PBPK modeling for drugs cleared by non-CYP enzymes: state-of-the-art and future perspectives.

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Administration; FMO, flavin-containing monooxygenase; \( f_m \), fraction metabolized by the specified enzyme; \( F_a \), fraction of compound absorbed; \( f_{u,p} \), free fraction in plasma; HLC, human liver cytosol; HLM, human liver microsomes; HLS9, human liver S9 fraction; IVIVE, \textit{in vitro-in vivo} extrapolation; \( k_a \), absorption rate constant; \( k_{cat} \), catalytic rate constant; \( K_i \), \textit{in vitro} inhibitory constant; \( K_p \), total concentration in tissue to plasma ratio; LC-MS/MS, liquid chromatography-tandem mass spectrometry; logP, partition coefficient between octanol and water at near infinite dilution or lipophilicity; MPPGL, microsomal protein per gram of liver; NAPQI, N-acetyl-p-benzoquinone imine; NSAID, non-steroidal anti-inflammatory drug; PBPK, physiologically-based pharmacokinetics; PK, pharmacokinetic; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; Qh, hepatic blood flow; RAF, relative activity factor; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase; \( V_{max} \), maximal rate of metabolism; \( V_{ss} \), volume of distribution at steady state.
Abstract

Physiologically-based pharmacokinetic (PBPK) modeling has become the established method for predicting human pharmacokinetics (PK) and drug-drug interactions (DDI). The number of drugs cleared by non-CYP enzyme metabolism has increased steadily and to date, there is no consolidated overview of PBPK modeling for drugs cleared by non-CYP enzymes. This review aims to describe the state-of-the-art PBPK modeling for drugs cleared via non-CYP enzymes, to identify successful strategies, to describe gaps and to provide suggestions to overcome them. To this end, we conducted a detailed literature search and found 58 articles published before the 1st of January 2023 containing 95 examples of clinical PBPK models for 62 non-CYP enzyme substrates. Reviewed articles covered the drug clearance by uridine 5'-diphospho-glucuronosyltransferases (UGTs), aldehyde oxidase (AO), flavin-containing monooxygenases (FMOs), sulfotransferases (SULTs) and carboxylesterases (CES), with UGT2B7, UGT1A9, CES1, FMO3 and AO being the enzymes most frequently involved. In vitro-in vivo extrapolation (IVIVE) of intrinsic clearance and the bottom-up PBPK modeling involving non-CYP enzymes remains challenging. We observed that the middle-out modeling approach was applied in 80% of the cases, with metabolism parameters optimized in 73% of the models. Our review could not identify a standardized approach used for model optimization based on clinical data, with manual optimization employed most frequently. Successful development of models for UGT2B7, UGT1A9, CES1, and FMO3 substrates provides a foundation for other drugs metabolized by these enzymes and guides the way forward in creating PBPK models for other enzymes in these families.
Significance Statement

Our review charts the rise of PBPK modeling for drugs cleared by non-CYP enzymes. Analyzing 58 articles and 62 non-CYP enzyme substrates, we found that UGTs, AO, FMOs, SULTs, and CES were the main enzyme families involved and that UGT2B7, UGT1A9, CES1, FMO3 and AO are the individual enzymes with the strongest PBPK modeling precedents. Approaches established for these enzymes can now be extended to additional substrates and to drugs metabolized by enzymes that are similarly well characterized.
1. Introduction

Physiologically-based pharmacokinetic (PBPK) modeling is established as a valuable tool to support drug development and its importance has increased over the last years (El-Khateeb et al., 2021). Due to the predictive performance of PBPK modeling and its ability to bring together information about drug properties and human biology, regulatory authorities encourage its use in drug development and regulatory filing. Indeed, between 2008 and 2018, the number of new drug applications submitted to the United States Food and Drug Administration (FDA) which contained PBPK analyses increased from 1 to 27 per year (Grimstein et al., 2019; Jamei, 2016; Maharaj et al., 2013). PBPK modeling supports drug development by predicting drug-drug interactions (DDI) (Wagner, Pan, et al., 2015), extrapolating pharmacokinetic (PK) data obtained in healthy adults to special populations (e.g., neonates, children, pregnant population, hepatically or renally impaired patients) (Alasmari et al., 2022; Cleary et al., 2021; Le Merdy et al., 2021) and predicting population variabilities (Chetty et al., 2014). So far, the use of PBPK modeling has been described mainly for drugs where clearance (CL) is mediated by cytochrome P450 enzymes (CYPs). This is due to the high number of drugs whose clearance is mainly CYP-mediated and the advanced status of in vitro tools to study CYPs (Saravanakumar et al., 2019; Wagner, Zhao, et al., 2015; Zhao et al., 2012). Reviews from Saravanakumar et al., Cerny et al. and Yu et al., (Cerny, 2016; Saravanakumar et al., 2019; Yu et al., 2019; Yu et al., 2016; J. Yu et al., 2017; Yu et al., 2018) have reported that ~30% of the FDA-approved small molecule drugs have a greater than 25% contribution of non-CYP enzymes to clearance and similar findings have been reported for the 200 most prescribed small molecule drugs (Saravanakumar et al., 2019). However, to date, there is no consolidated overview of PBPK modeling for non-CYP enzymes. Therefore, it is important to understand the state-of-the-art, to identify current capabilities, clarify and address current gaps and to propose methods to overcome them (Basit et al., 2020).

Bottom-up modeling, where human PK is predicted directly from non-clinical data remains challenging for drugs cleared by non-CYP enzymes due to multiple gaps. The main gap is the limitation of in vitro-in vivo extrapolation (IVIVE) methods, which are mostly established and validated for CYP-mediated metabolism (Argikar et al., 2016; De Sousa Mendes et al., 2020; V. P. Reddy et al., 2018). In theory, the IVIVE approach to scale in vitro intrinsic clearance to whole liver in vivo clearance is based on biochemical and
physiological scaling factors and should be applicable for all hepatic enzymes and multiple in vitro systems such as microsomes, hepatocytes or cytosolic fractions (Howgate et al., 2006; Vasilogianni et al., 2021). However, in the case of non-CYP enzymes, such scaling factors are not well-validated for each isoform (Takahashi et al., 2021). For instance, the fact that many drugs are substrates of multiple non-CYP enzyme isoforms and specific inhibitors are lacking for many enzymes complicates the estimation of the relative contributions of different enzymes to clearance, or fraction metabolized ($f_{\text{m}}$), and extrapolation of isolated enzyme experimental intrinsic clearance ($CL_{\text{int}}$) data (Argikar et al., 2016; Dhuria et al., 2021).

Furthermore, non-CYP enzymes may have a greater extra-hepatic expression than CYPs (e.g., aldehyde oxidases (AO) are also expressed in the kidney and intestine, carboxylesterase 1 (CES1) in the lung, uridine 5'-diphospho-glucuronosyltransferase 2B7 and 1A9 (UGT2B7 and UGT1A9) in the kidney), which complicates systemic IVIVE, especially in the absence of established cellular models for kidney or lung metabolism (Argikar et al., 2016; Fan et al., 2010; Garattini et al., 2009; Riches et al., 2009). Therefore, although reliable extra-hepatic IVIVE is important, it remains difficult (Argikar et al., 2016; De Sousa Mendes et al., 2020). Uncertainty exists in the population expression levels for some non-CYP enzymes (Takahashi et al., 2021) and also in reported liver abundances for some non-CYP enzymes, as shown by Wegler et al. (Wegler et al., 2017), UGT1A1, UGT1A3, UGT2B7 and UGT2B15 exhibited significant inter-laboratory differences. To review the current status of PBPK modeling for drugs with significant involvement of non-CYP enzymes, the literature was searched for reports of PBPK modeling for drugs cleared by the five non-CYP enzyme families most commonly involved in drug clearance, namely uridine 5'-diphospho-glucuronosyltransferases (UGTs), aldehyde oxidases (AOs), flavin-containing monooxygenases (FMOs), sulfotransferases (SULTs) and carboxylesterases (CES). The resulting papers have been summarized and the reported models, applications, drugs and populations were analyzed. Then, gaps and challenges in non-CYP enzyme PBPK modeling were evaluated and the merits of different PBPK modeling approaches were discussed. Finally, published PBPK models involving non-CYP enzymes were summarized and key articles highlighted.
2. Research method

2.1 Non-CYP enzyme selection

Saravanakumar et al. (Saravanakumar et al., 2019) reviewed the major enzymes involved in small molecule drug metabolism. They found that between 2005 and 2016, 28% of FDA-approved drugs had at least one non-CYP enzyme responsible for at least 25% of the drug’s elimination. For these drugs, 38% were UGT substrates, 10% were CES substrates, 6% were cleared by AO, 6% were FMO substrates and 3% were SULT substrates. Saravanakumar et al. (Saravanakumar et al., 2019) also observed similar proportions reported for the “Top 200 most prescribed drugs” and confirmed the findings of a previously published evaluation by Cerny et al. (Cerny, 2016) which indicated that between 2006 and 2015, non-CYP enzymes were responsible for the formation of major metabolites (≥10% of dose) for 29.9% of the FDA-approved small molecule drugs. Similar results were also presented in multiple surveys done by Yu et al. (Yu et al., 2019; Yu et al., 2016; J. Yu et al., 2017; Yu et al., 2018) on FDA-approved drugs from 2013 to 2017.

2.2 Search criteria

Searches were performed on PubMed (PubMed) until the 1st of January 2023 following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram for inclusion and exclusion of studies, as follows:

1. Identification of articles on PubMed with the following keywords:
   - ((PBPK) OR (Physiologically Based Pharmacokinetic)) AND ((UGT) OR (UDP-glucuronosyltransferase)), delivered 101 results.
   - ((PBPK) OR (Physiologically Based Pharmacokinetic)) AND ((AO) OR (aldehyde oxidase)), delivered 23 results.
   - ((PBPK) OR (Physiologically Based Pharmacokinetic)) AND ((FMO) OR (flavin-containing monooxygenases)), delivered 9 results.
   - ((PBPK) OR (Physiologically Based Pharmacokinetic)) AND ((SULT) OR (sulfotransferase)), delivered 20 results.
- ((PBPK) OR (Physiologically Based Pharmacokinetic)) AND ((CES) OR (carboxylesterase)), delivered 96 results.

- Since CES is known to be involved in the activation of multiple pro-drugs, further searches were made to find articles on CES-activated prodrugs by using the following keywords on PubMed: ((PBPK) OR (Physiologically Based Pharmacokinetic)) AND ([Name of the prodrug]), using a list of CES-activated prodrugs provided by Her and Zhu et al. (Her & Zhu, 2020), which delivered a total of 103 results.

Exclusion if:

- Articles are not about PBPK modeling
- Articles do not include a non-CYP enzyme
- Articles are not written in English

2. Screening for relevance on the abstract and the main text to determine if the article should be integrated or not.

Exclusion if:

- PBPK models are only developed on non-human populations
- Clinical data are not used to verify the PBPK models.
- Included non-CYP is not a primary metabolizing enzyme

3. Final set of reviewed articles.

After exclusions, 58 articles presenting 95 PBPK models for 62 compounds remained and are further described in this manuscript (more details are available in the Supp. Table 1). The source articles spanned publication years 2011–2022. Although no time limit was defined in the searches, no articles were identified that preceded 2011.
3. State-of-the-art, challenges and knowledge gaps

3.1 Overview of reviewed PBPK articles

Our review confirmed the increasing interest in non-CYP enzyme PBPK modeling. A review by El-Khateeb et al. (El-Khateeb et al., 2021) showed a trend of an increasing number of PBPK publications per year, which passed from 36 articles in 2011 to 104 articles in 2019. The non-CYP enzyme PBPK publications increased in a similar manner but started at a later date. The percentage increased from 3 to 6% of the total PBPK publications (Figure 1A). Additionally, a more detailed analysis indicated a disparity in the represented non-CYP enzymes. Among non-CYP enzyme families, UGTs were the most studied with 23 articles, closely followed by CES with 21 articles (Figure 1B). High UGT representation is not surprising since this enzyme family is the most important after CYPs (Bhutani et al., 2021; Saravanakumar et al., 2019). UGT2B7 and UGT1A9 were the main studied isoforms among UGT enzymes, in fact, of the 25 UGT substrates studied 20 were substrates of UGT2B7 or UGT1A9 or both and CES1 was the most studied CES isoform.

In addition to the evolution of non-CYP enzyme PBPK modeling with time, the studied populations were also reviewed. The most frequently studied population was healthy adults, closely followed by pediatrics (Figure 2) while modeling in special populations such as cancer patients and pregnant women is still developing, with relatively few published models.

Furthermore, we observed that a significant number of compounds were studied several times by different teams, for example six oseltamivir models, four lorazepam, morphine, raltegravir models and irinotecan models were observed (Table 1). Although previously published work may influence the approach taken in subsequent PBPK modeling, the new models can often be considered as further validation of the models they are based on, consolidating knowledge for the compound of interest and the non-CYP enzymes responsible for its metabolism. For example, in the case of morphine, a substrate of UGT2B7, the Emoto et al. (Emoto et al., 2017) initial bottom-up PBPK model was later used by Uchaipichat et al. (Uchaipichat et al., 2021) for additional DDI evaluation, which helped to validate and consolidate the model for morphine and UGT2B7. This same pattern exists for lorazepam (UGT2B7, UGT2B15), zidovudine (UGT2B7), clopidogrel (CES1), methotrexate (AO), ethionamide and itopride (FMO3), which are the most...
modeled compounds in this review. The complete list of compounds studied in the review is given in Table 1. More details on the studied compounds such as therapeutic class, molecular weights, lipophilicity (logP), pKa and free fraction in plasma ($f_{u,p}$) are presented in Supp. Table 2.

We were also interested in understanding how the non-CYP enzyme PBPK models had been applied. Of the presented 95 non-CYP enzyme PBPK models, our review highlighted DDI evaluation and pediatric PK prediction as the major application areas, each accounting for ~30% of the published models. These were followed by the evaluation of polymorphisms, accounting for 15% of models and extra-hepatic metabolism and organ-impairment accounting for 7% of models. The details of each application with the key comments are given in Table 2 and Figure 3. Additionally, the principal expression site of the main reviewed non-CYP enzymes are presented in Supp. Table 3.

3.2 PBPK modeling strategies: middle-out modeling to overcome bottom-up limitations

A bottom-up approach is the fundamental method to develop PBPK models based only on non-clinical data. Bottom-up modeling can be performed to predict human PK for drug candidates which can later be validated using clinical data. The middle-out approach starts with the bottom-up approach, but then proceeds to the optimization of one or more model parameters using clinical data and the optimized model is then used to simulate other clinical scenarios (Rostami-Hodjegan, 2018). In 45 of the 58 reviewed articles (78%) and 77 of the 95 (80%) PBPK models, the middle-out approach was used (Figure 4). 73% of the middle-out models optimized metabolism or excretion-related parameters (e.g., $CL_{int}$, renal clearance ($CL_{renal}$), biliary clearance ($CL_{bile}$), maximal rate of metabolism ($V_{max}$), catalytic rate constant ($k_{cat}$), $f_{m}$), 20% optimized distribution-related parameters (volume of distribution at steady state ($V_{ss}$), total concentration in tissue to plasma ratio ($K_p$)) and 20% optimized absorption-related parameters (e.g., absorption rate constant ($k_a$), fraction of compound absorbed ($F_a$), intestinal permeability). Optimizations were often justified by the uncertainties in the fitted parameters or the need to fit observed clinical data. The optimization of parameters was done either by using optimization tools integrated into the PBPK software or, more often, manually. We did not observe an established or standardized method to select the parameters to optimize or to perform the optimization process. Among UGTs, the middle-out approach
was usually applied for UGT2B7 and UGT1A9, with 14 of the 18 UGT2B7 models and 9 of the 11 UGT1A9 models using middle-out optimization. For CES1 20 of 33 PBPK models were middle-out. FMO3 was involved in the metabolism of all 10 FMO substrates studied in this review and all the FMO3 PBPK models applied middle-out optimization. Finally, the middle-out approach was applied in 7 of the 8 PBPK models for AO substrates. The high representation of a few isoforms in PBPK modeling can be explained by the high relevance of these isoforms for drug metabolism; for example, UGT2B7 and FMO3 are known to be the most abundant isoforms in the liver and are involved in the metabolism of many drugs (Cashman & Zhang, 2006; John O. Miners et al., 2010). In addition, according to a recent review by Miners et al. (J. O. Miners et al., 2023) clinical DDI studies on UGT1A9 and UGT2B7 substrates are facilitated by the existence of inhibitors for these enzymes, such as probenecid for UGT2B7 and mefenamic acid for UGT1A9.

At the heart of bottom-up modeling are IVIVE methods. In theory, bottom-up IVIVE can be performed for all drug-metabolizing tissues such as the intestines, kidneys or lungs (De Sousa Mendes et al., 2020; Nguyen et al., 2018) but the main drug-metabolizing organ is the liver. To scale apparent unbound intrinsic clearance (CL\textsubscript{int,u,in vitro}) of a hepatic enzyme measured in an \textit{in vitro} system to the hepatic clearance (CL\textsubscript{hep,in vitro}) (Espie et al., 2009; Gerlowski & Jain, 1983) the required scaling factors depend on the used \textit{in vitro} system and will adjust CL\textsubscript{int,u,in vitro} for the amounts of metabolizing enzymes in this system relative to the \textit{in vivo} situation. CL\textsubscript{int,u,in vitro} measured in recombinant systems, human liver microsomes (HLM) or hepatocytes can be extrapolated to CL\textsubscript{hep,in vitro} using the \textit{in vivo} hepatic specific enzyme abundances (pmol of enzyme per gram of tissue), the microsomal protein per gram of liver (MPPGL), or the hepatocellularity (number of hepatocytes per gram of liver), respectively. When using cytosol for enzymes such as AO or SULTs, the cytosolic protein per gram of liver (CPPGL) is needed. The adjusted CL\textsubscript{int,u,in vitro} is further scaled up to the whole liver based on liver weight, then a hepatic model, typically a well-stirred model, is applied to account for the combined effect of hepatic blood flow (Q\textsubscript{h}) and protein binding (f\textsubscript{u,p}), resulting to an estimation of the \textit{in vivo} hepatic clearance (CL\textsubscript{hep,in vivo}). Other hepatic disposition models, such as the dispersion model and parallel-tube model, exist but are less used (M. B. Reddy et al., 2021). The bottom-up approach can be applied to estimate the f\textsubscript{in} of enzymes from \textit{in vitro} DDI data and these estimations can then be used to simulate further DDI situations and can be validated with clinical data.
from DDI evaluation studies. Reliable f_m values are important for predictive DDI simulations and extrapolation of PK profiles from adults to children based on ontogenies of the relevant enzyme isoforms. Information on the impact of disease on non-CYP enzymes is as yet only scarce, limiting the simulation of the specific PK populations. Nonetheless, DDI and pediatric PK evaluation for non-CYP enzymes were highly represented in our review (Figure 3).

In the middle-out approach, clinical PK data are used to refine certain parameters, such as CL_{int,u,in vitro}. Naturally, as seen previously, the middle-out optimization process is not limited to metabolism. Other parameters like the F_a or the V_{ss} can be optimized to fit the observed concentration profiles. However, for CL optimization, the so-called “retrograde calculation” method can be highlighted. The retrograde calculation of CL_{int,u,in vivo} is a type of middle-out approach that is often used. CL_{hep,in vivo} is back-calculated from the observed systemic CL by first subtracting any potential extrahepatic CL and then using a hepatic model, such as the well-stirred model (Equation 1). The well-stirred model was systematically applied for retrograde calculations in the reviewed articles.

\[
CL_{hep,in vivo,plasma} = Q_h \left[ \frac{f_{a,p,m} \cdot CL_{int,u,in vivo}}{Q_h + f_{a,p,m} \cdot CL_{int,u,in vivo} / (C_{B/C_p})} \right]
\]

Note: the initial well-stirred model relies on blood concentrations with CL_{hep,in vivo} = CL_{hep,in vivo,blood} \cdot (C_B / C_p) and f_{a,p,m} = f_{a,blood} \cdot (C_B / C_p).

Furthermore, the retrograde calculation of enzymatic CL_{int,u} can be assigned to each enzymatic pathway by applying f_m values. For example, Zhou et al. (Zhou et al., 2017) back-calculated FMO3 CL_{int,u} values from clinical data using Equation 1 to build a model to evaluate the impact of FMO3 polymorphisms on itopride exposure. Yu et al. (Y. Yu et al., 2017) used the same approach to obtain SULT2A1 and CYP3A4 CL_{int,u} values to develop their palbociclib model and then evaluated DDI. For AO substrates, methotrexate and 6-mercaptopurine (6-MP), Ogungbenro et al. (Ogungbenro et al., 2014a, 2014b) applied the retrograde calculation to obtain hepatic and biliary CL_{int,u}. 

\[
\text{CL}_{\text{hep,in vivo,plasma}}: \text{in vivo hepatic clearance based on plasma concentration}; \text{CL}_{\text{int,u,in vivo}}: \text{apparent unbound intrinsic clearance}; Q_h: \text{hepatic blood flow}; f_{a,p,m}: \text{free fraction in plasma}; C_{B/C_p}: \text{total blood to total plasma drug concentration ratio.}
\]
In the case of non-CYP enzymes, the IVIVE has typically been performed following the process established for CYPs. However, in contrast to CYPs, IVIVE for non-CYP enzymes using isolated enzyme systems is limited by high variability in the activities of different enzyme preparations and uncertainty as to the correct enzyme abundance information for the metabolizing organs in vivo (M. B. Reddy et al., 2021) and the difficulty in quantifying most non-CYP enzyme concentrations in vitro. It is possible to obtain a direct and reliable measure of the enzyme’s abundance based on the quantification of specific peptides from proteomic measurement via liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Ahire et al., 2022). However, this method is not yet fully standardized for non-CYP enzymes because different peptide standards and bioanalytical methods may be used for measurements, which can lead to differences in the reported abundance values (Achour et al., 2014; Achour et al., 2018; Takahashi et al., 2021; Wegler et al., 2017). Antibody-based proteomic quantifications are also possible but suffer from the lack of isoform-specific antibodies.

We observed that the most modeled enzyme isoforms in this review were those where specific substrates are established as clinically used drugs, such as morphine or zidovudine for UGT2B7, dapagliflozin or propofol for UGT1A9, ethionamide for FMO3, methotrexate for AO or methylphenidate for CES1. When selective substrates appear for additional non-CYP enzymes, these can be used to validate scaling from in vitro to in vivo (J. O. Miners et al., 2023; M. B. Reddy et al., 2021; Takahashi et al., 2021). In addition to the lack of enzyme isoform-specific substrates, the lack of specific inhibitors complicates the estimation of reliable f_m values and limits the IVIVE for drugs that are substrates of multiple non-CYP enzymes. Therefore, IVIVE is performed with increasing confidence for drugs that are substrates of certain non-CYP enzymes but large uncertainty remains for many non-CYP enzyme substrates and our review showed that middle-out modeling was often applied in these cases. It is then interesting to analyze some of the bottom-up models to understand this more fully. For morphine, a large proportion of the models were bottom-up and predicted clinical data to within 2-fold (Emoto et al., 2017; Emoto et al., 2018). UGT2B7 is the main metabolizing enzyme, with only 10% of unchanged morphine eliminated by renal extraction (Hasselstrom & Sawe, 1993) and studies by Yue et al. (Yue et al., 1988) indicated that even though UGT2B7 has renal expression, the metabolism in human kidney microsomes is very minor. Therefore, morphine can be considered as a compound principally eliminated by one enzyme isoform, UGT2B7, in one tissue, the liver.
and this facilitates the IVIVE process. For example, the bottom-up model of Emoto et al. (Emoto et al., 2017; 2018) was used to evaluate the age-dependency of morphine PK. The CL\textsubscript{int} of morphine was obtained in HLM systems and scaled up to the liver in adults before translation to pediatrics. In the case of CES1, Mallick et al. (Mallick et al., 2020) developed bottom-up PBPK models for eight pyrethroids. They assumed that the metabolism of these compounds was only by hepatic CES1 and CYP enzymes and they generated CL\textsubscript{int} values in HLM and cytosol \textit{in vitro} systems. Again, the bottom-up IVIVE was facilitated by considering only one metabolizing tissue and a limited number of metabolizing isoforms. PBPK models developed for compounds metabolized by multiple enzyme isoforms in multiple tissues more often required optimization. For example, lorazepam, a substrate of UGT2B7 and UGT2B15, shows both liver and kidney metabolism (Docci, Umehara, et al., 2020) and the PBPK models developed for this compound by different teams (Docci, Umehara, et al., 2020; Maharaj et al., 2013; Mahmood et al., 2017; M. B. Reddy et al., 2021) all required optimization of the CL\textsubscript{int}. Therefore, it becomes clear that IVIVE for non-CYP enzymes is more challenging when multiple enzymes and metabolizing tissues are involved. Middle-out models then require more clinical data for verification such as DDI studies or studies in different enzyme polymorphic groups.

The middle-out optimizations are mostly applied at a drug-specific level. Nevertheless, it can be applied to derive scaling factors which can then be used at an isoform-specific level if a similar test system is used (eg.: pooled HLM or pooled human hepatocytes). Indeed, the gap observed between CL\textsubscript{int,u} obtained via retrograde calculation and CL\textsubscript{int,u} via \textit{in vitro} systems can be considered an empirical scaling factor (Howgate et al., 2006). If this scaling factor is calculated for a probe substrate known to be metabolized by a specific non-CYP enzyme, it can be considered to be an enzyme isoform-specific scaling factor and can then be used to correct the bottom-up CL\textsubscript{int,u} of other compounds that are cleared by the same enzyme, if data for these compounds has been generated using the same reagents under equivalent conditions. Since these scaling factors would be impacted by inter-laboratory variabilities, standardization of the selective substrates to use for each enzyme isoform and agreement on the experimental concentrations should be the aims rather than fixing scaling factors. Therefore, middle-out adjustment applied to models of selective substrates of non-CYP enzymes can be used to reduce the uncertainty in future bottom-up PK predictions for other substrates (Ogungbenro et al., 2014a; Rostami-Hodjegan, 2018). This was shown by
De Sousa Mendes et al. (De Sousa Mendes et al., 2020) who worked with six AO substrates and calculated \textit{in vitro} CL\textsubscript{int,u} in human liver cytosol (HLC) and human liver S9 fractions (HLS9) and used the retrograde CL\textsubscript{int,u} derivate from literature clinical data. They showed that applying an average scaling factor of 4.6 significantly improved the PBPK predictions. However, they also highlighted the need for each laboratory to calculate their own scaling factor to integrate laboratory-specific and protocol-specific variabilities. To further explore the possibility of using isoform-specific scaling factors to improve PBPK model development when clinical data are rare, we consulted the Certara\textsuperscript{®} Drug Interaction Data Base (DIDB) to obtain reported \textit{in vitro} CL\textsubscript{int} values for dapagliflozin and mirabegron, the substrate of two of the most studied UGTs, UGT1A9 and UGT2B7, respectively. A search with the name of both compounds was done on the \textit{in vitro} Parameter Query section (DIDB) and reported \textit{in vitro} CL\textsubscript{int} and V\textsubscript{max} in liver microsomes were observed. For mirabegron, three CL\textsubscript{int} results were found (11, 14.1 and 0.169 \(\mu\text{L/min/mg}\)) and for dapagliflozin, one CL\textsubscript{int} (3.48 \(\mu\text{L/min/mg}\)) and two V\textsubscript{max} (2.80 and 60.8 pmol/min/mg protein) results were found. First, a high variability in \textit{in vitro} measurement was seen depending on the experimental conditions. Secondly, a difference of \(\sim 10\) and \(\sim 3\) fold was observed between the retrograde CL\textsubscript{int} from clinical data (Callegari et al., 2021; Konishi et al., 2019b) and the \textit{in vitro} CL\textsubscript{int} (DIDB), respectively. With additional probe substrates these data could be used to obtain empirical scaling factors for different enzyme isoforms.

Finally, we can cite two alternative methods, which have sometimes been used to fill some of the observed gaps. First, the relative activity factor (RAF) approach can give more flexibility in IVIVE by assuming that a drug candidate interacts with the test systems in the same way as an enzyme-selective probe. Then, the RAF is calculated as the ratio of specific probe substrate activity in HLM to the activity in the recombinant system and used as a transition factor to calculate the drug candidate activity in one test system by knowing its activity in another (De Bruyn et al., 2016; Stringer et al., 2009). Even if the RAF approach is not always possible due to the absence of specific substrates for many non-CYP enzymes, it can be used to adjust for differences in intrinsic activity between recombinant systems and HLM and, therefore, is a good example of how standardized factors for a specific enzyme can facilitate the IVIVE of multiple drug candidates cleared by this enzyme (Gibson et al., 2013; Milani et al., 2020). Secondly, reverse transcription polymerase chain reaction (RT-PCR) can be used as an alternative to proteomic
measurement (Takahashi et al., 2021). This technique permits measurement of the amount of mRNA of each isoform instead of directly measuring the enzyme levels. However, as an assessment of protein expression this is limited since the correlation between mRNA and protein abundance is often poor.
4. Selected case studies in PBPK modeling, grouped by non-CYP enzyme

In the following sections, case studies are highlighted to show the performance of PBPK models for non-CYP enzymes with a focus on the gaps identified above including enzyme tissue abundance, IVIVE of CL and justification for model optimization. Additional information on all the reviewed articles is presented in Supp. Table 1.

**UDP-glucuronosyltransferase**

UDP-glucuronosyltransferases (UGTs) are microsomal glucuronosyltransferases and are one of the most important drug-metabolizing enzyme families besides CYPs (Bhutani et al., 2021; Saravanakumar et al., 2019). They are expressed in the liver but also have a significant extra-hepatic expression in tissues such as the intestines, kidneys and lungs (e.g. additionally to the liver UGT2B7 and UGT1A9 are expressed in the kidney and UGT1A1 in the gastro-intestinal tract) (Bigo et al., 2013). A total of twenty-three articles on PBPK modeling of drugs primarily metabolized by UGTs and published between 2013 and 2022 were reviewed. 21 of 23 articles were published between 2016 and 2022, indicating the recent development in this area. Morphine and lorazepam were the most studied compounds accounting for four papers each. Among the UGTs, UGT2B7 followed by UGT1A9 were the most studied isoforms. Fourteen papers described the use of Simcyp® (Simcyp®) for the modeling, six papers used PK-Sim® (PK-Sim®), two used GastroPlus® (GastroPlus®) and one used MATLAB® (MATLAB®). Fourteen of the articles used a middle-out approach with CL\textsubscript{int} and tissue/plasma partition coefficients (K\textsubscript{p}) or V\textsubscript{ss} most regularly being the parameters optimized to improve the fit with observed data. In these studies, in vitro CL\textsubscript{int} values in HLM and recombinant UGT isoenzymes were used as inputs for modeling. Healthy adults were the most commonly modeled population followed by the pediatric population.

Models for morphine (UGT2B7 substrate) were built by following a bottom-up approach in three out of four articles. The Emoto et al. (Emoto et al., 2017; 2018) model was also used by Uchaipichat et al. (Uchaipichat et al., 2021) to study morphine as a victim of DDI interaction due to the inhibitory effect of fifteen non-steroidal anti-inflammatory drugs (NSAIDs) on glucuronidation. The authors based their bottom-up model on literature HLM data and generated the in vitro inhibitory constant (K\textsubscript{i}) values for each NSAID. In the case of lorazepam, a substrate of UGT2B7 and UGT2B15, all articles used the middle-out
approach to achieve a better estimation of in vivo CL. Maharaj et al. (Maharaj et al., 2013) developed a lorazepam PBPK model where $CL_{\text{int}}$, logP and $K_p$ were manually optimized to improve the simulation of concentration-time data from four studies in adults. Ontogeny data were then integrated and the authors successfully predicted PK and age-dependent CL in a pediatric population. Docci et al. (Docci, Umehara, et al., 2020) combined in vitro $CL_{\text{int}}$ data obtained using HepatoPac® cell cultures (co-cultures of hepatocytes and stromal cells formed using a proprietary micro-patterning method) with clinical data to build a middle-out PBPK model. They acknowledged the challenge in modeling compounds metabolized by multiple UGTs due to the lack of isoform-selective inhibitors, which would help to estimate the $f_m$ of each isoform. The authors also indicated that bottom-up IVIVE of CL in microsomes and hepatocytes was challenging mainly due to an underprediction of hepatic CL but also due to the extra-hepatic expression of UGTs. They overcame this limitation, first by using HepatoPac® cells, which delivered a more accurate estimate of hepatic $CL_{\text{int}}$ compared to suspension hepatocytes (Docci, Klammers, et al., 2020) and secondly by applying a middle-out PBPK approach to estimate the extra-hepatic contributions. With this approach, $f_m$ values for each UGT isoform were derived from in vivo mass balance studies and were used to calculate isoform-specific $CL_{\text{int}}$ values. Then the isoform-specific $CL_{\text{int}}$ was attributed to extra-hepatic organs by using tissue-specific data on isoform expression for kidneys, liver and intestines assuming that all tissues have the same intrinsic activity per pmol of expressed UGT isoforms. The refined model was applied to predict the effect of UGT polymorphisms on the PK of lorazepam and its metabolite. Docci et al. estimated that the kidneys contribute 9% of the total systemic CL of lorazepam but that liver metabolism is predominant at 91%.

**Aldehyde oxidase**

Aldehyde oxidase (AO) is a molybdo-flavoenzyme that metabolizes aldehydes to carboxylic acids and also oxidizes N-containing heterocycles (Gordon et al., 1940; Montefiori et al., 2017; Pryde et al., 2010; Sodhi et al., 2015). AO is a cytosolic enzyme that is highly expressed in the liver and the kidneys, but can also be found in the respiratory system, epithelia of the small and large intestines, prostate and adrenal glands (Berger et al., 1995; Garattini et al., 2009). Three articles on PBPK modeling of drugs primarily metabolized by AO were reviewed. One paper described the use of MATLAB® (MATLAB®) for the modeling, one paper used Simcyp® (Simcyp®) and one used GastroPlus® (GastroPlus®). Methotrexate
was the most studied compound appearing in two articles. The main objective behind PBPK modeling was to improve AO IVIVE and then simulate PK in pediatric populations.

Ogungbenro et al. (Ogungbenro et al., 2014a, 2014b) developed PBPK models in MATLAB® in healthy adults and the pediatric population for methotrexate and 6-MP, medications used for autoimmune diseases and cancer, respectively. The authors applied a middle-out approach to back-calculate hepatic and biliary CL\text{int} from \textit{in vivo} data with the well-stirred model (Equation 1). Distribution parameters were also optimized, and the authors justified this by the non-linear distribution of methotrexate in some tissues, which is a source of variability. Finally, the final models were validated with additional clinical data. The authors showed a non-linear relationship between bioavailability and dose for methotrexate, which was caused by changes in the F_a. The adult model was scaled to the pediatric population by incorporating age-dependent changes in body size, organ volumes and blood flow. The elimination pathways in adults and children were assumed to be the same and allometric scaling of metabolic and biliary CL based on body size, organ volume and plasma flow was integrated. De Sousa Mendes et al. (De Sousa Mendes et al., 2020) presented PBPK models for six AO substrates: O6-benzylguanine, BIBX1382 (falnidadamol), carbazeran, zaleplon, ziprasidone and zoniporide. The objective of the study was to overcome a large underprediction of AO clearance when using IVIVE. First, the metabolism of substrates was measured in HLC and HLS9; the authors indicated that IVIVE using either system underestimated the \textit{in vivo} CL, but HLC provided better CL estimation than HLS9. Secondly, PBPK models were developed for each compound in Simcyp® and a laboratory-specific scaling factor was calculated with the ratio between predicted CL\text{int} and observed CL\text{int}, obtained from the literature review. An average scaling factor of 4.6, based on observed data, was reported to recover the observed \textit{in vivo} CL. The authors applied this scaling factor to optimized AO CL\text{int}, which significantly improved the model, with \textit{in vivo} CL predictions for all drugs within a 2-fold error. Therefore, De Sousa et al. recommend that each laboratory apply this method to determine their own AO laboratory-specific scaling factors. However, if no probe substrates are available, the authors recommend the use of the empirical scaling factor of 4.6 on AO CL\text{int} in HLC to predict \textit{in vivo} CL.
Flavin-containing monooxygenase

Flavin-containing monooxygenases (FMOs) are a subfamily of flavoprotein monooxygenases, belonging to the family of monooxygenase oxidoreductases (Hernandez et al., 2003; van Berkel et al., 2006). FMOs are involved in the oxidation of heteroatoms in a wide array of chemical groups such as amines, sulfides, and phosphates (Eswaramoorthy et al., 2006; Krueger & Williams, 2005). FMOs are predominately expressed in the liver (mainly FMO3) and kidneys (mainly FMO1) but also in the lungs, small intestine and brain (Cashman & Zhang, 2006). FMO1, 3 and 5 are the only functional isoforms in humans (Hines et al., 2002; Yeung et al., 2000). Four articles on PBPK modeling of drugs primarily metabolized by FMOs were reviewed. All presented models were built in Simcyp® (Simcyp®) between 2017 and 2019. FMO3 was the main studied isoform, all articles presented middle-out modeling strategies. Ethionamide and itopride were the most studied compounds, appearing in two articles each. The main objective behind PBPK modeling of FMO metabolized compounds was to have a better understanding of inter-individual variability by including the impact of FMO3 ontogeny and polymorphisms, especially the impact of the FMO3 null phenotype on PK, known to be responsible for the fish odor syndrome or trimethylaminuria disorder (Treacy et al., 1998).

Nguyen et al. (Nguyen et al., 2018; 2019) published two PBPK models for ethionamide, an antibiotic for tuberculosis treatment; one in healthy adults (Nguyen et al., 2019) and one in the pediatric population (Nguyen et al., 2018). Ethionamide is primarily eliminated by FMO3 and the authors wanted to study its ontogeny and the impact of FMO3 polymorphisms. A PBPK model for healthy adults was built with a middle-out approach. In vitro data from HLM incubations were used to obtain hepatic CL_{int} and pooled human lung microsomes were used to obtain the lung CL_{int} due to FMO3 and FMO2. Then, Caco-2 cell permeability was optimized using the sensitivity analysis and parameter estimation features in Simcyp® so that the simulations matched the clinical data. The adult model was validated with additional clinical data and then translated to the pediatric population (0-12 years old) using previously published FMO3 protein abundance data measured in microsomal samples taken from children aged from 8 weeks to 12 years old. In their second article, Nguyen et al. (Nguyen et al., 2019) reported inter-individual variability in ethionamide PK and characterized FMO3 polymorphisms in adults. The previously developed middle-out PBPK model was used to explore two common FMO3 polymorphisms using recombinant enzyme
systems. E158K and E308G genotypes were compared to the wild-type and the relative enzyme activities suggested that FMO3 activity would be approximately 60% lower in subjects with homozygous mutations in both alleles than in those carrying two wild-type alleles. Then, the DDI between ethionamide and methimazole (an FMO3 inhibitor) was simulated by integrating genotype-dependent enzyme abundance/activity and genotype frequency in their model. The \( K_i \) was generated by the authors by assuming a competitive inhibition model. An increase of ethionamide area under the curve (AUC) of 16% was predicted and was validated with clinical data. Zhou et al. (Zhou et al., 2017) also explored the impact of E158K and E308G polymorphisms using itopride – a treatment for functional dyspepsia and other gastrointestinal conditions. The authors developed a middle-out PBPK model in healthy adults where initial FMO3 CL\(_{\text{int}}\) values from HLM were obtained with retrograde calculation. The model was successfully able to predict observed AUC and \( C_{\text{max}} \) changes. Moreover, the model showed that FMO3 activity is 47% lower in subjects carrying the homozygous E158K/E308G mutation than in those who have two wild-type alleles. The authors highlighted that extrapolation from \textit{in vitro} CL\(_{\text{int}}\) obtained in HLM led to an under-prediction of 8-fold. According to the authors this can be due to the handling and storage of the tissue preparations. Indeed, FMO activity is sensitive to temperature, and HLM samples may have been compromised because of the storage conditions. Nevertheless, the misprediction via bottom-up methods could be corrected using the middle-out approach. Reddy et al. (V. P. Reddy et al., 2018) developed PBPK models for nine FMO1, FMO3, and FMO5 substrates using Simcyp® and calculated translational factors to convert kinetic values from recombinant FMO systems (rFMO) to HLM using \textit{in vitro} data obtained from the literature. The authors indicated that without using any scalar, the rFMO results deviated from observed data by 2.5-fold. According to the authors, it is necessary to compensate for differences in the intrinsic activity per unit enzyme of rFMO relative to HLM. Therefore, the authors applied a middle-out approach with clinical data from literature and automated sensitivity analysis or parameter estimation tools provided by Simcyp® in order to estimate the scaling factor to convert enzyme kinetic values obtained in different expression systems to the equivalent in HLM (rFMO scaling factor). The authors first calculated the rFMO scaling factors of 0.2 for itopride to recover the observed data. Subsequent application of this scaling factor to other FMO substrates resulted in better predictions.
Sulfotransferase

Cytosolic sulfotransferases (SULTs) catalyze sulfate conjugation of xenobiotics (B. H. Chen et al., 2015; Negishi et al., 2001; Rath et al., 2004). Six subfamilies of SULTs (SULT1 to SULT6) are identified in mammals, but SULT1 and SULT2 represent the two major subgroups in humans, commonly known as phenol (or aryl) and hydroxysteroid (or alcohol) SULTs respectively (B. H. Chen et al., 2015). SULTs are expressed in the liver and kidneys (mostly SULT1A1), but also in the small intestine and lungs (B. H. Chen et al., 2015; Fan et al., 2010; Riches et al., 2009). Metabolic SULT enzymes are expressed in the cytosol, and, therefore, S9 in vitro systems can be used to evaluate SULT-mediated metabolism. Recombinant in vitro systems are also used to study SULT polymorphisms (James & Ambadapadi, 2013). Four articles were found that focus on PBPK modeling of drugs metabolized by SULTs, developed on four different platforms: PK-Sim® (PK-Sim®), Phoenix® Winnonlin® (PhoenixWinnonlin®), Berkley Madonna® (BerkleyMadonna®) and Simcyp® (Simcyp®). 50% of the reviewed articles describe the scaling from rat to human while the middle-out approach was used in two articles to overcome gaps for CL estimation.

Mian et al. (Mian et al., 2020) identified a knowledge gap regarding paracetamol PK during pregnancy and developed a PBPK model in PK-Sim® for pregnant and non-pregnant women. The model was used to explore the impact of physiological changes and enzymatic modifications of UGT1A1, SULT1A1 and CYP2E1 during pregnancy. The PBPK model was first developed for non-pregnant women using a middle-out approach to refine drug-specific parameters (logP, intestinal permeability, and CLint of CYP2E1, UGT1A1 and SULT1A1). Thereafter, all the parameters were fixed and the model was extrapolated to pregnant women by incorporating changes in protein binding and UGT1A1 activity. The UGT1A1 expression increase during pregnancy was based on observations of free bilirubin concentrations in serum since this was assumed to be a surrogate for UGT activity. The authors did not input any expression change for SULT1A1 in pregnant women, arguing that the SULT1A1 activity in early pregnancy (10 weeks of gestational age) is similar to that in non-pregnant subjects and the expression of SULT1A1 is not affected by progesterone or estradiol. The PBPK models were able to simulate paracetamol PK successfully in both populations and confirmed the clinical observation of lower exposure in pregnant women. However, they did not recommend dose adjustment since the impact of pregnancy on the toxicodynamics of NAPQI (N-acetyl-p-benzoquinone imine; a toxic paracetamol metabolite) is...
unknown. Yu et al. (Y. Yu et al., 2017) developed a Simcyp® model for palbociclib – a medication against breast cancer, primarily metabolized by SULT2A1 and CYP3A4. The model was developed using a middle-out approach where four clinical studies were used to back-calculate CL_{int} values using the retrograde well-stirred model. An f_{m} of 53% was attributed to CYP3A4 based on in vitro data. Thereafter, the authors used the model to simulate the DDI potential and concluded that palbociclib PK would be strongly impacted by moderate and strong CYP3A inhibitors. Hao et al. (Hao, 2014) presented a bottom-up PBPK model for rhein in rats, developed in Phoenix® WinNonlin® and applied translation to humans to predict the first-in-human dose. In vitro rat liver microsomes and rat liver cytosol systems were used to obtain CYP, UGT and SULT CL_{int} values. Once the rat model was verified by comparing it to measured concentrations, the human PBPK model was built by applying human physiology, tissue distribution and drug-specific parameters. The metabolic CL values were obtained from in vitro HLM and HLC systems while the k_{a} and CL_{renal} were scaled from rats. The resulting simulations had a good prediction of experimental concentrations in Chinese adults and the authors’ conclusion highlighted the utility of PBPK to make PK predictions before dosing for first-in-human studies.

**Carboxylesterase**

Carboxylesterases (CES) are a multi-gene family of α,β-hydrolase-fold enzymes, which catalyze the hydrolysis of esters, amides, thioesters, and carbamates (Augusteyn et al., 1969; Laizure et al., 2013). Although CES often hydrolyzes drugs to inactive metabolites, they can also convert ester prodrugs into active metabolites (Laizure et al., 2013). CES are located mostly in the cytoplasm and endoplasmic reticulum of the liver and small intestine where they can contribute significantly to first-pass hydrolysis. They are also present in the kidneys and lungs (Hatfield et al., 2011; Holmes et al., 2010). In humans, CES1 and CES2 are the major isoforms. CES1 is mainly expressed in the liver while CES2 is expressed in both the liver and intestine. In the gut, CES2 can contribute significantly to the first-pass metabolic hydrolysis of substrate drugs and activation of prodrugs, while hepatic CES is more involved in systemic CL (Herbst et al., 2011; Hosokawa, 2008; Laizure et al., 2013; Taketani et al., 2007). Twenty-one articles focused on PBPK modeling of drugs metabolized by CES were reviewed; eight focused on drugs primarily metabolized by CES and thirteen focused on prodrugs activated by CES. Seven of these papers used PK-Sim® (PK-Sim®), six used Simcyp® (Simcyp®), three used GastroPlus® (GastroPlus®), three used
MATLAB® (MATLAB®), one used Napp® (Napp®) and one used acsIX®. The main objectives varied: authors used PBPK modeling to evaluate PK profiles in the pediatric population, to explore the impact of CES tissue abundance, and to investigate the impact of polymorphisms or the effects of disease (cirrhosis or influenza). In the current review, clopidogrel was the most studied CES substrate and oseltamivir was the most studied CES-activated prodrug, appearing in four and five of the twenty-one articles, respectively. The middle-out approach was used in seventeen of the twenty-one articles.

Loer et al. (Loer et al., 2022) used PK-Sim® to evaluate the DDI of clopidogrel in healthy adults. They developed a whole-body PBPK model including the metabolites: clopidogrel carboxylic acid, clopidogrel acyl glucuronide, 2-oxo-clopidogrel, and the active thiol metabolite. LogP, CES1/2 and CYP2C19/3A4 k_{cat}, intestinal permeability and dissolution were optimized based on multiple clinical datasets. The model was able to predict the PK and DDIs with bupropion, omeprazole, montelukast, pioglitazone, repaglinide, and rifampicin. Duong et al. (Duong et al., 2022) developed a PBPK model of clopidogrel in healthy adults. A minimal PBPK model was developed, followed by a middle-out approach with optimization of the absorption pathway and metabolism. The model was first developed to capture the clopidogrel metabolic pathways (CES1 and CYPs) for a European population and was then extrapolated to a Japanese population. The effects of fluvoxamine co-administration on CYP2C19 inhibition were captured, and population-specific factors such as age, sex, body mass index, body weight, cancer status, hepatic impairment and renal dysfunction were characterized. Hu et al. (Hu et al., 2014) used PK-Sim® to study oseltamivir CES1 hydrolysis in adults, looking into its inhibition by ethanol and the impact of a partial loss-of-function polymorphism (428G.A) associated with decreased in vitro hydrolysis. They used recombinant systems to generate data on ethanol inhibition and the impact of polymorphism, and then middle-out optimization was performed by running a sensitivity analysis and optimizing cellular permeability, intestinal permeability, dissolution parameters and V_{max} of CES1 against observed clinical data. They simulated changes in oseltamivir exposure when CES1 metabolism was impaired by ethanol or polymorphism. Parrott et al. (Parrott et al., 2011) investigated the PK of oseltamivir in infants and neonates with influenza using a middle-out PBPK strategy. First, a model for adult marmoset monkeys was built and refined using observed PK data. Then, human PK was predicted using the same refinements. The prediction of PK in children followed the same principle leveraging PK data in juvenile monkeys. Boberg et al. (Boberg et al.,
2017) explored the variability in CES tissue abundance by developing an oseltamivir PBPK model in Simcyp® based on proteomics data. CES1 and CES2 were quantified using LC-MS/MS in adult and pediatric livers. The abundance of CES1 showed an age-dependent maturation increasing by 5-fold in adults compared to neonates. In addition, CES-activity was correlated with protein abundance in pediatric and adult HLM. Oseltamivir’s physicochemical properties were obtained from Hu et al. (Hu et al., 2014) and Parrott et al. (Parrott et al., 2011).
5. Conclusion and perspective on future directions

The number of reported PBPK models for non-CYP enzyme substrates is growing steadily, reflecting trends in PBPK modeling use and the involvement of non-CYP enzymes in drug clearance. Bottom-up modeling was mainly performed for substrates of UGT2B7 (5 models) and CES1 (10 models), indicating precedence for bottom-up modeling for substrates of these enzymes. However, we observed that direct scaling from \textit{in vitro} remains challenging and that middle-out optimization was highly used. Optimizations were often done manually on an individual drug basis without apparent consensus on the optimal method, which can be a source of variability in resulting models. Systematic use and reporting of model sensitivity analysis and automated parameter optimization may help in developing systematized approaches and understanding the importance of individual assumptions in the final model. Increased publication of IVIVE for non-CYP enzyme metabolism will help to create a consensus about scaling factors. As shown by De Sousa Mendes et al. (De Sousa Mendes et al., 2020) for AO, it is possible to use isoform-specific empirical \textit{scaling factors} to improve bottom-up IVIVE. The availability of such standardized scaling factors would strengthen the basis upon which to develop a new drug PBPK model during drug development when clinical data for the new drug candidates are limited and, therefore, classical middle-out optimizations are difficult. Finally, we observed limited diversity in the modeled drugs. Therefore, we encourage more sharing of examples to cover an increased diversity of chemical classes. This would strengthen the field and facilitate the establishment of generalized modeling approaches and scaling factors for the non-CYP enzymes involved in their metabolism.

At present, the gaps in \textit{in vitro} experiments and IVIVE have resulted in a reliance on middle-out approaches for modeling non-CYP enzyme substrates in PBPK studies. This middle-out methodology inherently requires access to clinical data, preventing PBPK modeling during preclinical development. In the future, gaps in \textit{in vitro} experiments and IVIVE are likely to decrease. This will be aided by the progress in LC-MS-based proteomics methods, leading towards a consensus on non-CYP enzyme tissue expression, but also by the development of novel \textit{in vitro} methods such as co-culture systems (Kratochwil et al., 2017) which may allow better estimation of \textit{CL\_int} via non-CYP enzymes (Docci, Klammers, et al., 2020; Docci, Umehara, et al., 2020). It is to be expected that non-CYP enzyme PBPK modeling will continue to develop, with priority on enzymes highly involved in drug metabolism and where \textit{in vitro} tools
and clinical datasets facilitate the modeling. Future enzymes where PBPK modeling advances will be particularly valuable and those involved in metabolizing multiple marketed drugs such as UGT2B4 & UGT2B15 (Cashman & Zhang, 2006; John O. Miners et al., 2010). Furthermore, the existence of enzyme-specific inhibitors such as atazanavir (UGT1A1), probenecid and valproic acid (UGT1A4) or probenecid (UGT2B15) may facilitate progress in PBPK modeling to predict DDI involving these enzymes (J. O. Miners et al., 2023). We can also expect a growth in FMO3 studies, involved in the oxidation of multiple drugs and CES1 and CES2, responsible for the activation and metabolism of several substrates (Hernandez et al., 2003). The contribution of extrahepatic tissues to clearance of some drugs may be significant and needs to be predicted better. We anticipate that the growing number of individual drug case studies will help to validate the modelling approaches (e.g., accuracy of enzymes tissue abundances and scaling factors used) and that improved cellular models for measuring drug clearance in vitro will help improve intrinsic clearance estimates. Moreover, it's crucial to scrutinize the default PBPK assumption of uniform enzyme activity across tissues, a presumption that doesn't always hold for certain UGTs. In a recent study by Ahmed et al. (Ahmed et al., 2022), UGTs exhibited varying activity per unit of enzyme, even with higher abundances in the liver. For instance, UGT1A9 and UGT2B7 had a lower $k_{\text{cat}}$ value in the liver compared to the kidney, and UGT1A1 showed lower liver $k_{\text{cat}}$ values compared to the intestine or kidney. This tissue-specific variation may be linked to differing co-factor concentrations, such as UDP-glucuronic acid, which varies across tissues. Consequently, these findings challenge the accuracy of assigning liver metabolism contributions for UGT substrates, highlighting potential tissue-dependent $k_{\text{cat}}$, even within similar tissues under different disease states (Ahmed et al., 2022).

We also expect that with increased data and agreement on non-CYP enzyme IVIVE, non-CYP enzyme PBPK modeling can expand, this would include studying various populations, predicting polymorphism effects, and assessing perpetrator drug-drug interactions (DDIs).
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Data Availability Statement:

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

Authorship Contributions:


Performed data analysis: Agustos C. Ozbey.

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Legends for Figures and Tables

Figure 1 (A) Graph showing the cumulative number of PBPK publications per year (black squares and left axis) as reported by El-Khateeb et al. (El-Khateeb et al., 2021) and a cumulative number of PBPK articles on non-CYP enzymes (this study, blue circles and right axis). (B) Chart showing the number of articles, models and compounds identified in this review between 2011 and 2022, total and per non-CYP enzyme family. Note: Multiple models are presented per reviewed article. Consequently, the total count of models exceeds the number of articles, and similarly, the total number of compounds reviewed surpasses the number of articles included in the analysis.

Figure 2 Bar graphs presenting (A) the number of models per studied population and (B) the number of models per non-CYP enzyme family and per studied population. Note: For pediatric, organ-impaired and cancer populations a healthy adult model was first developed and then extended to these populations.

Figure 3 Bar chart presenting the number of articles per application grouped by non-CYP enzyme family. Note: “other” corresponds to various applications including extra-hepatic metabolism prediction, prediction of human PK from animal PK, bioequivalence study, prediction of the human first dose, adjustment of scaling factors to improve IVIVE, description of non-hepatic metabolism or prediction of PK in pregnant women (more details are available in Supp. Table 1).

Figure 4 PBPK modeling approaches per non-CYP enzyme in the 95 models reviewed. PBPK models where in vivo clinical data were used to optimize parameters were classified as “middle-out”. If no clinical data were used or were only used for validation, models were classified as “bottom-up”. NB: The capability of models to correctly predict observed data (e.g., within 2-fold) was not considered.
### Table 1 List of the compounds appearing in the reviewed articles per non-CYP enzyme family

<table>
<thead>
<tr>
<th>Number of models</th>
<th>UGT</th>
<th>CES</th>
<th>AO</th>
<th>FMO</th>
<th>SULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td></td>
<td>oseltamivir*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>lorazepam, morphine, raltegravir</td>
<td>irinotecan*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>zidovudine</td>
<td>clopidogrel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>buprenorphine, dolutegravir, mirabegron, naloxone, oxazepam, propofol, valproic acid</td>
<td>dabigatran etexilate, methylphenidate</td>
<td>methotrexate</td>
<td>ethionamide, itopride</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>bictegravir, cabotegravir, chloramphenicol, dabigatran, dapagliflozin, ertugliflozin, GDC-0810 (brilanestrant), haloperidol, ibuprofen, indomethacin, lamotrigine, midazolam, vericiguat, vorinostat</td>
<td>cyfluthrin, cyhalothrin, cyphenothrin, cis-permethrin, deltamethrin, enalapril*, esfenvalerate, MGS0274, mycophenolate</td>
<td>BIBX1382, (falnidamol), carbazeran, O6-benzylguaniline, zaleplon, ziprasidone, mofetil*, trans-permethrin, vicagrel**</td>
<td>benzydamine, clozapine, imipramine, moclobemide, palbociclib, tamoxifen, tozasertib</td>
<td></td>
</tr>
</tbody>
</table>

*CES-activated prodrugs (Her & Zhu, 2020), ** CES-activated prodrug, acetate derivate of clopidogrel

**Abbreviations:** AO = aldehyde oxidase; CES = carboxylesterase; FMO = flavin-containing monooxygenase; non-CYP = non-cytochrome P450; SULT = sulfotransferase; UGT = 5'-diphospho-glucuronosyltransferase.
Table 2: Major applications of non-CYP enzyme PBPK modeling

<table>
<thead>
<tr>
<th>Major PBPK Application</th>
<th>Minimal required data</th>
<th>Comments</th>
<th>Compounds modeled</th>
</tr>
</thead>
</table>
| Victim DDI prediction  | - PK profiles for victim drug alone  
- *In vitro* inhibition potency data for perpetrator drug,  
- \( f_m \) for victim drug (in the case of multi-enzyme metabolism) | - Most common application of non-CYP enzyme PBPK modeling.  
- Mechanistic prediction of DDI is very valuable for the quantification of DDI risk and potential waiver of clinical DDI studies.  
- Evaluations are done mainly in healthy adult volunteers with human PK data.  

*Non-CYPs: UGT, FMO, SULT, CES*                                                                 | 6-mercaptopurine (Ogungbenro et al., 2014b)  
dapagliflozin (Jo et al., 2021),  
eritugliflozin (Callegari et al., 2021),  
ethionamide (Nguyen et al., 2018),  
GDC-0810 (Y. Chen et al., 2018),  
lorazepam (M. B. Reddy et al., 2021),  
mirabegron (Konishi et al., 2019b),  
morphine (Uchaipichat et al., 2011),  
raltegravir (Stader et al., 2021),  
vericiguat (Frechen et al., 2020) |
| Pediatric PK prediction| - Adult PK profiles to validate the adult model  
- Ontogeny profiles of isoforms involved in the metabolism | - Second most common non-CYP enzyme PBPK application.  
- Helps to enhance and to some extent, replace pediatric clinical trials.  
- First, an adult model is developed and then an extrapolation to the pediatric population is done by incorporating ontogeny data.  

| | | | deltamethrin (Mallick et al., 2020),  
lorazepam (Maharaj et al., 2013; Mahmood et al., 2017),  
methotrexate (Ogungbenro et al., 2014a),  
morphine (Emoto et al., 2017; Emoto et al., 2018; Mahmood et al., 2017),  
oseltamivir (Bhatt et al., 2019; Boberg et al., 2017; Parrott et al., 2011),  
valproic acid |
The main goal is to propose a dose for the pediatric population. (Conner et al., 2018), vorinostat (Moj et al., 2017)

Non-CYPs: UGT, FMO, AO, CES

| Polymorphism-evaluation | Clearance data for each polymorphic enzyme isoform | Limited by sparse data available on non-CYP enzyme polymorphisms and their frequency in different populations | ethionamide (Nguyen et al., 2019), itopride (Zhou et al., 2017), lorazepam (Docci, Umehara, et al., 2020), oseltamivir (Hu et al., 2014) |
| | Frequency of the polymorphic enzyme expression in the studied population | Non-CYPs: UGT, FMO, CES |

| Evaluation of the impact of organ impairment | Physiological changes in the organ-impaired population | Renal or hepatic impairment is mainly evaluated. | dabigatran (Moj et al., 2019), mirabegron (Konishi et al., 2019a), oseltamivir (Y. Chen et al., 2020) |
| | Enzyme- and transporter-expression changes | The main goal is to propose a dose for the impaired population. | Non-CYPs: UGT, CES |

Abbreviations: AO = aldehyde oxidase; CES = carboxylesterase; DDI = drug-drug interaction; FMO = flavin-containing monooxygenase; non-CYP = non-cytochrome P450; PBPK = physiologically-based pharmacokinetics; PK = pharmacokinetics; SULT = sulfotransferase; UGT = 5’-diphospho-glucuronosyltransferase.
Figure 1

A

Cumulative PBPK Report Incidence

B

Cumulative non-CYPs PBPK publications

- Total PBPK
- non-CYPs

Number of articles
Number of PBPK models
Number of compounds

UGT AO FMO SULT CES Total

0
50
100


0
200
400
600
800
1000

0
20
40
60
80
100

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Figure 2

A

Number of published Models

Healthy adults
Pediatrics
Cancer
Renal or hepatic impaired
Pregnant women

B

UGT  AO  FMO  SULT  CES

Healthy adults
Pediatrics
Cancer
Renal or hepatic impaired
Pregnant women

Legend:
- Black: Healthy adults
- Red: Pediatrics
- Green: Renal or hepatic impaired
- Purple: Cancer
- Blue: Pregnant women
Figure 3
Figure 4

The figure shows a bar chart comparing the number of models for different categories (UGT, AO, FMO, SULT, CES, Total) between Middle-out and Bottom-up approaches. The Middle-out approach has significantly more models for each category, with a particularly large difference for the CES category.