the survey was conducted prior to the focus group discussions. The response rate was 100%. Six out of nine companies (67%) claimed to have experience in developing AO/mixed AO and CYP substrates, although no compound had received marketing authorization. All nine companies used *in vitro* systems for the prediction of *in vivo* CL including human hepatocytes, human liver cytosols and human liver S9. However, there was no consensus on which *in vitro* system is the best when predicting *in vivo* CL. Reaction phenotyping using chemical inhibitors (e.g., hydralazine, raloxifene or menadione) was the most common *in vitro* method for predicting fm_{AO} (Fig. 1). Only one company considered storage instability of human tissue samples or extrahepatic contribution of AO for total clearance predictions, although 78% of the companies have quantified AO abundance or variability in various human tissues. Over half of the companies had some experience in developing PBPK and/or static mechanistic models for translational prediction of human PK and victim DDIs of AO or mixed AO/CYP substrates. One third of the companies applied modeling in limited cases or used only static models for worst-case DDI predictions.

Database analysis

A systematic literature search of AO substrates yielded 37 compounds with diverse pharmacokinetic properties for which *in vitro* studies in human hepatocytes, human liver cytosols and/or human liver S9 were available. Among these compounds, $CL_{int,u,in\ vitro}$ data could not be calculated for 9 compounds (A7701, bafetinib, CL387785, INCB28060, lapatinib, LDN193189, ML347, SB525344, and VX509) (Dick, 2018). Three compounds (phthalazine, ripasudil, VU0409106) did not have available *in vivo* iv or oral PK data. The AO contribution to hepatocyte CL_{int} was insufficient to allow a reliable calculation of fm_{AO} for imatinib (Toselli et al., 2022), and AMG900 and favipravir had missing measured fu_p or B/P data. Only CL_{int} data measured in the absence of NADPH were included in the human liver S9 dataset, to reduce potential confounding impact of CYP-mediated metabolism in this system. In-house

data for human hepatocytes (Suppl. Table S1) were included in the dataset after correcting for non-specific binding using the literature data. CL_{int} measurements of compounds were sensitive to assay conditions especially in case of low turnover compounds such as XK469. After excluding data that were below limits of quantification, the final database consisted of 100 $CL_{int,\textit{in vitro}}$ measurements for 22 AO substrates (Table 2). Among those, 10 compounds were also metabolized by CYPs to varying degrees. The largest dataset was for human hepatocytes (n=19 compounds), followed by human liver cytosols (n=16) and liver S9 (n=11), with 9 compounds overlapping across all *in vitro* systems. Table 3 lists the fu_{inc} values, either corrected from literature (n=16) or predicted from logP data (n=6), that were used in the calculation of $CL_{int,u,\textit{in vitro}}$.

Mean fu_p and B/P values from literature and in-house data (Table 3) were used in the back-calculation of observed $CL_{int,u}$. Despite the similar *in vitro* methodology between studies, fu_p values of DACA, fasudil, idelalisib and ziprasidone showed %CV over 30%. B/P data were more consistent between different studies.

Plasma clearance data after iv administration, and apparent clearance data after oral drug administration, were collated for the investigated AO substrates from 25 reports (Table 4). The iv plasma clearance (n=10) ranged from 0.04 to 73.2 mL/min/kg for XK469 and fasudil, respectively. Six compounds had CL_H over 80% of average Q_H, of which BIBX1382, carbazeran and fasudil had CL_H exceeding Q_H suggesting non-AO metabolic pathways (Table 4) may be involved in extrahepatic metabolism, since AO clearance of extra-hepatic tissues (kidney, lung, vasculature and intestine) predicted from S9 fractions were to be <1% of the liver (Kozminski et al., 2021). For the compounds with CL_H exceeding Q_H, the cut-off CL_H of 90% of Q_H was applied (Cubitt et al., 2009) in the calculation of $CL_{int,u}$ via the well-stirred liver model. Oral plasma clearance (n=12) ranged between 2.05 to 17,857 mL/min/kg for lenvatinib and LuAF09535, respectively. The correction for f_a was applied for 6-deoxypenciclovir (Filer et al., 1994), capmatinib (Glaenzel et al., 2020), idelalisib (TGA, 2015) and lenvatinib (EMA, 2015) with respective f_a values of 0.8, 0.67, 0.91 and 0.97.

All available data on $fm_{AO,}$ from *in vitro* hepatocyte or S9 inhibition studies, PBPK model-based predictions in literature, ADME studies and previous literature were collated, with up to 10 different fm_{AO} values reported for individual compounds (Suppl. Table S3). Mean fm_{AO} ranged from 0.2 to 1, with compounds grouped as low (0.2-0.49: n=6) medium (0.5-0.79: n=6) and high (0.8 and over: n=10) AO-metabolized compounds. However, large variability was noted for some compounds, including ziprasidone, capmatinib and lenvatinib with %CV in fm_{AO} of 117%, 76% and 39%, respectively, due to the discrepancy between *in vitro* and *in vivo* data. Considering this high uncertainty, human mass balance data were considered as a primary input for fm_{AO} when reported (available for 6 compounds in the database). For the remaining compounds (n=16), mean and range of fm_{AO} from all reported values were used (Suppl. Table S3).

Predictive performance of *in vitro-in vivo* extrapolation

IVIVE using purely physiological scaling factors was applied to predict $CL_{int,u}$ (hepatocytes) and $CL_{int,u,AO}$ (human liver cytosols and S9) (Table 2). Mean predicted $CL_{int,u,AO}$ using human liver cytosols or S9 data was higher than the mean of predicted total $CL_{int,u}$ in human hepatocytes for 6 compounds having data from all *in vitro* systems. Mean predicted $CL_{int,u,AO}$ between human liver cytosol and S9 were correlated with R^2 of 0.5 (n=11 compounds), and most of these compounds (n=7) had higher predicted mean $CL_{int,u,AO}$ using human liver S9 than human liver cytosols.

Large inter-assay variability of predicted $CL_{int,u}$ was observed for all three *in vitro* systems. For compounds with data from multiple studies, the %CV ranged 26-91% for human hepatocytes, 22-73% for human liver cytosols, and 5-98% for human liver S9 (Table 2). Potential contributions of factors such as lot-to-lot variability, data analysis methodology and publication date to the inter-assay variability were investigated. For most of the compounds, *in vitro* data were generated using hepatocytes or subcellular fractions from different commercial sources, with lot numbers and supplier information on AO activity or assay sensitivity typically not reported in literature studies. The predicted $CL_{int,u}$ for 8 compounds from a lot of cryopreserved hepatocytes pooled from individual donors with relatively high AO activity were comparable with corresponding data in lots for which AO activity of

donors was unknown. The highest $CL_{int,in\ vitro}$ for carbazeran in human liver S9 (2-fold higher than mean value) came from measurement of the early fast rate of the metabolite formation using the modulated activity model (Abbasi et al., 2019). Data were also analyzed to explore whether *in vitro* systems/methods that had been refined over the last decade (e.g., due to improved tissue processing, modified assay formats, etc.) would result in higher predicted $CL_{int,u}$. However, there were insufficient data for each substrate to assess potential trends between $CL_{int,in\ vitro}$ and publication date.

The observed $CL_{int,u}$ values back-calculated from iv clinical data ranged between 3.17 and 5012 mL/min/kg, for methotrexate and ziprasidone, respectively (Table 4). Our static approach did not consider differences in physiological parameters (i.e., variability in liver blood flow) between study groups. The back-calculated $CL_{int,u}$ was highly sensitive to the variability in plasma and blood binding especially for high clearance compounds, therefore the mean fu_p and B/P values from various literature was taken when applicable. From oral data, the lowest and the highest $CL_{int,u}$ were observed for RS8359 (17.1 mL/min/kg) and LuAF09535 (68,681 mL/min/kg), respectively. Overall, observed $CL_{int,u}$ values covered a wide range, with compounds evenly distributed across low (<100 mL/min/kg: n=6), medium (101-1000 mL/min/kg: n=10) and high (>1000 mL/min/kg: n=6) $CL_{int,u}$ groups. After applying mean fm_{AO} values, the mean of observed $CL_{int,u,AO}$ in the complete dataset ranged from 0.63 to 59,753 mL/min/kg for methotrexate and LuAF09535, respectively (Table 4).

The predictive performance of IVIVE based solely on physiological scalars was assessed with the bias and precision of the predictions for each *in vitro* system (Table 5). The overall trend of underprediction of *in vivo* $CL_{int,u}$ was prominent in all systems with the highest gmfe of 10.4 noted in human hepatocyte dataset (n=19 substrates). There was no correlation (R^2= 0.02) between observed and predicted $CL_{int,u}$ from human hepatocytes (Fig. 2). FK3453 and LuAF09535 were outlier compounds in the human hepatocyte dataset with predicted $CL_{int,u}$ below 1% of observed values, whereas 11% of the compounds were predicted within 2-fold. Outliers were not excluded from the dataset and the distribution of gmfe values were assessed via the L-O-O approach. The arithmetic mean±SD (range) of L-O-O gmfe values of predictions in human hepatocytes was 10.4±0.8 (8.4-11.7) (Fig. 3).

The predictive performance of $CL_{int,u,AO}$ from human liver cytosol and S9 were comparable, with gmfe of 5.6 (n=16) and 5.0 (n=11), respectively. The observed and predicted $CL_{int,u,AO}$ in human liver cytosols and human liver S9 were weakly correlated with R^2 of 0.28 and 0.37, respectively. Majority of compounds were underpredicted using data from human liver cytosols and human liver S9, with $CL_{int,u,AO}$ overpredicted for only one (different) compound in each dataset, by 1.6- and 2.4-fold, respectively. The corresponding underpredictions in these systems were up to 29- and 18-fold, respectively. The arithmetic mean±SD (range) of L-O-O gmfe values in human liver cytosol and S9 datasets was 5.6±0.4 (5.1-6.1), and 5.0±0.5 (4.4-5.7), respectively (Fig. 3). The predictive performance of $CL_{int,u,AO}$ using human liver cytosols and S9 was dependent on the fm_{AO} estimates with associated uncertainty.

Considering the general lack of measured fu_{cyt} in the database, assumption of fu_{cyt} of 1 was explored as alternative approach to predicted fu_{cyt} from microsomal data, and to evaluate sensitivity of fu_{cyt} towards IVIVE performance. For most of the compounds, the impact of assuming fu_{cyt} of 1 on predicted $CL_{int,u,AO}$ was minimal. However, for highly protein bound compounds BIBX1382, lenvatinib, ziprasidone and methotrexate predicted $CL_{int,u,AO}$ was 2-fold lower when assuming fu_{cyt} of 1 compared with using predicted fu_{cyt} from available fu_{mic} or logP/D data (Suppl. Fig. S1), causing an increase in gmfe to 7.7 (Fig. 3).

The predictive performance of all *in vitro* systems showed high variability between compounds, even when considering groups with similar observed $CL_{int,u}$ or $CL_{int,u,AO}$. In human liver cytosols and S9, there was no clear trend between the observed $CL_{int,u}$ and the fold underprediction. Conversely, in the human hepatocyte dataset, the IVIVE performance improved with decreasing observed $CL_{int,u}$. The gmfe of predictions including low (n=3), medium (n=10) and high clearance (n=6) compounds were 3.4, 7.6 and 30, respectively. For the high clearance group, predictive performance was still the worst (gmfe of 10.4 (n=4)) even after excluding the two outliers with the highest fold underprediction (FK3453 and LuAF09535). The extent of underprediction of $CL_{int,u}$ in human hepatocytes increased with higher fm_{AO}. The gmfe of predictions for low, medium, and high fm_{AO} compounds were 6.1 (n=5),

10.5 (n=6), and 14.2-fold (n=8) respectively. However, when the two outlier compounds (FK3453 and LuAF09535) were excluded, the gmfe was 5.4 (n=6) for high fm_{AO} compounds.

Application of empirical scaling factors

The gmfe values in Table 5 were applied as system-specific empirical scaling factors (ESFs) to further scale predicted $CL_{int,u}$ and $CL_{int,u,AO}$. In the absence of ESFs, 11-27% of the data were predicted within 2-fold, compared with 45%-57% after application of ESFs (Fig. 4). In each dataset, $CL_{int,u}$ or $CL_{int,u,AO}$ were underpredicted for less than 20% of the compounds. 6-deoxypenciclovir, FK3453 and LuAF09535 in human hepatocytes; PF4217903, zaleplon and ziprasidone in human liver cytosols, and DACA and zaleplon in human liver S9 still showed more than 2.8-fold mean underprediction. Overall, the prediction bias was reduced to ≤ 2.5-fold for all three *in vitro* systems in the presence of ESFs. The rmse of predictions was also reduced in human hepatocytes and human liver S9, but slightly increased in human liver cytosols due to overpredictions after empirical scaling. The performance of system specific ESFs remained similar by leave-one out cross-validation (Table 5).

Discussion

Early planning of clinical pharmacology studies during drug development requires accurate prediction of human clearance, typically performed using IVIVE, allometry, and translational modeling. However, relatively few AO substrate compounds are progressed into clinical studies, leading to limited clinical datasets and lack of harmonized best practices for suitable *in vitro* tools (Fig. 1D). Unresolved challenges in developing *in vitro* assays of AO metabolism (e.g., loss of enzyme activity, inter-individual variability, lack of robust reaction phenotyping tools), compared with CYP substrates have been widely reported (Hartmann et al., 2012; Barr and Jones, 2013; Fu et al., 2013; Zientek and Youdim, 2015).

Improving prediction intrinsic clearance for AO substrates

Our *in vitro* and *in vivo* (iv/oral) dataset of AO metabolized compounds (n=22) has enabled a comprehensive analysis of inter-assay variability in $CL_{int,u,in\ vitro}$ and uncertainty of fm_{AO}. Importantly, our study used a consistent set of physiological scalars and approaches for *in vivo* $CL_{int,u}$ calculations. The underprediction of hepatic $CL_{int,u}$ was system-dependent ranging from 10.4-fold in human hepatocytes to 5.3-fold in human liver cytosols, and was in agreement with previously reported $CL_{int,u,AO,}$ of 10-fold in pooled hepatocytes (n=6 compounds (Akabane et al., 2012a)) and 6.5-fold in human liver cytosols (n=5; considering extrahepatic AO (De Sousa Mendes et al., 2020)), respectively. The clearance-dependent underprediction of $CL_{int,u}$ was only observed in human hepatocytes (gmfe; 3.4, 7.6 and 30, for low, medium, and high clearance compounds, respectively), which was in agreement with previous studies (Wood et al., 2017), supporting possible experimental causes such as passive permeation mechanism, integrity of hepatocyte membrane, and cofactor depletion, which may not be relevant for cytosols and S9 as the clearance-dependent prediction of $CL_{int,u,AO}$ was not noted in our dataset (Fig. 2). The underprediction of AO $CL_{int,u}$ in hepatocytes is apparently higher than for non-AO-mediated CL, suggesting enzyme specific factors affecting AO activity *in vitro*. For example, consideration of hepatocyte data for compounds with low AO contribution (fm_{AO}; 0.2-0.4; n=5) decreased the ESF from 10.4 to 6.2, which was closer to a human hepatocytes ESF of 4.2 reported for mostly non-AO compounds (n=101 (Wood et al., 2017)).

Loss of AO enzyme activity during tissue processing and preparation of isolated hepatocytes or subcellular fractions is likely to contribute to the observed underprediction and variability of human *in vivo* AO clearance. For example, there was greater underprediction of human $CL_{int,in\ vivo}$ by isolated human hepatocytes from humanized mice compared to predictions with *in vivo* clearance measured in the same animals (Sanoh et al., 2012). Large, donor-dependent loss of AO activity was reported within 24 hours following isolation of human hepatocytes (N = 75, 15-81% loss) (Hutzler et al., 2014b). As commercially obtained fresh hepatocytes are usually more than 24 hours old, use of well-characterized cryopreserved hepatocyte lots is recommended (Akabane et al., 2012b; Hutzler et al., 2014b). The current study, which only included cryopreserved hepatocytes data, highlights the high inter-assay variability (26-91% CV) in AO activity.

Higher metabolic activity with prolonged incubations have been shown in hepatocyte co-cultures (e.g., HepatoPac® and HμREL) and microphysiological systems for many CYP and UGT substrates compared to suspended human hepatocytes (Hultman et al., 2016; Da-Silva et al., 2018; Docci et al., 2022). However, reported data on AO activity in these systems are limited (Docci et al., 2022) and their performance for AO substrates is yet to be established. Correction of *in vitro* CL_{int} for fu_{inc} is an important step in IVIVE. When fu_{inc} data are not available for the specific experimental conditions, fu_{inc} for human liver microsomes and hepatocytes can be estimated from measurements at different protein concentrations or predicted from phys-chem data (Suppl. Fig. S1). Although cytosol and microsomes have different levels of lipid and protein, our data suggest that these equations may be used for estimation of fu_{inc} in cytosol, although a larger dataset is needed for confirmation. Since the dog is a natural knockout for AO in the liver, fu_{cyt} measured in dog liver cytosols could be used for future studies. Alternatively, substrate depletion assay can be performed with varying cytosolic protein concentration with back-extrapolation to zero protein level to estimate fu_{cyt} (Giuliano et al., 2005). However, this method may be challenging for low clearance compounds due to the assay sensitivity.

How to improve confidence in fm_{AO} assessments

Quantitative understanding of clearance mechanisms and fractional contributions of metabolic enzymes and transporters to drug disposition is critical for assessment of DDI risk (Rowland Yeo and Venkatakrishnan, 2021). The experimental techniques for reaction phenotyping and scaling factors to determine the fraction metabolized by individual enzymes (fm_i) have been more firmly established for CYPs compared to non-CYP enzymes (Zhang et al., 2007; Houston and Galetin, 2008; Bohnert et al., 2016).

The most common *in vitro* method for estimating fm_{AO} was using human hepatocytes in the presence or absence of a selective inhibitor (Strelevitz et al., 2012; Argikar et al., 2016; Toselli et al., 2022), where the relative contribution of AO to overall metabolism can be estimated without the need for separate assays to investigate clearance via parallel pathways (e.g. CYPs).

We noted considerable inter-study variability, for example fm_{AO} of carbazeran ranged 0.49-0.89 (Suppl. Table S3). Uncertainty in fm_{AO} may come from loss in AO abundance and activity during isolation and preparation of *in vitro* systems, instability of AO during activity assays, lack of specificity in reaction phenotyping for AO or potential contamination of AO in human liver microsomes (Table 6). Simultaneous monitoring of parent depletion and AO metabolite formation could improve accuracy of fm_{AO} determination (Dick, 2018).

Later in clinical drug development, more definitive estimates of fm_{AO} are expected from reverse translation of human CYP DDI study (for dual AO-CYP substrates) and mass balance study data. AO DDI study data to inform fm_{AO} are limited by lack of clinically relevant DDIs attributed to AO inhibition and availability of established clinical strong index inhibitors, despite several potent *in vitro* AO inhibitors (e.g., raloxifene, hydralazine, estrogen). Hence, appropriately designed human ADME mass balance/absolute bioavailability study and metabolite profiling of excreta become critical enablers of *in vivo* parameters (Ma et al., 2019; Zamek-Gliszczynski et al., 2021) including fm_{AO}. However, in the current analysis fm_{AO} data from ADME studies were available for only 27% of compounds (n=6), highlighting limited examples upon which a translational framework can be developed and validated. Many AO substrates are mixed substrates with metabolism by parallel pathways (e.g., CYP), as

reflected by the dataset in current analysis (45% of compounds were mixed AO-CYP substrates). For multi-metabolic pathway compounds, a DDI study with a strong index inhibitor of the parallel metabolic pathway (e.g., with itraconazole for a mixed AO-CYP3A substrate) may enable indirect refinement of fm_{AO}.

For a limited number of compounds (e.g., capmatinib, lenvatinib, zaleplon and ziprasidone) fm_{AO} from both *in vitro* and human mass balance studies were available, but did not show consistent trends (Suppl. Table S3). For capmatinib and ziprasidone, fm_{AO} estimated by *in vitro* inhibition studies were 5- and 10-fold lower than the human mass balance determinations, whereas for lenvatinib *in vitro* fm_{AO} was up to 2-fold higher than the human mass balance data (Suppl. Table S3). Recommendation to obtain *in vivo* fm_{AO}, supported by *in vitro* reaction phenotyping data, can be exemplified using ziprasidone as a case study. A clinical ADME mass balance study with dual-labeled (^{14}C and ^{3}H) ziprasidone found that reductive cleavage of the benzisothiazole moiety accounted for >60% of the dose (Prakash et al., 1997). Early *in vitro* reaction phenotyping using human liver microsomes and recombinant CYPs suggested CYP3A was the primary CYP for ziprasidone metabolism (Prakash et al., 2000). However, clinical DDI studies conducted with ketoconazole, a strong CYP3A inhibitor, showed a marginal increase (<35%) in exposure (AUC and C_{max}) indicating contribution of non-CYP3A enzymes (Miceli et al., 2000). Subsequent *in vitro* studies revealed the reductive cleavage reaction was not CYP-mediated, allowing the ketoconazole DDI and mass balance study data to be explained together. The exact role of AO remained unclear; although the reductive cleavage reaction exhibited saturable kinetics and was inhibited by menadione (a known AO inhibitor) in human liver cytosols, the reaction could also occur by GSH-mediated chemical reduction (Obach et al., 2012).

Implications for clinical pharmacology strategies: Future perspective

Recent reviews have highlighted the growing interest and emerging importance of AO within drug development (Dalvie and Di, 2019; Manevski et al., 2019; Beedham, 2020). Uncertainty in fm_{AO} values reflects disconnect between different approaches and inconsistency in the type of studies used to estimate fm_{AO}, precluding recommendations for a particular approach. Lack of confidence in fm_{AO}

predictions in part explains the limited PBPK modeling efforts, and subsequent uncertainty for appropriate clinical pharmacology strategies for AO substrates (Fig. 1). Therefore, research teams should aim towards thorough reaction phenotyping data from *in vitro* assays, chimeric or humanized mouse models, clinical mass balance and DDI studies for compounds currently progressing through the drug development pipeline. Such data generation efforts will enable a holistic and iterative characterization and refinement of understanding of fm_{AO} (as summarized in the workflow in Fig. 5) and will advance PBPK modeling upon experience gained with other enzymes (e.g., CYPs) (Kilford et al., 2022) and transporters (Guo et al., 2018; Taskar et al., 2020). PBPK models could then become a useful tool for DDI risk assessment for AO substrates. With expedited sharing of data and case studies for validation and qualification efforts, PBPK models are anticipated to enable prediction of clinical DDI risk for AO inhibitors, e.g., oral contraceptive steroids (Obach et al., 2004). Such advances, along with clinical considerations (e.g., pharmacokinetic variability, exposure-safety relationships) would empower model-informed clinical trial design for the enrollment of women of childbearing potential, enable diversity and inclusion in clinical development, and inform rational decisions around exclusion of specific concomitant medications. Such a holistic approach based on totality of evidence principles (Venkatakrishnan and Cook, 2018) can be valuable in enabling patient-focused and inclusive drug development, also obviating the need for unnecessary prospective clinical DDI studies with AO inhibitors.

Conclusion

Successful drug development relies on accurate human dose projections, which is currently challenging for AO substrates. Our study has established the most comprehensive assessment of empirical scalars for human liver cytosol, S9, and hepatocytes to-date. These ESFs support a pragmatic approach to mitigate against underprediction of *in vivo* $CL_{int,u,AO}$ via IVIVE, and may be useful to predict fm_{AO} in the early stages of drug development. Understanding and reducing uncertainty in fm_{AO} through a holistic approach (Fig. 5), using data from *in vitro* assays, chimeric mice models, and clinical mass balance studies, should be a focus in drug development to enable rational AO clinical pharmacology strategies, PBPK model development and prediction of DDI liabilities.

Data Availability Statement

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

Statement of Financial Interest

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Human Samples Source Statement

The human biological samples were sourced ethically, and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol.

Authorship Contributions

Participated in research design: N.I., J.B.H., A.G., D.S.

Performed data analysis: N.I., D.S.

Wrote or contributed to the writing of the manuscript: N.I., J.Bolleddula, A.A., L.C., R.S.J., D.M., F.O.M., Y.P., V.C.P., D.-D.T., K.V, M.A.Z., J.Barber, J.B.H., A.G., D.S.

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Footnotes

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Figure Legends

Fig. 1. Survey responses from pharmaceutical companies (n=9). Panel A, method(s) in use for the prediction of *in vivo* clearance for aldehyde oxidase/mixed aldehyde oxidase and CYP substrates; Panel B, *in vitro* system(s) in use by companies among human hepatocytes (HH), human liver cytosols (HLC), and human liver S9 (HLS9); Panel C, method(s) used for predicting fraction metabolized by aldehyde oxidase vs. fraction metabolized by CYPs; Panel D, favoured method for *in vivo* clearance predictions for aldehyde oxidase and mixed aldehyde oxidase and CYP substrates. Different colour bars differentiate multiple choice answers.

Fig. 2. Comparison of predicted and observed hepatic unbound intrinsic clearance ($CL_{int,u}$) for 22 aldehyde oxidase substrates. IVIVE of *in vitro* data from human hepatocytes, cytosol and S9 was performed using corresponding physiological scaling factors for each *in vitro* system. Panel A, comparison of observed and predicted $CL_{int,u}$ from *in vitro* data generated in human hepatocytes (n=19, red square). Panel B, comparison of observed and predicted $CL_{int,u,AO}$ from human liver cytosols (n=16, green circle) and human liver S9 (n=11, blue triangle) *in vitro* data. Dashed lines represent the 2-fold deviation from the line of unity. Vertical and horizontal error bars represent inter-assay variability and uncertainty in fm_{AO}, respectively (min-max). Compounds are numbered in the following order: 1, 6-deoxypenciclovir; 2, BIBX1382; 3, capmatinib; 4, carbazeran; 5, DACA; 6, fasudil; 7, FK3453; 8, idelalisib; 9, JNJ38877605; 10, lenvatinib; 11, LuAF09535; 12, methotrexate; 13, O6-Benzylguanine; 14, PF4217903; 15, PF5190457; 16, PF6273340; 17, PF-945863; 18, RS8359; 19, XK469; 20, zaleplon; 21, ziprasidone; 22, zoniporide.

Fig. 3. Box plot of geometric mean fold error (gmfe) values calculated after leaving one compound out from each dataset. *In vitro* data includes human hepatocytes (HH; n=19), human liver cytosols (HLC; n=16), and human liver S9 (n=11). Within each box, horizontal black lines denote median values and + denote mean values; boxes extend from the 25^{th} to the 75^{th} percentile of each group's distribution of values; vertical extending lines denote the minimum and maximum values; dashed lines represent the geometric mean of gmfe calculated in each dataset (overall gmfe) as 10.4, 5.6, 7.7 and 5.0 for HH, HLC, HLC (fu_{cyt}=1 approach) and HLS9, respectively.

Fig. 4. Comparison of predicted and observed $CL_{int,u}$ and $CL_{int,u,AO}$ in the without (closed symbols) and with (open symbols) application of system specific empirical scaling factors. Panel A, human hepatocytes (n=19, red square); Panel B, human liver cytosols (n=16, green circle); C, human liver S9 (n=11, blue triangle) datasets. The solid and dashed lines represent the line of unity and 2-fold difference, respectively.

Fig. 5. A holistic approach for the quantitative understanding of the clearance mechanisms and drug-drug interactions for aldehyde oxidase substrates. CL_{Total}; total clearance, CL_{Renal}; renal clerance, DDI; drug-drug interaction, F; absolute bioavailability, fa; fraction absorbed, F_G; fraction escaping gut metabolism, F_H; fraction escaping hepatic metabolism, $fm_{parallel\ pathways}$; fraction metabolized via non-AO pathways, fm_{AO}; fraction metabolized by aldehyde oxidase, PBPK; physiologically-based pharmacokinetic, V_{ss}; the volume of distribution at steady state.

Tables

TABLE 1

Substrate dependency in species differences, inhibition and enzyme kinetics for aldehyde oxidase

Aldehyde oxidase substrate	**Substrate dependent difference in intrinsic clearance (µL/min/mg protein) between species**	**Substrate-dependent difference in IC50 values (µM) and mode of inhibition**	**Substrate dependency in non-linear enzyme kinetics [a] (Abbasi et al., 2019)** ***Modulated activity model*** **$k_{cat,fast}$ / $k_{cat,slow}$**
DACA	Guinea pig> Rhesus monkey>human (Choughule et al., 2013) Rabbit≈human (Choughule et al., 2015)	- 17β-estradiol: *Mixed inhibition*[b]*; more competitive* *K_{is} 0.87, K_{ii} 4.4; K_{ii}/K_{is} 5.1* (Barr and Jones, 2013) - Menadione: 1.4[b] (Schofield et al., 2000) *Mixed inhibition*[b]*, more competitive* *K_{is} 0.47, K_{ii} 1.5; K_{ii}/K_{is} 3.2* (Barr and Jones, 2013) - Raloxifene: *Competitive inhibition*[b]; *K_{is} 0.002* (Barr and Jones, 2013))	5.4[b] – 6.5[c]
Phthalazine	Rhesus monkey>guinea pig>human (Choughule et al., 2013) Rabbit>human (Choughule et al., 2015)	- 17β-estradiol: 2.4[c] (Takaoka et al., 2018) *Mixed inhibition*[b]*, more uncompetitive inhibition* *K_{is} 0.9, K_{ii} 0.13; K_{ii}/K_{is} 0.14* (Barr and Jones, 2013) - Menadione: 0.6[c] (Takaoka et al., 2018) *Mixed inhibition*[b]*, more uncompetitive inhibition* *K_{is} 0.76, K_{ii} 0.12; K_{ii}/K_{is} 0.16* (Barr and Jones, 2013) - Raloxifene: 0.06[c] (Takaoka et al., 2018) *Uncompetitive inhibition* [b]; K_{ii} 0.0009 (Barr and Jones, 2013)	32[c] - 55[b]

O6-benzylguanine	Human>domestic pig (Hu, 2018) - Rats≈PXB mice>cynomolgus monkeys[d]monkeys[d] (Miyamoto et al., 2017)	- 17β-estradiol: 12[c] (Takaoka et al., 2018) - Hydralazine: 0.08[c] (Takaoka et al., 2018) - Menadione: 6[c] (Takaoka et al., 2018) - Raloxifene: 0.2[c] (Takaoka et al., 2018)	1.9[b] - 3.1[c]
Carbazeran	- PXB mice≈cynomolgus monkeys>rats[d]rats[d] (Miyamoto et al., 2017)	- 17β-estradiol: 1[g] (Toselli, et al., 2022) - Hydralazine: 0.8[b] (Nishinoaki et al., 2017) - Menadione: 12[g] (Toselli, et al., 2022) - Raloxifene: 0.028[b] *Noncompetitive inhibition* (Chen et al., 2019)	41[e] (Kozminski et al., 2021)
BIBX1382	- Cynomolgus monkey≈rhesus monkey≈human (Hutzler et al., 2014a) - PXB mice>cynomolgus monkeys>rats[d] (Miyamoto et al., 2017)	- n/a	2.8[c, f] - 2.9[b, f]
Zoniporide	- Rabbit>Gottingen minipig>mouse≈human≈SD rat≈Wistar rat> Fischer rat≈cynomolgous monkey≈rhesus monkey (Dalvie et al., 2013) - PXB mice≈rats>cynomolgus monkeys[d] (Miyamoto et al., 2017)	- Hydralazine: 0.8[b] (Nishinoaki et al., 2017) *Time dependent inhibition by hydralazine* (Strelevitz et al., 2012) - Menadione: 10[b] (Dalvie et al., 2010) - Raloxifene: 0.01[b] (Dalvie et al., 2010)	0.23[c, f] - 0.50[b, f]

Abbreviations: K_{ii}, the inhibition constant for the enzyme-substrate complex; K_{is}, the inhibition constant for the free enzyme

[a]Based on the ratio of the initial fast AO catalytic rate ($k_{cat, fast}$) /slow AO catalytic rate constant ($k_{cat, slow}$) in the modulated activity model (MAM)

[b]Human liver cytosol assay

[c]Human AOX1 assay

[d]Based on total clearance mL/min/kg

[e]Human liver S9 assay

[f]Rate constants were based on peak area ratio

TABLE 2

Predicted unbound intrinsic clearance ($CL_{int,u}$) obtained from scaling in vitro data measured in human hepatocytes, human liver cytosols and human liver S9

References for all *in vitro* studies are available in the Suppl. Table S2.

No	Substrate	Predicted $CL_{int,u}$ (mL/min/kg)								
		Human hepatocytes			Human liver cytosols			Human liver S9		
		Mean	CV%	n	Mean	CV%	n	Mean	CV%	n
1	**6-Deoxypenciclovir**	5.75	-	1	25.6	22	2	124	-	1
2	**BIBX1382**	241	-	1	1306	57	2	441	5	2
3	**Capmatinib**	32.6	-	1	7.16	-	1	-	-	-
4	**Carbazeran**	183	26	5	653	67	7	799	89	4
5	**DACA**	133	26	3	264	73	2	92.2	-	1
6	**Fasudil**	385	-	1	583	-	1	-	-	-
7	**FK3453**	33.6	-	1	-	-	-	-	-	-
8	**Idelalisib**	22.0	33	2	-	-	-	-	-	-
9	**JNJ38877605**	13.8	-	1	-	-	-	-	-	-
10	**Lenvatinib**	15.04	-	1	7.64	-	1	-	-	-
11	**LuAF09535**	154	-	1	-	-	-	-	-	-
12	**Methotrexate**	-	-	-	0.06	-	1	-	-	-
13	**O6-Benzylguanine**	48.0	60	7	28.5	34	3	26.6	98	4
14	**PF4217903**	-	-	-	2.42	-	1	7.21	-	1
15	**PF5190457**	14.5	-	1	4.96	-	1	-	-	-
16	**PF6273340**	6.70	-	1	-	-	-	-	-	-
17	**PF-945863**	73.1	60	3	57.5	-	1	92.9	-	1
18	**RS-8359**	-	-	-	8.03	-	1	37.6	-	1
19	**XK-469**	1.50	-	1	-	-	-	-	-	-
20	**Zaleplon**	18.4	82	6	4.31	52	3	7.66	41	2
21	**Ziprasidone**	587	91	2	118	72	2	389	-	1
22	**Zoniporide**	35	63	6	48.6	40	5	28.7	75	3
	Number of data points			45			34			21
	Number of studies			7			13			5

TABLE 3

Octanol–water partition coefficient (logP), unbound fraction in incubations (fu_{inc}), human fraction unbound in plasma (fu_p) and blood-to plasma ratio (B/P) data used in the IVIVE analysis.

References for human plasma protein binding and blood-to plasma ratio data are available in the Suppl. Table S2.

No	Substrate	logP	fu_{inc}		fu_p Mean (n; CV%)	B/P Mean (n; CV%)
			fu_{hep} (10^6 hepatocytes)	fu_{mic} (1 mg/mL protein)		
1	**6-deoxypenciclovir**	-2.15[a]	0.99 (Akabane et al., 2012a)	1 (Wilkinson et al., 2017)	0.897 (2; 16)	1.14 (2; 7)
2	**BIBX1382**	3.97 (De Sousa Mendes et al., 2020)	0.46[b] (Predicted)	0.347[b] (Predicted)	0.108 (2;15)	1.35 (2; 10)
3	**Capmatinib**	1.2 (Novartis, 2020)	0.67[c] (Predicted)	0.56 (Toselli et al., 2022)	0.045 (2; 16)	1.57 (3; 33)
4	**Carbazeran[d]**	1.83 (De Sousa Mendes et al., 2020)	0.86[c] (Predicted)	0.85 (Toselli et al., 2022)	0.093 (4; 14)	0.803 (4; 15)
5	**DACA**	1.35[a]	0.93[b] (Predicted)	0.89[b] (Predicted)	0.051 (2; 84)	0.965 (2; 17)
6	**Fasudil**	1.19[a]	1[c] (Predicted)	1 (Toselli et al., 2022)	0.660 (2; 32)	1.59 (1; -)
7	**FK3453**	1.09[a]	0.82[e] (Akabane et al., 2012a)	0.74[c] (Predicted)	0.195 (1; -)	0.86 (1; -)
8	**Idelalisib**	2 (Gilead, 2017)	0.84[c] (Predicted)	0.76 (Toselli et al., 2022)	0.086 (3; 75)	0.879 (3; 18)
9	**JNJ38877605**	3.13[a]	0.82[c] (Predicted)	0.74 (Toselli et al., 2022)	0.080 (1; -)	0.770 (1; -)
10	**Lenvatinib**	3.3 (FDA, 2015)	0.14[c] (Predicted)	0.09 (Toselli et al., 2022)	0.023 (2; 44)	0.728 (2; 26)
11	**LuAF09535**	2.4 (Jensen et al., 2017)	0.86[c] (Predicted)	0.80 (Toselli et al., 2022)	0.260 (1; -)	0.860 (1; -)
12	**Methotrexate**	-2.52 (logD7.4) (Benet et al., 2011)	0.92[b] (Predicted)	0.87[b] (Predicted)	0.660 (1; -)	1.00 (1; -)

13	**O6-Benzylguanine**[d]	1.04 (Liu et al., 2005)	0.74[e] (Akabane et al., 2012a)	0.94 (Toselli et al., 2022)	0.120 (5;21)	0.979 (5; 18)
14	**PF4217903**	0.3[a]	0.95[b] (Predicted)	0.93[b] (Predicted)	0.160 (1; -)	0.900 (1; -)
15	**PF5190457**	4.8[a]	0.85[c] (Predicted)	0.78 (Toselli et al., 2022)	0.120 (1; -)	1.10 (1; -)
16	**PF6273340**	0.92[a]	0.84[c] (Predicted)	0.77 (Toselli et al., 2022)	0.160 (1; -)	0.760 (1; -)
17	**PF945863**	3.14[a]	0.72[c] (Predicted)	0.62 (Toselli et al., 2022)	0.550 (2; 3)	1.13 (2; 4)
18	**RS8359**	1.12[a]	0.94[b] (Predicted)	0.9[b] (Predicted)	0.490 (1; -)	1.00 (1; -)
19	**XK469**	4.05[a]	0.98[e] (Akabane et al., 2012a)	1 (Toselli et al., 2022)	0.006 (3;35)	0.703 (2; 37)
20	**Zaleplon**	1.3 (De Sousa Mendes et al., 2020)	0.83 (Akabane et al., 2012a)	0.9 (Toselli et al., 2022)	0.562 (5; 23)	0.889 (5; 14)
21	**Ziprasidone**	4.53 (De Sousa Mendes et al., 2020)	0.26[b] (Predicted)	0.18[b] (Predicted)	0.002 (2; 47)	0.757 (2; 24)
22	**Zoniporide**	1.15 (De Sousa Mendes et al., 2020)	0.89 (Akabane et al., 2012a)	0.82 (Toselli et al., 2022)	0.359 (5; 16)	0.954 (5; 21)

a Predicted logP (ACD Chem)

b Predicted fuinc using logP data (Hallifax and Houston, 2006; Kilford et al., 2008)

c Predicted from measured fumic/ fuhep based on relative protein concentration and cell volume

d Measured fucyt (0.1 mg/mL) and fuS9 (0.3 mg/mL) are 0. 94 and 0.96 for carbazeran and 1.01 and 0.96 for O6-benzylguanine, respectively (Xie et al., 2019)

e Normalised from measured fu based on relative cell volume

TABLE 4

Intravenous or oral (apparent) plasma clearance, observed unbound intrinsic clearance ($CL_{int,u}$), fraction metabolized by aldehyde oxidase (fm_{AO}) and observed unbound intrinsic clearance by aldehyde oxidase ($CL_{int,u,AO}$)

Weighted mean of plasma clearance for compounds for which more than one clinical study or multiple dose studies were available. Number of studies and between study coefficient of variation are given in parenthesis. References for fm_{AO} data are available in the Suppl. Table S3. When fm_{AO} data from human mass balance studies were available, only those were used as the input in the $CL_{int,u,AO}$ calculation; otherwise, all fm_{AO} values in the Suppl. Table 3 were considered.

No	Substrate	Primary metabolic pathways	Dose (mg)	Subjects	Plasma clearance (mL/min/kg) (n; CV%)	Observed $CL_{int,u}$ (mL/min/kg)	fm_{AO}[a] Mean (min-max)	fm_{AO}[b] Mean (min-max)	Observed $CL_{int,u,AO}$ (mL/min/kg) Mean (min-max)
1	**6-deoxypenciclovir**	AO (Filer et al., 1994)	500[c] (Filer et al., 1994)	Healthy	209 (oral)	178	1	-	178
2	**BIBX1382**	AO, CYP2D6 (Hutzler et al., 2014a)	25-200 (Dittrich et al., 2002)	Cancer patients	25-55 (iv)	2327[d]	0.95 (0.92-0.97)	-	2199 (2141-2257)
3	**Capmatinib**	AO, CYP3A4 (Glaenzel et al., 2020)	200 (Chen et al., 2022), 400 (FDA, 2019), 600 (Glaenzel et al., 2020)	Healthy	8.59 (oral) (3; 19)	128	0.38 (0.08-0.65)	0.4	51.2[e]
4	**Carbazeran**	AO, UGT (Kaye et al., 1984)	1.28 mg/kg (Kaye et al., 1984)	Healthy	37.6 (iv)	1613[d]	0.77 (0.49-1)	-	1239 (790-1613)
5	**DACA**	AO, FMO, CYPs (Schofield et al., 1999)	18-1000 mg/m^2 (Kestell et al., 1999)	Cancer patients	13.3 (iv) (9; 5.73)	2135	0.76 (0.53-1)	-	1629 (1131-2135)
6	**Fasudil**	AO (Sanoh et al., 2012)	n/a (Nakajima, 1992; Sanoh et al., 2012)	Healthy	73.2 (iv)	449[d]	0.8	-	359

7	**FK3453**	AO (Akabane et al., 2011)	0.5-10 (Akabane et al., 2011)	Healthy	962 (oral) (3; 13)	4933	1	-	4933
8	**Idelalisib**	AO, CYP3A4, UGT1A4 (FDA, 2014)	150 (Jin et al., 2015a; Jin et al., 2015b)	Healthy	3.57 (oral)	36.7	0.29	0.29	10.6[e]
9	**JNJ38877605**	AO, CYP3A4 (Lolkema et al., 2015)	10-60 (Lolkema et al., 2015)	Cancer patients	12.0 (oral) (4; 22)	149	0.7	-	105
10	**Lenvatinib**	AO, CYP3A4, CYP1A2, CYP2B6, GSH (FDA, 2015)	5 (Shumaker et al., 2015b), 10 (Shumaker et al., 2015a), 24 (Shumaker et al., 2014)	Healthy	2.05 (oral) (3; 14)	86.5	0.27 (0.17-0.38)	0.22 (0.17-0.26)	18.6 (14.7-22.5)[e]
11	**LuAF09535**	AO (Jensen et al., 2017)	75 (Jensen et al., 2017)	Healthy	17857 (oral)	68681	0.87	-	59753
12	**Methotrexate**	AO, Folylpolyglutamate synthase (Choughule et al., 2015; Yamamoto et al., 2016)	3.6-4.8 (Kristensen et al., 1975)	Other[f]	1.90 (iv)	3.17	0.2	-	0.63
13	**O6-Benzylguanine**	AO, CYP1A2, CYP3A4(Roy et al., 1995)	10-80 mg/m^2 (Dolan et al., 1998)	Cancer patients	13.3 (iv) (4; 0.77)	320	0.81 (0.63-1)	-	259 (202-320)
14	**PF4217903**	AO (Cheng et al., 2013)	n/a (Zientek et al., 2010)	n/a	6.00 (oral)	37.5	1	-	37.5
15	**PF5190457**	AO, XO, CYPs (Adusumalli et al., 2019)	50- 300 (Denney et al., 2017)	Healthy	18.8 (oral) (4; 5)	156	0.25	-	39.1
16	**PF6273340**	AO and others (Skerratt et al., 2016)	50, 400 (Loudon et al., 2018)	Healthy	23.1 (oral) (2; 4)	144	0.27	-	39.0

17	**PF945863**	AO, N-demethylation (Strelevitz et al., 2012)	n/a (Zientek et al., 2010)	n/a	108 (oral)	196	0.83 (0.61-1)	-	162 (120-196)
18	**RS8359**	AO (Itoh et al., 2005)	50-200 (Püchler et al., 1997)	Healthy	9.28 (oral) (3; 23)	17.1	0.9	-	15.4
19	**XK469**	AO, conjugation (with glycine, taurine, and glucuronic acid) (Anderson et al., 2005)	9-346 mg/m^2 (Alousi et al., 2007)	Cancer patients	0.04 (iv) (11; 0.01)	6.26	0.98	-	6.13
20	**Zaleplon**	AO, CYP3A4 (Lake et al., 2002)	5 (Rosen et al., 1999)	Healthy	15.67 (iv)	187.9	0.72 (0.55-1)	0.66 (0.57-0.74)	123 (107-139)[e]
21	**Ziprasidone**	AO, CYP3A4 (Prakash et al., 1997; Obach et al., 2012)	5 (Miceli et al., 2005)	Healthy	5.08 (iv)	5012	0.37 (0.064-0.67)	0.67	3358[e]
22	**Zoniporide**	AO, hydrolysis (Dalvie et al., 2010)	80 (Dalvie et al., 2010)	Healthy	20.77 (iv)	344	0.68 (0.52-0.9)	0.61 (0.52-0.69)[g]	253 (217-238)[e]

Abbreviations: $CL_{int,u}$, unbound intrinsic clearance; $CL_{int,u,AO}$, unbound intrinsic clearance by aldehyde oxidase; fm_{AO}, fraction metabolized by aldehyde oxidase.
Observed $CL_{int,u}$ values after intravenous and oral administration were calculated using eqs. 2 and 3, respectively, after plasma clearances were corrected for renal/biliary excretion and blood/plasma ratio ($CL_H = (CL_{plasma} - CL_{renal/biliary})/B/P$).
a All fm_{AO} data
b fm_{AO} data from human mass balance/ADME studies
c Assuming all dosed famciclovir converted to 6-deoxypenciclovir
d The hepatic blood clearance was set at 90% of hepatic blood flow (i.e., 18.63 mL/min/kg)
e Only fm_{AO} data from human mass balance/ADME studies were utilized in $CL_{int,u,AO}$ calculations.
f Patients with arteriosclerotic disorders
g Considering the non-hepatic elimination (18%), the percentage of hepatic metabolic CL by AO (63-84%) have been used to calculate $CL_{int,u,AO}$. For other compounds with *in vivo* fm_{AO} data, renal CL was negligible.

TABLE 5

Bias and precision of in vitro to in vivo human hepatic unbound intrinsic clearance (for human hepatocytes) and human unbound intrinsic clearance by aldehyde oxidase (for human liver cytosols and human liver S9) predictions

Mean predicted and observed unbound intrinsic clearance values were used in these predictions.

	Human hepatocytes	**Human liver cytosols**	**Human liver S9**
Number of substrates	19	16	11
% Points out of 2-fold range	89.5	75.0	72.7
% Points out of 5-fold range	73.7	62.5	54.6
Geometric mean fold error (gmfe)	10.4	5.6	5.0
root mean squared error (rmse)	15812	925	1152
	After application of gmfe as an empirical scaling factor:		
Number of substrates	19	16	11
% Points out of 2-fold range	42.9	50.0	54.6
% Points out of 5-fold range	22.2	18.8	9.1
Geometric mean fold error (gmfe)	2.2	2.3	2.5
root mean squared error (rmse)	15451	1741	1018
Leave-one out cross validation (gmfe)	2.7	2.4	2.7

TABLE 6

Current status of in vitro and clinical methodology to estimate in vivo fraction metabolized by aldehyde oxidase fm_{AO}

	Description	**Current challenges**	**Key references**
In vitro	Estimating fm_{AO} by the ratio of $CL_{int,u,AO}$ to total $CL_{int,u}$ following physiological scaling of *in vitro* systems data.	Lack of reproducibility between studies AO activity thought to decline faster than that of CYPs following tissue isolation and processing, potentially leading to underestimation of fm_{AO} Inconsistencies relating to importance of aerobic vs anaerobic conditions particularly for reduction reactions	(Strelevitz et al., 2012; Abbasi et al., 2019; De Sousa Mendes et al., 2020; Toselli et al., 2022)
Human hepatocytes ± AO inhibitor	CL_{int} measured in human hepatocytes in the presence or absence of a selective AO inhibitor. Inhibited fraction will be fm_{AO}. Should be used in conjunction with inhibitors of other enzymes to delineate parallel pathways (e.g. 1-aminobenzotriazole for CYPs)	Hydralazine, the most used inhibitor: - mixed competitive (IC50= ~5 µM) and time-dependent inhibitor (K_i = 83 µM; k_{inact} = 0.063/min) of AO activity, thus AO inhibition may be sensitive to *in vitro* experimental conditions (e.g., pre-incubation or relay stage; length of co-incubation time) - Lacks specificity towards AO (e.g., IC50 for CYP2D6 = 18 µM), potentially leading to overestimation of fm_{AO} - AO inactivation by hydralazine may be reduced by glutathione Substrate dependent inhibition of AO may require use of multiple AO inhibitors Application of empirical scaling factor (10.4) for IVIVE of $CL_{int,u}$ is suggested and other pathways should be delineated to address uncertainty in fm_{AO}.	(Moriwaki et al., 2001; Nishimura and Naito, 2006; Hartmann et al., 2012; Strelevitz et al., 2012; Barr and Jones, 2013; Zientek and Youdim, 2015; Argikar et al., 2016; Dalvie and Di, 2019; Yang et al., 2019; Limaye, 2021; Toselli et al., 2022; Barnes et al., 2023)
Human liver S9 ± NADPH	CL_{int} measured in human liver S9 fraction in the presence or absence of CYP cofactor NADPH used to delineate between CYP and AO involvement. Potential contributions of other enzymes in S9 (e.g., xanthine oxidase) should be ruled out using specific inhibitors for robust fm_{AO}	Few studies to demonstrate validity. Presence/absence of cofactor alone is indicative but not conclusive Application of empirical scaling factors for IVIVE of AO (5.0) and non-AO-mediated $CL_{int,u}$ (2.8 (Wood et al., 2017)) from human liver S9 (without cofactor) and microsomes, respectively are suggested to improve fm_{AO} predictions.	(Crouch et al., 2018)

Human liver cytosol	CL_{int} measured in human liver cytosol and microsomes for AO and non-AO contributions, respectively. Fm_{AO} estimated following physiological scaling of CL_{int} data. Potential contributions of other enzymes in cytosol (e.g., xanthine oxidase) should be ruled out using specific inhibitors for robust fm_{AO}	CL_{int} in human liver microsomes may be partially due to AO due to presence of AO in this fraction, most likely due to residual contamination. Potentially underestimates fm_{AO} Application of empirical scaling factors for IVIVE of AO (5.6) and non-AO-mediated $CL_{int,u}$ (2.8 (Wood et al., 2017)) from human liver cytosols and microsomes, respectively are suggested to improve fm_{AO} predictions.	(Barr et al., 2013; Xie et al., 2019)
Clinical study with reverse translation	Building a mechanistic static or PBPK model that utilizes prior clinical information of the drug/system by reverse translation	May depend on high fidelity PBPK model, requiring suitable clinical pharmacokinetic data (e.g., after intravenous administration) Delineation of hepatic vs. extrahepatic contributions	(Rostami-Hodjegan, 2018; Kozminski et al., 2021)
DDI study	Reverse translation of clinical pharmacokinetics data in absence and presence of selective AO or CYP inhibitor using mechanistic static models or PBPK modelling	Lack of selective clinical index inhibitors for AO Lack of clinically relevant DDIs attributed to AO inhibition. Use of indirect evidence with CYP inhibitor requires assumption that AO is the only non-inhibited pathway, or other means to delineate other metabolic routes	(Miceli et al., 2000).
ADME/ mass balance study	Metabolite profiling of excreta following administration of radiolabelled drug Possibility to combine with an absolute bioavailability investigation using microtracer approach	Incomplete collection of excreta can confound interpretation. *In vitro* reaction phenotyping required to identify enzyme(s) mediating each specific pathway, including sequential metabolism	(Prakash et al., 1997)