Evaluation of a New Molecular Entity as a Victim of Metabolic Drug-Drug Interactions - an Industry Perspective

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Victim Drug-Drug Interactions Working Group
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List of Abbreviations

ABT - 1-Aminobenzotriazole
ACAT - Advanced Compartmental Absorption and Transit
ADAM -Advanced Drug Absorption and Metabolism
ADME - Absorption Distribution Metabolism Excretion
AMS- Accelerator Mass Spectrometry
AO - Aldehyde Oxidase
AUC – Area Under Curve
BCS – Biopharmaceutical Classification System
BDC – Bile Duct Cannulated
B/P – Blood/Plasma Partition Ratio
CES - Carboxylesterase
cDNA – complementary DNA (recombinant enzyme)
CL - Clearance
CLint – Intrinsic Clearance
CYP – Cytochrome P450
DDI – Drug-drug Interactions
DME- Drug Metabolizing Enzyme
DNA - Deoxyribonucleic Acid
DPM - Disintegrations Per Minute
EM – Extensive Metabolizer
EMA – European Medicines Agency
F_A – Fraction Absorbed
FAD - Flavin Adenine Dinucleotide
f_{CL} – Fraction of Clearance
FDA – US Food and Drug Administration
F_G – Fraction Escaping Gut Metabolism
FIH – First in Human
f_m – fraction metabolized
FMO - Flavin-containing Monooxygenase
f_u – Fraction Unbound
$f_{u,gut}$ – Fraction Unbound Enterocytes
GSH - Glutathione
HLM – Human Liver Microsomes
IQ - International Consortium for Innovation and Quality in Pharmaceutical Development
ISEF- Intersystem Extrapolation Factor
IV - Intravenous
IVIVC – *In vitro In vivo* Correlation
k_a – Absorption Rate Constant
k_{deg} – Enzyme Degradation Rate Constant
k_i - Enzyme Inhibition Constant
K_I - Inhibitor Concentration At 50% of k_{inact}
k_{inact} - Maximal Inactivation Rate Constant
K_m - Substrate Concentration at which the Reaction Rate is Half of Maximal Velocity
LC/MS - Liquid Chromatography–Mass Spectrometry

MAO – Monoamine Oxidase
MAD - Multiple Ascending Dose
NADPH - Nicotinamide Adenine Dinucleotide Phosphate reduced
NAT – N-acetyl Transferase
NME – New Molecular Entity
NDA – New Drug Application
PAPS - 3'-Phosphoadenosyl-5'-Phosphosulfate
PBPK - Physiologically-Based Pharmacokinetic
PK - Pharmacokinetics
PM – Poor Metabolizer
PO – Oral (Per Os)
PoC – Proof of Concept
Q_h – Hepatic Blood Flow
RAF – Relative Activity Factor
rh – recombinant CYP
[S] – Substrate Concentration
SAD – Single Ascending Dose
SULT - Sulfotransferase
SSRI - Selective Serotonin Re-uptake Inhibitors
TI – Therapeutic Index
UDPGA - Uridine Diphosphoglucuronic acid
UGT – Uridine Diphosphoglucuronosyltransferase
UV - Ultraviolet
V_{dss} – Steady-state Volume of Distribution
V_{max} - Maximum Reaction Velocity
XO – Xanthine Oxidase

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Abstract

Under the guidance of the International Consortium for Innovation and Quality in Pharmaceutical Development (IQ), scientists from 20 pharmaceutical companies formed a Victim Drug-Drug Interactions Working Group. This working group has conducted a review of the literature and the practices of each company on the approaches to clearance pathway identification (f_{CL}), estimation of fractional contribution of metabolizing enzyme towards metabolism (f_m), along with modeling and simulation-aided strategy in predicting the victim drug-drug interaction (DDI) liability due to modulation of drug metabolizing enzymes. Presented in this perspective are the recommendations from this working group on 1) strategic and experimental approaches to identify f_{CL} and f_m, 2) whether those assessments may be quantitative for certain enzymes (e.g. cytochrome P450, CYP and limited Uridine Diphosphoglucuronosyltransferase, UGT enzymes) or qualitative (for most of other drug metabolism enzymes), and the impact due to the lack of quantitative information on the latter. Multiple decision trees are presented with stepwise approaches to identify specific enzymes that are involved in the metabolism of a given drug and to aid the prediction and risk assessment of drug as a victim in DDI. Modeling and simulation approaches are also discussed to better predict DDI risk in humans. Variability and parameter sensitivity analysis were emphasized when applying modeling and simulation to capture the differences within the population used and to characterize the parameters that have the most influence on the prediction outcome.

Introduction

Metabolism-based drug-drug interactions (DDI) remain a safety concern, as observed by the number of drugs that have been withdrawn from the market due to severe toxicities arising from metabolic DDIs (http://www.fda.gov/Drugs/DrugSafety/DrugRecalls/default.htm). It is therefore extremely important to assess and avoid if possible, the potential of a drug to cause or be affected by such DDIs. Regulatory agencies such as the US Food and Drug Administration (FDA) (FDA, 2012) and European Medicines Agency (EMA) (EMA, 2012) require that potential drug interaction risks be investigated before large scale clinical trials are conducted. During the assessment of metabolism-based DDIs, new molecular entities (NME) are classified as *perpetrators*-drugs (inhibitor/inducer) that alter the metabolic clearance of another co-administered drug and *victims*- co-administered drugs whose metabolic clearance is affected.

In 2003, the Pharmaceutical Research and Manufacturers of America (PhRMA) consortium published a comprehensive DDI manuscript, which provided best practices for the conduct of *in vitro* and clinical DDI studies required for registration dossiers of drugs in clinical development (Bjornsson et al., 2003). The manuscript described technical and strategic aspects of experimental assays pertaining to both perpetrator and victim DDI assessment, with a focus on CYP-mediated DDIs. The current working group comprised of pharmaceutical company members of the International Consortium for Innovation and Quality in Pharmaceutical Development (IQ), was formed with a similar intent but with the focus to expand on the solid foundation of the earlier manuscript with regard to *victim* DDI risk assessment. Every NME has the potential to be a victim of some DDI (drug-drug, genetic polymorphism, food-drug, disease-drug interactions) since it has to be cleared by certain pathway. Unlike the liability of a perpetrator drug that can potentially be dialed out before final clinical candidate nomination via structural modifications, victim drug liability can at best be managed by thorough assessment of a NME's clearance pathways and identifying those that will be most sensitive to modulation. Additionally, definitive clinical studies to confirm the victim DDI risk of a NME are usually not done till later stages (post proof of concept clinical studies) of development. However, early evaluation of victim DDI risk of a NME is deemed crucial and is a routine practice amongst companies as part of the drug development paradigm. Therefore, a manuscript outlining holistic strategies dedicated to victim DDI risk assessment of NMEs, through various stages of preclinical to clinical development was merited.

Drug metabolizing enzymes (DME) and transporters have both emerged to play a prominent role in clearance of NMEs, and modulation of either of these can result in exposure change of a victim NME. Due to the expansive scope of both DMEs and transporters in drug disposition and to avoid duplication of efforts by other transporter-focused working groups (Hillgren et al., 2013; Tweedie et al., 2013), current working group's focus was on metabolism-based victim DDI assessment only. Researchers are also encouraged to read comprehensive recent publications on victim DDIs resulting from modulation of transporters (Lai and Hsiao, 2014; Nakanishi and Tamai, 2015). Other topics not within the scope of this manuscript but comprehensively covered in recent publications include evaluation of NMEs as perpetrators of DDIs (Zhao et al., 2014; Varma et al., 2015) and assessment of performance of static and dynamic models commonly used for successful prediction of clinical DDIs (Vieira et al. 2014).

This manuscript will summarize the commonly adopted industry practices which include *in vitro* methods, in combination with *in vivo* preclinical and clinical studies, along with modeling and simulation, to best estimate the potential of a NME to be a *victim* of CYP and non-CYP-mediated metabolic DDIs in the clinic. The compilation of non-CYP enzymes discussed here is not an exhaustive list of reported non-CYP enzymes, rather a list of non-CYP enzymes that are commonly evaluated and have been encountered in recent years by working group member companies during assessment of NME metabolism. The intent of the manuscript is not to define the timing or stage of drug development when certain studies need to be conducted but to recommend what studies may provide the best estimate of the experimentally determined parameters viewed as the key elements of a victim DDI liability. Challenges commonly encountered in the estimation of the key parameters including limitations in evaluating non-CYP mediated metabolic DDIs, will be discussed. Application of modeling and simulation via case studies to highlight the importance and the impact of victim DDI input parameters on clinical DDI study design and outcome, as well as strategies for managing uncertainties in key parameters will be presented.

Substantial amount of detailed information on RAF/ISEF scaling method for the CYP and IVIVC of overall clearance prediction via UGT and AO/XO enzymes that the working group compiled during their review has been intentionally placed in the Supplemental section.

Including such compiled up-to-date information in Supplemental is deemed advantageous to maintain optimum length of main manuscript, while providing such useful information to interested readers without having to do exhaustive literature search. Analysis of optimization of experimental assay conditions required for CYP and non-CYP enzymatic reactions were also not the intent of this manuscript and key references have been provided for interested readers in appropriate sections of the main text. This working group of pharmaceutical companies acknowledges the need for such a comprehensive manuscript which summarizes universal strategies and currently available tools that are routinely used to guide decision-making through various stages of preclinical and clinical development, encompassing both CYP and non-CYP mediated metabolic victim DDI risk potential of NMEs.

Metabolic victim DDIs discussed in this manuscript represent pharmacokinetic DDIs, resulting in exposure change of the victim drug. Due to complex underlying mechanisms for pharmacodynamic drug-drug interactions (e.g. synergistic, additive, or antagonistic effect of co-administered drugs on a target receptor), this manuscript does not attempt to address these type of interactions. It is worthwhile to note that in cases where a drug forms pharmacologically active metabolites (e.g acebutalol, alprenolol, diltiazem; carbamazepine, valproic acid, diazepam, fluoxetine), pharmacokinetic change in exposure of parent drug may not reflect parallel change in overall pharmacodynamic response.

A victim drug’s metabolic clearance and exposure may undergo significant fluctuation due to modulation of the DME primarily responsible for its clearance. Modulation of a DME can result from either inhibition and/or induction of its enzyme activity in the presence of a co-administered drug, in certain disease states (Cheng and Morgan, 2001; Harvey and Morgan, 2014; Jiang et al., 2015) or due to inherent variability associated with the abundance or polymorphic nature of the DMEs [e.g. CYP2D6, *N*-acetyltransferase1 (NAT1), UDP-glucuronosyltransferase (UGT)1A1]. Several representative examples are presented in Supplemental Table 1S. For instance, a drug primarily metabolized by a polymorphic DME is more susceptible to substantial exposure change in poor metabolizers (PMs), in the presence of inhibitors of a minor non-polymorphic pathway (Collins et al., 2006).

To accurately assess the magnitude of exposure change of a victim NME due to modulation of its metabolic clearance it is important to understand the role of metabolism towards the victim NME’s overall clearance and contribution of a DME(s) to its overall metabolic clearance. These parameters are represented by the terms f_{CL} and f_m throughout this manuscript. The term f_{CL} is defined as the *fraction of drug cleared by a pathway* representing its *route of clearance*, where $f_{CL,metabolism}$ represents the fraction of drug cleared through metabolism, $f_{CL,renal}$ represents the fraction of drug cleared *unchanged* renally, and $f_{CL,biliary}$ refers to the fraction of drug cleared *unchanged* via biliary excretion. For any drug systemic clearance is the sum of metabolism, renal excretion, and hepatic uptake by transporters followed by metabolism and/or subsequent excretion into bile (and then feces): $f_{CL,metabolism} + f_{CL,renal} + f_{CL,biliary} = 1$. Some drugs may also be cleared via intestinal secretion but it is often not possible to distinguish this from biliary clearance. The term f_m is defined as the *fraction of drug metabolized by an enzyme*. To illustrate, a drug that is metabolically cleared primarily by CYP3A4, CYP2C9, and UGT2B7 can be illustrated as: $f_{m,CYP3A4} + f_{m,CYP2C9} + f_{m,UGT2B7} = f_{CL,\ metabolism}$, where sum total of fractional metabolism via each of these enzymes is equal to the total fraction of a NME cleared by metabolism.

The parameters $f_{CL,metabolism}$ and f_m are considered key for a victim DDI risk assessment since the sensitivity/dependency of the AUC ratio for a victim NME, significantly depends on $f_{CL,metabolism}$ x f_m (Zhang et al., 2007). It is widely recognized, that when $f_{CL,metabolism}$ and/or f_m attain higher values such that $f_{CL,metabolism} \times f_m \geq 0.5$, the victim drug AUC ratio is predicted to be ≥ 2, and increases with f_m exponentially. The predicted AUC ratio of a victim drug also increases sharply with increasing potency and concentration of the inhibitor (e.g. $[I]/K_i$ = 5, 10, 20, 50, 100), especially when $f_{CL,metabolism} \times f_m > 0.8$. In the absence of well-defined clinical safety margins and for compounds with narrow therapeutic indices (TI), when the NME is cleared by a single metabolic pathway, impairment of that pathway can result in a potentially undesirable adverse outcome especially when value of $f_{CL,metabolism}$ x f_m of the NME ≥ 0.5. Most definitive human studies to quantitatively determine $f_{CL,metabolism}$ and f_m are not conducted until later stages of clinical development (e.g. post- proof of concept, mostly Phase II or Phase III). It is therefore necessary to obtain best estimates of $f_{CL,metabolism}$ and f_m early on to allow investigators to ensure the safe conduct of Phase I and Phase II studies with consideration of certain clinical exclusion criteria. Clinical exclusion criteria to mitigate victim DDI risk in Phase I/first in human (FIH) studies are especially important in cases where FIH studies are conducted in patients compared to healthy volunteers (who are not on any medications) and in Phase II in patients who are taking concomitant medications.

Metabolism continues to be the predominant route of clearance of small molecule NMEs as experienced by pharmaceutical companies. This is also confirmed via assessment of clearance information of NMEs approved over the years between 2010-2014, as available from New Drug Applications (NDAs), which reveal that metabolism accounted for at least 25% of total clearance (Figure 1) for >80% of the 95 approved NMEs (assessment included NMEs administered via oral, intravenous, or inhalation route and excluded imaging agents, enzyme replacement therapies, sclerosing agents and topical applications). Of the drugs that were primarily cleared by metabolism, CYPs were involved in the metabolism of approximately 75% of the NMEs approved during 2010-2014 (Figure 1), supporting the current view of a potentially higher risk of CYP-mediated victim DDI compared to other non-CYP enzymes. Hence, it is routine practice amongst pharmaceutical companies to start preliminary assessment of overall role of metabolism and understand DMEs involved in a NME's clearance, starting in the preclinical stages of drug development. This is commonly done via evaluation of 1) *in vitro* f_m in human-derived matrices to understand whether one or multiple, CYP or non-CYP enzymes are involved in a NME's metabolism and 2) *in vivo* f_{CL} information in preclinical species and whether *in vitro*: *in vivo* correlation (IVIVC) holds in preclinical species, to gain qualitative understanding of whether metabolism or biliary or renal excretion is predominant. The combined information obtained is used as an early guide to evaluate victim DDI risk in the clinic using various predictive models (Vieira et al., 2014). Once the $f_{CL,metabolism}$ is available from human ^{14}C-ADME study and $f_{m,enzyme}$ is quantitatively available from a clinical DDI study (or PK study in genotyped population), the victim DDI predictions are further refined to predict additional and/or potentially complex DDIs prior to the NME being administered in larger clinical trials (Lu et al., 2014). The next two sections will focus on discussing how companies rely on currently available *in vitro* assays and *in vivo* data to best estimate f_m and $f_{CL,metabolism}$ along with some of the pitfalls encountered through various stages of drug development.

IN VITRO DETERMINATION OF THE ENZYMES INVOLVED IN METABOLIZING OF A NME AND THEIR RELATIVE CONTRIBUTION $f_{m,enzyme}$

Quantitative determination of f_m in the clinic is obtained with studies conducted either (1) in a genotyped population where f_m can be estimated from the change in exposure of a victim drug in extensive metabolizers compared to poor metabolizers (Ito et al., 2005) or (2) with selective

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inhibitors of a metabolizing enzyme (responsible for the primary metabolic pathway of victim drug) (Shou et al., 2008):

$$f_m = 1 - \frac{AUC_{\text{no inhibitor}} \text{ or } AUC_{\text{extensive metabolizer}}}{AUC_{\text{with inhibitor}} \text{ or } AUC_{\text{poor metabolizer}}} \qquad \text{Equation 1}$$

Before such definitive clinical studies are conducted, reliance on *in vitro* methods to estimate f_m is common during early stages of development. “Enzyme mapping” is commonly the first step conducted to identify DMEs involved in the major metabolic pathways of a NME. The second step often referred to as “reaction phenotyping” allows for the quantitative determination of the fraction metabolized (f_m) by a specific enzyme or isoform in appropriate human liver matrices. After preliminary *in vitro* enzyme mapping, detailed reaction phenotyping experiments are recommended when the contribution of a particular enzyme family is estimated to be ≥ 25% towards overall metabolism of a NME. In the absence of quantitative information on $f_{CL,metabolism}$ from ^{14}C-ADME studies, worst-case scenario for victim DDI risk assessment is the assumption that metabolism is responsible for 100% of the NME clearance (consistent with current observed trend of metabolism accounting for >80% clearance of NMEs registered between 2010-2014). The conservative ≥ 25% cutoff from *in vitro* studies is deemed to be a reasonable starting point to warrant further investigation of f_m. Some of the reasons behind this approach include a consideration for the experimental errors in *in vitro* determination of f_m due to un-optimized assay conditions, reduced enzyme activity in *in vitro* systems, difficulty of extrapolation of *in vitro* f_m to *in vivo* f_m, especially in cases where extra-hepatic metabolizing enzymes are involved.

General Guidelines for Enzyme Reaction Phenotyping

Comprehensive metabolite profiling in complete human liver systems such as hepatocytes to capture major metabolic pathways should be obtained before conducting detailed f_m studies. Whether a NME is cleared via oxidation, reduction, hydrolysis, or direct conjugation guides the selection of metabolizing enzymes to be prioritized for phenotyping studies. After major metabolic pathways have been identified it is recommended to determine metabolite formation kinetics over a range of NME concentrations to establish $K_{m,apparent}$ (apparent/observed Michaelis-Menten constant) value for the metabolic pathways of interest, particularly if using chemical inhibition method. Once $K_{m,apparent}$ has been established f_m experiments are conducted maintaining NME/substrate concentration at approximately 1/10th value of $K_{m,apparent}$ to capture accurate contribution of the physiologically relevant high-affinity enzymes towards the metabolic pathway of interest. Since most of the CYP substrates have $K_{m,apparent}$ values in 1-100 μM range, hence 0.1- 10 μM is an appropriate concentration range to investigate metabolite formation kinetics while maintaining linear first-order kinetics. Investigators are encouraged to consider non-specific binding of a NME to microsomes (and hepatocytes) in incubation mixture. For NMEs with high non-specific binding (as in cases of lipophilic basic drugs), while the V_{max} values are unaffected, the observed $K_{m,apparent}$ can be substantially higher than $K_{m,unbound}$,($K_{m,apparent} = f_{unbound, incubation} * K_{m,unbound}$), which represents the true unbound/pharmacologic drug concentration at the enzyme active site and is key determinant of *in vitro* CL_{int}. Challenges in assay sensitivity of metabolite monitoring at such low $K_{m,unbound}$ concentration is a practical hurdle and most *in vitro* experiments are conducted at approximately 1/10 th value of $K_{m,apparent}$ or in cases where $K_{m,apparent}$ has not been fully characterized, at three concentrations typically 0.1, 1, and 10 μM. Generally, under optimal/low protein (e.g. microsomal) concentration (ideally 0.05-0.2 mg/mL), linear first-order kinetics are still maintained in these $K_{m,apparent}$ concentration ranges. Graphical analysis of metabolite formation kinetics (using Eadie-Hofstee or Lineweaver-Burke plots) may allow one to determine involvement of multiple enzymes and saturable high affinity pathway in some cases. Enzyme-selective inhibitor or RAF/ISEF scaling methods (Supplemental) are then applied to quantitatively determine f_m for each of the major metabolic pathways tested. This overall process is best done quantitatively, with radiolabeled NME or with authentic metabolite standards if they are available. During early stages of drug discovery, radiolabeled NME or metabolite standards are rarely available and reliance on identifying metabolic pathways using un-labeled NME is commonly used for decision-making and early victim DDI risk assessment.

When un-labeled NME is used “relative” contribution of metabolic pathways is estimated utilizing either UV-absorption or LC/MS/MS methods. Underlying assumption is that either the UV-absorption (when using UV) or the ionization efficiency (in LC/MS/MS) of the metabolite(s) is similar to the parent NME. Both the assumptions warrant cautious evaluation (Dahal et al., 2011) depending on the nature of the biotransformation of the NME.

When radiolabeled NME is available the contribution of various metabolic pathways can be quantitatively assessed when radiolabeled NME is available. Metabolites can be separated and quantified via HPLC- radiometric detection device while LC/MS techniques can be used to

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confirm the identity of the metabolites. If several minor metabolites are formed the sensitivity of an inline radiochemical detector may not be sufficient to provide a quantitative measurement of all the metabolites and a scintillation counter is often used after fraction collection from the HPLC separation. For extremely low turnover NMEs, a combined approach of radiometric detector and LC/MS/MS can be used (Yi and Luffer-Atlas, 2010)(discussed further in Section for Special Considerations: Low Turnover, Extra-Hepatic Metabolism, Inhibition of Parallel Metabolic Pathways).

When biotransformation pathways have not been fully characterized, monitoring total parent NME disappearance is also commonly done. Enzyme-selective inhibitors or RAF/ISEF scaling methods (Supplemental) are then applied to semi-quantitatively determine f_m for overall NME disappearance. The parent disappearance approach is limited by the challenge to accurately monitor depletion of NME especially when % depletion is low with assays usually being conducted under initial rate conditions (NME depletion is maintained at $\leq$ 15-20% to ensure metabolite formation is linear with protein concentration and incubation time).When using enzyme- selective inhibitors, at least 40% of parent NME disappearance has been reported to be desirable (Lu et al., 2008) to capture maximum sensitivity of inhibition of NME metabolism. In comparison when using the RAF or ISEF approaches, ~15-20% of parent NME disappearance has been demonstrated to give acceptable results (Uttamsingh et al., 2005). These are in contrast to the level of sensitivity achieved when monitoring metabolites which can be measured at ~5% of parent turnover with reasonable accuracy. The parent disappearance approach provides an advantage that allows the researcher to easily monitor the total NME metabolism rather than individual metabolite-formation kinetics, which is significantly more labor-intensive and usually not done until a candidate is identified for clinical development. However monitoring NME disappearance does not yield a complete picture of metabolic pathways and enzymes involved in each of the pathways which could have important implications in victim DDI risk predictions. This can be demonstrated with an example of a NME metabolized predominantly through a major metabolic pathway which - has significant contribution from two distinct isoforms CYP3A4 and CYP2C9, each with different kinetic behaviors ($K_{mCYP2C9} = 5$ μM, $V_{maxCYP2C9} = 18$ pmol min^{-1} mg^{-1}; $K_{mCYP3A4} = 100$ μM, $V_{maxCYP3A4} = 170$ pmol min^{-1} mg^{-1}). When overall NME disappearance is monitored, the apparent observed kinetic parameters obtained for the NME will be a result of hybrid of the kinetic parameters for both isoforms. At low NME concentration (usually therapeutically relevant concentrations) contribution of the high affinity isoform CYP2C9 will be predominant towards NME metabolism. At high NME concentration due to higher V_{max} of CYP3A4-mediated pathway, contribution of CYP3A4 will be primary. Several drugs demonstrate such biphasic kinetics (bufuralol, dextromethorphan, diazepam, omeprazole, lansoprazole, voriconazole) (Pelkonen et al., 2008; Griffin and Houston, 2004) where *in vivo* metabolism is dominated by the high affinity/low capacity enzymes (e.g. 2D6 or 2C19) although *in vitro*, contribution of low affinity/high capacity enzyme (e.g. 3A4) may be predominant at high substrate concentrations. It is important to understand such mechanistic aspects in DDI risk assessment at clinically efficacious doses of a NME so as to not underestimate or miss contribution of the physiologically relevant high affinity enzyme under supra-therapeutic *in vitro* assay conditions. In the next few sections summary of current tools and approaches for f_m determination of CYP and non-CYP enzymes will be provided. The approaches can be evaluated by monitoring CL_{int} of a NME via either major metabolite formation or overall NME depletion, depending on the sensitivity of assays available to investigators that imparts data interpretation with high level of confidence. Technical details of typical incubation conditions for CYP and non-CYP mediated *in vitro* metabolism assays are beyond the scope of this manuscript. Interested readers are referred to past reviews of this topic (Johnson and Waterman, 1996, Venkatakrishnan et al., 2001; Kramer and Tracy 2008, Ogilvie et al., 2008; Sensenhauser, 2014; Korzekwa, 2014) and citations made throughout this document.

Early Enzyme Mapping: Identifying CYP and Non-CYP Enzymes Involved in NME Metabolism

CYP and non-CYP enzymes such as flavin-containing monooxygenase (FMO), monoamine oxidase (MAO), and aldehyde oxidase (AO)/xanthine oxidase (XO), all catalyze oxidative metabolism of xenobiotics with differential preference based on the chemical nature of their substrates. AO/XO preferentially oxidize electron deficient carbons (Beedham 1985; Pryde et al., 2010) compared to the CYPs that prefer electron rich C-oxidations. Hence aldehyde, imine, or heteroaromatic functional groups are typical substrates of AO/XO. FMO is another NADPH-dependent oxidative enzyme like CYP enzymes but preferentially mediates *N*- and *S*-oxidations, typical peroxy acid reactions (e.g. Baeyer-Villiger oxidation) but not C-oxidation or heteroatom-dealkylations like CYP enzymes (Hines et al., 1994; Cashman 1995; Ziegler 2002;

Harper and Brassil 2008; Lang and Kalgutkar 2003). MAO-mediated metabolism often occurs on compounds containing basic amine (Geha et al., 2001). Glucuronidation and sulfation commonly occurs on phenol and aliphatic alcohol groups forming O-glucuronides and O-sulfates, respectively. Glucuronidation may also occur on amines and carboxylic acids forming *N*-glucuronides and acyl glucuronides, respectively, while NAT has been observed to acetylate both aromatic and aliphatic amines. Carboxylesterase (CES) is usually involved in ester hydrolysis although some amide and ketones with α-CF3 have been reported to be substrates of CES (Wadkins et al., 2007).

Knowledge of the NME structure combined with information about major metabolic pathways (oxidation, reduction, hydrolysis, or direct conjugation) guides the nature of enzyme mapping assays. High level outlines of these studies are described in Figures 2-4. If major metabolic pathways are identified to be phase 1 in nature then involvement of NADPH-dependent enzymes such as CYP and FMO (Scheme A/Figure 3) and the NADPH-independent enzymes such as MAO or AO/XO (Scheme B/Figure 4) are evaluated. When the NME undergoes direct conjugation involvement of enzymes such as UGT, SULT, NAT, or GST is evaluated depending on the major metabolites identified. For a NME that primarily undergoes hydrolysis enzymes such as carboxylesterases (CES) and amidases are further investigated. These studies may be conducted in conjunction with preliminary assessments in recombinant enzymes when available to better identify the enzymes responsible for major metabolic pathways. In the case of AO/XO since commercial availability of recombinant enzymes is very limited, assessments are best made in cytosol (details in AO/XO section).

Preliminary assessment of relative contribution of FMO and CYP enzymes towards *N*- or *S*- oxidation (Figure 3) can be assessed by: (1) treating HLM with a non-ionic detergent (e.g. Triton X-100) or pan-CYP inhibitor (e.g. 1-aminobenzotriazole, ABT) (both of which selectively suppress the majority of CYP activities but not FMO activity); or (2) incubating HLM at 50 ºC for 2-3 min without NADPH. FMO is heat inactivated in the absence of NADPH but CYP activity is not significantly compromised. While none of these methods are ideal (e.g. CYP and FMO activity may not be fully inhibited with ABT and heat inactivation, respectively), results obtained from combined studies are acceptable for early estimates of the relative contribution of CYP vs FMO to then initiate detailed studies described in sections for CYP and FMO below.

Identification of NADPH-independent oxidative enzymes AO/XO and MAO is outlined in Figure 4. Significant oxidative metabolism of a NME in human liver cytosol is indicative of metabolism by AO or XO. Inhibition of overall metabolism in cytosol by hydralazine or allopurinol would implicate AO or XO, respectively, in the metabolism of the NME. Inhibition of metabolism in the mitochondrial fraction with tranylcypromine or pargylline is indicative of MAO involvement although if the NME does not possess a primary, secondary or tertiary amine functional group, there is a very high probability that MAO will not be involved.

Teasing out the contribution of CYP vs. non-CYP oxidative enzymes (AO/XO, FMO, and MAO) can be challenging in cases where the same oxidative metabolite may be formed via multiple enzymes (e.g. CYP and FMO, CYP and AO/XO, or CYP and MAO). Based on the reported incidence and magnitude of victim DDIs in the clinical setting (Yu et al., 2014) as well as the continued trend of CYP being the major enzyme involved in metabolic clearance, CYP stands out as the high risk enzyme class compared to others. Careful assessment of contribution of individual CYP isoforms towards overall metabolism of a NME is therefore crucial to accurately assess the victim DDI liability of a NME in the clinic.

Strategies for f_m determination discussed for the non-CYP enzymes are based on literature reported cases or current practices within member companies. Since known cases of drugs where non-CYP enzymes are the primary metabolizing enzyme is low, experience in determination of f_m and extrapolation to *in vivo* outcome for non-CYP enzymes is not extensive. It is therefore recommended to thoroughly assess and optimize conditions for f_m of non-CYP enzymes when using the strategies described for the non-CYPs as good starting guidelines.

Determining Fractional Metabolism by CYP: CYP Reaction Phenotyping

Relative contributions of individual CYP enzymes to total human hepatic microsomal clearance can be assessed *in vitro* by some commonly used techniques (Rodrigues, 1999; Zhang et al., 2007; Zientek et al., 2015; Wienkers 2003; Ogilvie et al., 2008; Korzekwa, 2014): (1) Recombinant CYP kinetics scaled to HLM: Commonly referred to as the RAF/ISEF method (Supplemental), this involves determination of enzyme kinetic parameters for the metabolism of the NME in a panel of recombinant human (rh) CYP (with pre-determined specific activity and normalized for protein content) and scaling the rhCYP intrinsic clearance (CL_{int} or V_{max}/K_m) to HLM CL_{int} via a RAF/ISEF approach. When the contributions of specific CYP enzymes are scaled to HLM CL_{int} then the percent contribution of each CYP enzyme towards the total HLM CL_{int} can be estimated. It is not uncommon that the absolute value of the HLM CL_{int} and rhCYP-scaled HLM CL_{int} may differ. Several factors could contribute to the differences including potential involvement of enzymes other than CYP enzymes in HLM in the formation of the same metabolite(s), lack of 100% specificity of probe substrates used to calculate the scaling factors, and the potential for compound-specific (probe vs. NME) differences in metabolism in HLM vs. rhCYP enzymes. However, with respect to determining the relative contributions of individual CYP enzymes to the overall CYP-mediated oxidative clearance in HLM, RAF/ISEF methods are deemed very useful. (2) Chemical Inhibition Method: This approach involves the use of CYP-isoform selective chemical inhibitors. After major metabolic pathways are identified and their kinetics evaluated, the effect of CYP-isoform-specific chemical inhibitors on a particular metabolic pathway (in HLM) is assessed. Percent inhibition of a metabolic pathway by a known isoform-selective chemical inhibitor reflects contribution of the isoform towards that pathway. Ideally chemical inhibitors should be potent, selective and metabolically stable. Sometimes the balance between potency and selectivity can be challenging to attain since in reaction phenotyping assays the concentration of inhibitors should be such that they inhibit the desired isoform >90%. This is usually achieved at approximately 10-fold of inhibitor K_i values. However at such high inhibitor concentrations some CYP inhibitors may demonstrate non-selectivity where slight inhibition towards other un-intended CYP isoforms may exist and hence overestimate percent contribution via intended CYP pathway. A mathematical correction has been proposed using CYP probe substrates to correct for the cross reactivity and partial inhibition towards the target CYP (Lu et al., 2007), but this has not been widely qualified. Non-specific binding of chemical inhibitors ($f_{u,incubation}$) also needs to be carefully evaluated (Waters et al., 2014), especially in cases where phenotyping studies are conducted at high microsomal protein concentration >0.5 mg/mL, where certain chemical inhibitors (e.g. α-napthaflavone, ketoconazole, benzylnirvanol, quinidine) show substantial non-specific binding (Nirogi et al., 2014). Due to high non-specific binding unbound concentration of an inhibitor at the enzyme active site, may be sub-optimal to attain the desired >90% inhibition of an intended enzyme. Furthermore, when using irreversible inhibitors it is recommended that the experimental conditions including pre-incubation times be carefully optimized. Based upon a literature search of reported K_i or IC_{50} values for the inhibition of individual CYP enzymes and compilation of the information, a recommended concentration range of inhibitors to use is shown in Table 1. This range is intended to maximize the selectivity of the inhibitor for the specific CYP isoforms listed. The extent of inhibition by the CYP-selective inhibitor provides information regarding the contribution of the inhibited enzyme to the total HLM oxidative metabolism. It is also recommended to maintain the NME concentrations below their $K_{m,apparent}$ values, so the IC50 value that is produced would be closer to the actual K_i value for the specific enzyme inhibition. Hence it is a good practice to establish $K_{m,apparent}$ values of a NME when using the chemical inhibition method.

Combined use of CYP-selective inhibitors in HLM and rhCYP scaling methods (RAF/ISEF) provides investigators with high level of confidence in estimating the primary CYP isoforms involved in the hepatic oxidative clearance of a NME. In majority of cases of CYP-mediated oxidative metabolism f_m values obtained via either of these two methods are not expected to be substantially different but on occasions, differences may arise. Potential reasons include (1) involvement of uncommon CYP isoforms (2J2, 4A11) or non-CYP enzymes (2) Non-ideal experimental conditions where sub-optimal inhibition is obtained from chemical inhibitors. Scenarios include high protein concentration resulting in high non-specific binding of inhibitor resulting in < 90% inhibition of the intended CYP – this underestimates f_m of intended CYP; use of excessive inhibitor concentration resulting in substantial cross-reactivity which leads to over-estimation of f_m of intended CYP; high substrate/ NME concentration $>>K_m$, resulting in overwhelming the inhibitory effect of reversible inhibitors and underestimating f_m of intended CYP (3) the lack of absolute specificity of the probe substrate and/or inhibitor for CYP (4) potential difference in CYP binding site for the probe substrate and the NME, where the NME may interact with the CYP at a binding site, different from that of the probe substrate (Mathur et al., 2013). Large discrepancies in f_m values obtained from chemical inhibition and rhCYP scaling methods, often result in considerable differences in the magnitude of the predicted victim DDI liability for a NME and warrant a systematic look into factors discussed above

Correlation analysis is another method that has been assessed for the CYP isoforms (and infrequent cases of UGT: Kamdem et al., 2010), however, review of current industry practices amongst the working group member companies, did not advocate this to be a method of choice for phenotyping of drug candidates, especially when there is discrepancy in the f_m values from RAF/ISEF and chemical inhibitor methods. A reason maybe that a pre-requisite for correlation

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analysis is the maintenance of a consistent collection of individual human liver microsomes, with a wide range of activity characterized for the metabolizing enzymes of interest. This is probably less practical/attractive for industry laboratories, which routinely use commercially available pooled human liver microsomes from 100-150 donors, for RAF/ISEF and chemical inhibitor studies. Correlation analysis is also substantially more labor-intensive than RAF/ISEF and chemical inhibitor methods, and when multiple CYP isoforms are involved, data interpretation may not be straight forward and often yields only qualitative information on f_m.

Successful examples of predictions of exposure change of victim drug in the clinic based on experimentally determined *in vitro* $f_{m,CYP}$ have been reported. These examples use the methods described in the CYP phenotyping section as the standard approach (Youdim et al., 2008; Lu et al., 2008) and in theory can also be applied to non-CYP enzymes when appropriate tools are available.

Identification of Flavin-Containing Monooxygenases FMO 1, 3, 5

The flavin-containing monooxygenase (FMO) family comprises a group of flavin adenine dinucleotide (FAD)-containing microsomal enzymes which catalyze oxidation of compounds containing soft nucleophilic groups. Typical FMO-catalyzed reactions are mono-oxygenation of heteroatoms such as nitrogen and sulfur (Hines et al., 1994; Cashman 1995; Ziegler 2002; Lang and Kalgutkar 2003). Since the key intermediate of the FMO catalytic cycle is a reactive peroxide intermediate C(4α)-hydroperoxyflavin, the FMO-mediated biotransformation is typically consistent with chemical reactions that involve peroxides or peroxyacids. Similar to CYP enzymes NADPH and oxygen are required for FMO-mediated reactions. However, unlike CYP enzymes FMO enzyme activities are generally greater at higher pH and can be inactivated by a brief heat treatment in the absence of the cofactor, NADPH (Cashman 2008). These methods are often applied *in vitro* to indicate and assess potential involvement of FMO in drug metabolism. The commercial availability of recombinant FMO1, 3, 5 isoforms has facilitated rapid identification of FMO-mediated pathways since negative results from properly conducted recombinant enzyme experiments generally allows exclusion of FMO involvement in metabolism. Due to the lack of selective inhibitors and limited information on tissue abundance for various FMO isoforms quantitative f_m determination via RAF/ISEF or chemical inhibitors for FMO has not been established. Nevertheless, a set of properly designed *in vitro* experiments

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may still provide a good estimate of the relative contribution of FMO to overall oxidative metabolism (Figure 5).

FMO3 and FMO5 are both expressed at high levels in adult human liver (Cashman 2012; Overby et al., 1997; Zhang and Cashman 2006). Methimazole (Table 2) is a well-characterized substrate/inhibitor of FMO1 and FMO3, but displays very low affinity for FMO5 (Overby et al., 1995). Therefore methimazole can be used to assess the relative contributions of FMO3 and FMO5 in HLM to overall FMO-mediated metabolism (Figure 5). However, this approach must be used in conjunction with the thermal inactivation approach because methimazole is also known to inhibit several CYP isoforms (Guo et al., 1997). FMO1 is highly expressed in human adult kidney. Therefore incubations in human kidney microsomes in the presence or absence of FMO1 inhibitor methimazole, can be used to assess involvement of FMO1 (Figure 5). Unlike human liver microsomal systems, heat inactivation of FMO has not been well-studied or documented in human kidney microsomes.

In contrast to the CYP enzymes, FMO activity is not susceptible to induction or inhibition (Cashman 2012) and known instances of drugs whose metabolism is primarily mediated by FMO is scarce (ranitidine, nicotine). Hence from the viewpoint of potential DDIs, FMO-mediated pathways are generally of less concern relative to CYP-mediated pathways.

Estimating Fractional Contribution of Aldehyde Oxidase/Xanthine Oxidase

AO and XO are cytosolic molybdoflavoproteins that catalyze NADPH-independent oxidation of a wide range of substrates. Typical reactions include oxidation of aldehydes to the corresponding carboxylic acids and hydroxylation of aromatic *N*-heterocycles at the electron-deficient carbon atom adjacent to the heteroatom (Beedham 1985; Pryde et al., 2010). Unlike CYP enzymes the source of the oxygen atom that is added by AO or XO is from water. Therefore incubations with appropriate matrices can be carried out under $^{18}O_2$ or in the presence of H_2O^{18} to further investigate the enzymes involved, if necessary.

The involvement of AO or XO in oxidative metabolism of a NME can be determined directly by monitoring the metabolism of a NME in liver cytosol or S9 fraction in the absence of NADPH (Hutzler et al., 2013; Barr et al., 2014a). It is important that the source of liver tissue used in these studies was not derived from liver that had been perfused with solutions containing allopurinol, a common practice following full hepatectomy, since that tissue would be expected to have little or no XO activity due to residual allopurinol, an inhibitor of XO (Barr et al., 2014).

If oxidative metabolism is observed in human liver cytosol the enzyme(s) involved can be elucidated by the use of chemical inhibitors. Hydralazine and allopurinol (~50 μM) have been identified as selective inhibitors of AO and XO (Table 2), respectively, and, therefore, inhibition of oxidative metabolism by either of these inhibitors would implicate the corresponding enzyme (Johnson et al., 1985; Elion 1988). Raloxifene or menadione can also be used to inhibit AO in incubations with liver cytosol but these inhibitors are less suitable for studies in liver S9 fractions or hepatocytes due to either their instability or potential to inhibit CYP enzymes (Obach 2004; Strelevitz et al., 2012).

Fractional metabolism by AO can be determined using hydralazine in human hepatocytes as has been established (Strelevitz et al., 2012). The difference in intrinsic clearance with and without hydralazine (Table 2) divided by intrinsic clearance in the un-inhibited state gives the fractional metabolism via AO. Conceptually this same approach could be applied to determine the fractional metabolism via XO by using allopurinol in place of hydralazine but the authors are not aware of examples where this has been done.

Few marketed drugs are metabolized primarily by, or potently inhibit, either AO or XO so examples of drug interactions are rare. One notable example is allopurinol, an XO inhibitor designed to treat gout, which is contraindicated for co-administration with azathioprine, an immunosuppressant that is cleared via XO (Gearry et al, 2010). To date there is no example of drug interaction that occurs via inhibition of AO but the potential exists. Famciclovir is an antiviral prodrug that is converted to its active form penciclovir by AO (Clarke et al., 1995). Co-administration of famciclovir with a potent inhibitor of AO such as raloxifene could potentially lead to a reduction in antiviral efficacy (Obach 2004) but studies to determine the magnitude of this interaction have not been conducted. It has recently been demonstrated that inhibition of AO may be substrate-dependent and occur by mixed modes of reversible and irreversible inhibition so care should be exercised in predicting drug interactions based on *in vitro* data (Barr and Jones 2013a; Barr and Jones 2011, Barr et al., 2014).

Because of species differences in AO isoforms and activities, good preclinical models for human AO activity have yet to be identified (Choughule et al., 2013), so caution should be exercised in extrapolating AO-mediated overall metabolic clearance from animals to humans. *In vitro-in vivo* correlation (IVIVC) assessed for set of 11 drugs (Zientek et al., 2010) (Supplemental) predominantly metabolized by this AO suggested that the IVIVC is typically poor and the AO-mediated *in vivo* clearance is usually underestimated. Reasons for this poor correlation have been attributed to the extra-hepatic contribution of AO to total clearance and the potential lability of AO in liver preparations used for *in vitro* studies. Recently the protein content of AO has been quantified in human liver cytosol (Barr et al., 2013a, 2013; Fu et al., 2013). Use of these methods and their extension to extra-hepatic tissues may help to better define our ability to predict contribution of human metabolic clearance via AO in the future.

Identification of Monoamine Oxidases MAO-A and MAO-B

Monoamine oxidase (MAO) is a flavin-containing protein located on the outer mitochondrial membrane of a wide range of mammalian cells in various tissues (Saura et al., 1996). MAO catalyzes the oxidative deamination of biogenic amines as well as basic amine-containing xenobiotics. MAO exists in two forms encoded by separate genes with 70% sequence homology (Weyler et al., 1990). MAO-A is found in the liver, gastrointestinal tract, and placenta, while MAO-B is found primarily in blood platelets.

When a NME contains a primary, secondary or tertiary amine evaluation of the NME as substrates of MAO-A or MAO-B is important for potential DDI risk assessment especially if the NME may be used concomitantly with MAO inhibitors in the clinic. The liver mitochondrial fraction where MAO activity is the highest may be used as an appropriate system to evaluate the contribution of MAO-A or MAO-B. The overall strategy to identify MAO-A or MAO-B is outlined in Figure 6. When a basic amine-containing NME exhibits NADPH-independent metabolism in a mitochondrial fraction, it is warranted to further evaluate its metabolism in recombinant MAO-A and MAO-B (both are commercially available). If no metabolism is observed in either of the recombinant MAO isoforms, the probability of MAO involvement is low and no further evaluation is warranted. MAO-A preferentially oxidizes serotonin and is inhibited by low concentrations of clorgyline (Table 2) whereas MAO-B preferentially oxidizes β-phenylethylamine and is inhibited by low concentrations of (-) deprenyl (Geha et al., 2001; Kalgutkar and Castagnoli 1995; Moussa et al., 2006). Therefore the relative contribution of MAO-A and MAO-B towards the overall oxidative metabolism of a NME may theoretically be assessed in hepatocytes using these isoform-selective inhibitors. However use of chemical inhibition to assess contribution of MAO-A and MAO-B is not routinely done amongst companies and literature cases of this approach are rare (Erickson D et al., 2007). A RAF approach similar to that for CYPs has been reported (Pybus et al., 2012) to evaluate the relative contribution of MAOs towards overall NME metabolism. But since the presence of MAO in microsomes is due to contamination during preparation, levels of MAO vary significantly between microsomal lots, microsomes are not recommended for MAO f_m determination, thereby limiting the utility of this approach.

MAO enzymes play a vital role in the inactivation of neurotransmitters (serotonin, noradrenaline, epinephrine, noepinephrine, dopamine), and a number of psychiatric and neurological disorders are attributed to MAO dysfunction. MAO inhibitors are one of the major classes of drugs prescribed for the treatment of depression and anxiety (MAO-A inhibitors) and are used alone or in combination to treat Alzheimer’s and Parkinson’s disease (MAO-B inhibitors). Several potent MAO inhibitors are marketed drugs, primarily to treat depression, such as (Azilect, Nardil, Parnate, Eldepryl, Marplan, Zelapar). It is therefore useful to assess whether MAO-A/B has a major role in the metabolic clearance of an amine-containing NME to better understand potential victim DDI risk when co-administered with known MAO inhibitors.

Determination of Fractional Metabolism by Uridine 5'-diphospho-Glucuronosyltransferase

Commonly used methods for UGT reaction phenotyping are similar to those of CYPs (albeit less well-established for several UGTs still) and include the use of human recombinant enzymes and selective chemical inhibitors in human liver microsomal incubations (Court 2005; Miners et al., 2010a, 2010b).

Recombinantly expressed UGT isoforms

Advances in the availability of human recombinant UGT isoforms, identification of selective probe substrates for several of the major UGT isoforms, and information of UGT protein quantification in various organs, has allowed for f_m estimation of UGT via RAF/ISEF methods. Commercially available human recombinant UGT isoforms include the major hepatic UGTs, such as UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15, as well as UGTs that are expressed predominantly in the intestine such as UGT1A8 and 1A10. Selective substrates for the major hepatic UGT isoforms have been identified (Table 2) which may be suitable for reaction phenotyping studies include: 17β-estradiol (3-glucuronide formation) for 1A1, trifluoperazine for 1A4, 5-hydroxytryptophol for 1A6, propofol for 1A9 and zidovudine for 2B7 (Itaaho et al,, 2008; Lepine et al., 2004; Uchaipichat et al., 2006a, 2006b; Krishnaswamy et al. 2004; Court., 2005; Manevski et al., 2011; Walsky et al., 2012). Availability of selective probe substrates for the different UGT isoforms has allowed the determination of RAFs that have been useful in determination of relative contribution of different UGT isoforms in drug's metabolic clearance, as exemplified by recent publications on haloperidol (Kato et al., 2012) and laropiprant (Gibson et al., 2013). Information on protein quantification of several UGT isoforms has enabled determination of ISEF values to allow improved prediction of UGT reaction phenotyping. Protein abundance of various UGT isoforms including 1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, 2B10, 2B15 and 2B17 were quantified in human liver, intestine and kidney primarily by LC/MS/MS (Fallon et al., 2013a, 2013b; Milne et al., 2011; Harbourt et al., 2012; Sato et al., 2012; Schaefer et al., 2012; Sridar et al., 2013). Due to differences in the protein abundance values across different studies caution should be taken when these values are used for the generation of ISEF values. It is advisable that either the median values or a range of values be used.

Chemical Inhibitors of UGT

Incubations in microsomes in the presence of selective UGT inhibitors may also be useful as a complementary method to assess the fractional metabolism by UGT. Table 2 summarizes examples of proposed selective substrates and inhibitors of five major hepatic UGT isoforms and recommended inhibitor concentrations relative to their inhibition potency (IC_{50} values). Examples of selective inhibitors are atazanavir for UGT1A1/1A3 (Zhang et al., 2005), hecogenin for UGT1A4 (Uchaipichat et al., 2006a, 2006b; Walsky et al., 2012), troglitzone for UGT1A6 (Ito et al., 2001), digoxin, transilast and niflumic acid for UGT1A9 (Vietri et al., 2002; Lapham et al., 2012) and β-phenyllongifolol-2 for 2B7 (Bichlmaier et al., 2007). Careful optimization of experimental conditions (Court et al., 2014; Zhou et al., 2014) (also more in Supplemental) should be done for the selection of suitable inhibitor concentrations, e.g. when considering the presence of BSA, in particular for UGT1A9 and 2B7 reactions. These methods have not been well-established yet for several other UGT isoforms. As with any chemical inhibitor (described in CYP inhibitor section), chemical inhibitors of UGT are rarely absolutely specific at higher concentrations used for phenotyping studies. It is therefore warranted to carefully assess and account for cross-reactivity when using chemical inhibitors. Additional tools appropriate for *in vitro* reaction phenotyping for an extended number of UGT enzymes considered to be important in drug metabolism need to be developed.

The use of selective inhibitors in liver microsomal incubations together with commercially available recombinant human UGT isoforms, selective probe substrates, and protein abundance information in intestine, liver, and kidney, for some of the major UGT isoforms has led to the emergence of a more semi-quantitative approach towards determination of the contribution of these UGT isoforms to overall drug clearance. However, current tools only allow quantitative scaling for limited UGT isoforms (UGT1A1, 1A6, 1A9, and 2B7) as this area remains an area of active growth. For drugs metabolized by both CYP and UGT, incubations in human liver, kidney, and intestinal microsomes together with CYP and UGT cofactors in the presence of BSA, to improve $f_{m,CYP}$ and $f_{m,UGT}$ prediction has been proposed and is also an emerging area (Cubitt et al., 2011)(Supplemental). IVIVC analyses (Miners et al., 2006) (Supplemental) of limited dataset of UGT-mediated drugs suggest mixed success in the prediction of total UGT-mediated whole body clearance of drugs. Under-predictions were observed in cases like zidovudine and morphine, while reasonable success was demonstrated for drugs like laropiprant (Gibson et al., 2013), where inclusion of BSA was suggested to improve the predictions. Low incidence of UGT-mediated DDI in the clinic has also made it challenging to extrapolate *in vitro* $f_{m,UGT}$ values to those in the clinic. Analysis of limited cases of UGT-mediated DDIs as in lamotrigine-valproic acid (Rowland et al., 2006), zidovudine-fluconazole (Uchaipichat et al., 2006b), zidovudine-vaproic acid interactions also suggest improvement in predictions of DDI magnitude with addition of BSA. Addition of BSA to improve predictions of UGT-mediated clearance and for determining $f_{m,UGT}$ has not been standardized across companies and continues to be an area of further research.

Relative Contribution of Glutathione-S-Transferases

Fractional metabolism by GST may be assessed by intrinsic clearance in hepatocytes or human liver S9 fractions fortified with reduced glutathione (GSH). GST-mediated metabolism may also be qualitatively assessed by determining intrinsic clearance in the presence of GSH versus intrinsic clearance in cytosol in the presence of other cofactors that may be needed for other known or suspected pathways of metabolism (e.g. NADPH for CYP-mediated oxidation or UDPGA for UGT). Intrinsic non-enzymatic reactivity of NME with GSH in buffer needs to be determined and factored in while establishing GST mediated metabolism. Inhibitors such as ethacrynic acid may be used to confirm the involvement of GST isoforms (Ploemen et al., 1990;

Ahokas et al., 1985), though sufficiently selective inhibitors for the different GST enzymes are needed to effectively conduct reaction phenotyping in subcellular or cellular systems.

Human recombinant GST isoforms such as GSTA1, A2, M1, M2, P1 and T1 are available commercially and may be used to assess the qualitative contribution of the different GST isoforms in GSH-conjugation. However, tissue abundance of various GST isoforms (including different GST genotypes) and GST isoform-selective substrates (Ginberg et al., 2009) need to be further evaluated to develop scaling factors for reaction phenotyping using human recombinant GST isoforms.

Due to the expression of many GST isoforms in extra-hepatic tissues (e.g. GSTP1 in erythrocytes) (Awasthi et al., 1994; Whalen and Boyer 1998), *in vitro-in vivo* extrapolation of human clearance needs to take into consideration the contribution from extra-hepatic organs (including blood). *In vitro-in vivo* extrapolation of GST-mediated metabolic clearance remains to be established and hence data from *in vitro* experiments should be interpreted with caution when assessing overall metabolic clearance by GST. There are no reported metabolism-based DDIs via GST that this working group is aware of.

Estimating Relative Contribution of Sulfotransferases

Sulfotransferases (SULTs) are cytosolic enzymes that transfer a sulfonate group from 3’-phosphoadenosine-5’-phosphosulfate (PAPS) to a drug molecule thereby making it more polar and readily excreted mostly in urine (Pakinson and Ogilvie 2007). In addition to biotransformation of many endogenous substrates such as cholesterol, dehydroepoandrostrerone (DHEA), or estradiol and other estrogens, several hepatic SULTs are responsible for biotransformation of xenobiotics. These SULTs include SULT1A1, SULT1A2, and SULT2A1. Phenols and aliphatic alcohols are the major sites of sulfation on drug molecules. Other sites such as aromatic amines and primary amines can also undergo sulfation. However, unlike glucuronidation, sulfation usually does not occur on carboxylic acids. SULT and UGT often share similar substrate properties, such as conjugation on phenol groups. However, SULT is easily saturable, high affinity low capacity enzyme class, whereas UGT is a low affinity high capacity enzyme class (Pakinson and Ogilvie 2007), due to which UGTs are often the predominant enzymes if phase 2 conjugation is the major metabolic pathway. *In vitro* studies need to be carefully designed to elucidate the sulfation pathway since sulfation is easily saturated at low concentrations (Li et al., 1999; James 2014).

Relative contribution by SULT for a given compound can be done using recombinant SULT isoforms to determine the intrinsic clearance value of a NME. The abundance of SULT isoforms in human liver, kidney, intestine, and lung has been reported using Western blot quantification (Riches et al., 2009) (Supplemental Table 2S), though the current literature is limited. The intrinsic clearance values in theory can be normalized by the reported relative abundance of SULT in human tissues (e.g. ng SULT/g cytosol) to obtain the relative contributions of each SULT toward the total clearance (assumption is that relative abundance of various SULT isoforms determined by Western blot is reflective of relative activity). 2,6-Dichloro-4-nitrophenol has been reported to inhibit various SULTs with varying potency (Wang et al., 2006). Using a combination of rhSULT and inhibition by 2,6-dichloro-4-nitrophenol (Table 2) one can study the relative contribution of SULT isoforms in liver S9, cytosol, or hepatocytes. Improved predictions of $f_{m,SULT}$ utilizing hepatic and intestinal scaling factors are also emerging (Gertz et al., 2011) and will be an area of continued research to see whether additional incorporation of $f_{m,SULT}$ will also refine predictions of $f_{m,CYP}$ and $f_{m,UGT}$ for NMEs that are metabolized by all the three enzymes (e.g. troglitazone). This working group is not aware of any reported cases of metabolic DDIs via SULT.

Identifying Role of N-acetyltransferases NAT1 and NAT2

The N-acetyltransferases (NATs) are an important family of metabolizing enzymes that catalyze direct conjugation of aryl and alkyl amine compounds such as hydrazine with the acetyl group of acetyl-CoA (Sim et al., 2008). These cytosolic enzymes have been shown to be important in arylamine-containing drug detoxification and carcinogen activation and are present in liver, esophagus, stomach, small intestine, colon, ureter, bladder, and lung (Windmill et al., 2000). In humans, there are two functional NAT genes encoding two isoenzymes, NAT1 and NAT2. A genetic polymorphism at the NAT2 gene locus encoding for polymorphic NAT2, results in individuals with rapid, intermediate or slow acetylator phenotypes (Sim et al., 2008a). NAT1 which also metabolizes a variety of arylamines including p-amino benzoic acid but not isoniazid, is also polymorphic although the clinical effect of NAT1 polymorphism is less well-defined compared to NAT2 polymorphisms. NAT polymorphism leads to different rates of inactivation of drugs, such as isoniazid, hydralazine, and sulphonamides, which undergo NAT-mediated metabolism.

NAT1 and NAT2 have distinct substrate specificities (Wu et al., 2007): NAT2 acetylates hydralazine, isoniazid, and sulfamethazine, while human NAT1 acetylates sulfamethoxazole, *p*-aminosalicylate and *p*-aminobenzoylglutamate (folate catabolite). Human NAT2 is mainly in the liver and gut while human NAT1 is expressed in many tissues (Windmill et al., 2000). Caffeic acid (Table 2) ferulic acid, and gallic acid have been shown to selectively inhibit NAT1 whereas scopuletin and curcumin (Table 2) have been shown to selectively inhibit NAT2 (Kukongviriyapan et al., 2006).

Once involvement of NAT1 or NAT2 is confirmed using recombinant NAT1 or NAT2, the relative contribution of NAT1 or NAT2 may be assessed in human hepatocytes, using selective chemical inhibitors, as outlined in Figure 7. A RAF-type approach, using NAT1 or NAT2 probe substrates in commercially available NAT1 and NAT2 (fortified with cofactors acetyl-CoA and an acetyl-CoA regenerating system composed of acetyl-*dl*-carnitine and carnitine acetyltransferase) system can theoretically be scaled to total metabolism in human hepatocytes. However, such scaling approaches are not well-established and should be interpreted with caution.

Since only a few drugs have *N*-acetylation as the major metabolic clearance pathway documented incidences of NAT-mediated DDI are rare. However, potential inhibitions of NAT activity were reported by gypenosides, cisplatin, and nitrosoarene metabolites of carcinogenic arylamines (Chiu et al., 2004; Ragunathan et al., 2008; Liu et al., 2008). When NAT is identified to be the primary enzyme responsible for the metabolic clearance of a NME it is advisable to assess the contributions of the NAT and isoforms using currently available tools.

Determining Relative Role of Carboxylesterase CES1 and CES2

Carboxyesterase (CES) enzymes are serine esterases responsible for the hydrolysis of esters, amides, thioesters and carbamates (Laizure et al., 2013). In humans the two major carboxyesterases known are human carboxyesterase-1 (hCES1) and human carboxyesterase-2 (hCES2). CES1 is primarily expressed in the liver where it plays an important role in the metabolism of many prescribed medications including clopidogrel (Kazui et al., 2010; Hagihara et al., 2009) and methylphenidate (Nemoda et al., 2009). CES2 is present predominately in the intestine where it has been shown to hydrolyze anti-cancer pro-drugs gemcitabine (Pratt et al., 2013), capecitabine (Ribelles et al., 2008) and irinotecan. (Humerickhouse et al., 2000).

During *in vitro* assessment if NADPH-independent metabolism is observed with a NME exhibiting an ester or amide-linkage, metabolism using rhCES1 and rhCES2 (commercially available) should be investigated. If no metabolism is observed in the recombinant CES the probability of CES involvement is low and no further evaluation is warranted. If NME metabolism is observed in the recombinant CES system further assessment can be conducted using sub-cellular fractions e.g. human liver (CES1 enriched, low CES2 levels) and intestinal (CES2 only) microsomal and/or S9systems, in combination with specific substrates and inhibitors listed in Table 2, to evaluate the relative contribution of CES to overall clearance (Nishimuta et al., 2014; Ross et al., 2012; Shimizu et al., 2014; Zhu et al., 2009). There are no known CES-mediated metabolic DDIs reported making this enzyme class as a potentially low risk for causing metabolic DDIs.

Special Considerations: Low Turnover, Extra-Hepatic Metabolism, Inhibition of Parallel Metabolic Pathways

Approaches for the Determination of Metabolic Pathways for Low Clearance Compounds: The determination of fractional metabolized in subcellular systems or in recombinant enzymes by metabolite formation kinetics or substrate depletion approaches may become problematic for compounds that exhibit very low intrinsic clearance. An example is ertugliflozin (PF-04971729), a selective inhibitor of the sodium-dependent glucose co-transporter 2 (SGLT2). Reaction phenotyping studies indicated that both CYP (CYP3A4/3A5) and non-CYP pathways (UGT1A9 and UGT2B7) are involved in the metabolism of this compound (Kalgutkar et al., 2011). However, quantitative assignment of each of these pathways was not feasible due to low *in vitro* turnover in hepatic microsomes and hepatocytes. Subsequent to the *in vitro* evaluation, human ^{14}C-ADME study using ^{14}C-ertugliflozin showed that UGT was the major metabolic pathway whereas CYP metabolism was minor (Miao et al., 2013). In general, upon understanding the total picture of metabolism from the ^{14}C-ADME study, quantitative *in vitro* delineation of the enzymes responsible for metabolic clearance can be done by measuring the rates of metabolite formation using HPLC-MS with authentic metabolite standards. Recently, approaches including the hepatocyte relay method (Di et al., 2013) and co-culture models, such as HepatoPac or Hurel (Chan et al., 2013) have been evaluated and have shown promising results to better determine CL_{int} via increasing the amount of substrate

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depletion or metabolite formation. While these systems have gained considerable popularity in generating metabolites and determining intrinsic clearance of very low clearance compounds, the use of these models for phenotyping studies is under development (Yang et al., 2015).

With radiolabeled NME, when metabolites generated during a microsomal incubation under linear kinetics are too low in quantity to be quantified accurately by a radioactivity detector, a more sensitive LC/MS/MS method may provide an alternative for quantification. In early development when synthesized metabolite standards are usually not available, a radioactive calibration approach (Yi et al., 2010; Yu et al., 2007) can be applied. In this approach, bio-reactors (concentrated pool of enzymes), can be used to generate a sufficient amount of metabolites. Metabolites generated via this method usually follow non-linear kinetics. Also, since in majority of the cases metabolites are formed under supra-physiological NME concentrations, they may not reflect the high affinity low capacity pathway, predominant *in vivo*. The samples can be injected in parallel into a radioactivity detector / LC/MS/MS system to determine the ratio of radioactive DPM and LC/MS/MS peak area for each metabolite and a calibration ratio can then be calculated for each metabolite, as:

*Metabolite * (DPM) = LC/MS/MS peak area from kinetic incubation * DPM/peak area ratio from the bio-reactor incubation*

Further calculation can be applied to obtain the absolute concentration of a metabolite of interest by using parent drug as an additional calibration (Yi et al., 2010). Thus, the metabolites can be detected using the LC/MS/MS and quantified using the calculation above.

Extra-Hepatic Extraction

Several CYP and non-CYP enzymes discussed above are expressed intestine and kidney in addition to liver, which is recognized to be the major metabolizing organ. So for refined assessments of victim DDI interactions primary site of metabolism needs a case by case consideration once the major DMEs involved have been identified. Intestinal CYP3A4-mediated metabolism has been implicated to be major contributor of the overall first pass metabolism of a large number of CYP3A4 substrates, such as cyclosporine, tacrolimus, midazolam, felodipine, simvastatin, atorvastatin, verapamil, and nifedipine, where inhibition of intestinal CYP3A4 may significantly contribute to the overall magnitude of a DDI. Analysis and comparison of observed and estimated ratio change of fraction extracted in gut (F_G) $F_{G,inhibited}/F_G$ for CYP3A4 victim drugs have highlighted the need to accurately estimate and incorporate $F_{G,inhibited}/F_G$ ratio for prediction of a DDI magnitude of a victim drug (Galetin et al., 2007, 2008), in particular for highly extracted victim drugs whose $F_G \leq 0.5$. For such drugs that undergo high first pass intestinal extraction ($\geq 50\%$), magnitude of DDI will be very sensitive to inaccuracies in estimating the $F_{G,inhibited}/F_G$ ratio. Since F_G is not a commonly measured parameter in clinic it is strongly recommended to understand the impact of uncertainty of $F_{G,inhibited}/F_G$ ratio via sensitivity analysis modeling (more in modeling and simulation section). Metabolism-based DDIs in kidneys have not been reported.

Impact of Simultaneous Inhibition of Parallel Metabolic Pathways/Enzymes

It is generally desirable for a NME to have multiple metabolic pathways of clearance so when one pathway is inhibited the impact on its exposure change is not drastic. However, investigators should appreciate cases where parallel metabolic pathways of a victim NME are simultaneously inhibited (Ogilvie and Parkinson, 2014). This can potentially occur due to simultaneous loss of activity of multiple DMEs involved in the parallel metabolic pathways of the victim DME or DME and transporters, via any combination of factors such as genetic polymorphism, chemical inhibition, and disease state. Result is a dramatic change in exposure of a victim NME. Several victim DDI examples are well-documented including those of propranol (genetic polymorphism); omeprazole, lansoprazole (genetic polymorphism + CYP inhibitor); telithromycin (disease state + CYP inhibitor); and ramelteon (multi-CYP inhibitor) (Obach and Ryder, 2010). Investigators are encouraged to read some excellent publications for detailed understanding of the complex nature of such victim DDIs (Ogilvie and Parkinson, 2014; Isoherranen et al., 2012; Ito et al., 2005) and consider this on a case by case basis during NME development, as also recommended in regulatory guidances (FDA 2012 and EMA 2012).

Current Status for Phenotyping of CYP and non-CYP enzymes

Substantial research over the past several years to mitigate CYP-mediated victim DDI in the clinic has enhanced our ability to predict $f_{m,CYP}$ of NMEs with high confidence, utilizing commonly used methods of RAF/ISEF and use of CYP-specific chemical inhibitors. Several of these examples have been summarized in literature publications (Youdim et al., 2008; Lu et al., 2008) and in recent regulatory submissions as in the case of ibrutinib (http://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm432240.html). Substantial higher incidence of CYP-mediated compared to non-CYP-mediated DDI cases in the clinic has allowed scientists to assess the IVIVC of the observed CYP-mediated victim DDIs and therefore, to gain better confidence in the currently available *in vitro* tools, such as scaling methods, recombinant enzymes, probe substrates and selective chemical inhibitors of CYPs. The knowledge gained from the CYPs has been applied to other non-CYP enzymes, which has led to tremendous growth in the understanding of the non-CYP enzymes and identification of selective inhibitors and probe substrates for several of the non-CYP enzymes, as discussed in this section and summarized in Table 2. This has remarkably increased the confidence amongst researchers to predict overall CYP vs non-CYP contribution towards the hepatic metabolism of a NME (Table 3). However, extra-hepatic tissue abundance and activity information for majority of the non-CYPs (including several UGT isoforms), is still largely absent. This poses a challenge in estimating $f_{m,non\text{-}CYP}$ towards NME clearance if extra-hepatic enzymes are substantially involved as primary metabolic pathways. This is demonstrated from lack of predictive power to accurately assess contribution of non-CYP enzymes AO and UGT towards overall drug clearance, observed in the analysis of known, limited set of drugs metabolized by AO (Zientek et al., 2010) and UGT (Miners et al., 2006). Scaling factors for human liver, kidney, and intestinal matrices are emerging and will continue to be an area of active research to refine predictions of $f_{m,CYP}$ and $f_{m,non\text{-}CYP}$ enzymes. Due to scarce incidence of reported non-CYP-mediated clinical DDIs such as those of XO-mediated allopurinol- azathioprine, UGT-mediated lamotrigine-valproic acid, zidovudine-fluconazole and, zidovudine-vaproic acid), extrapolation of *in vitro* $f_{m,non\text{-}CYP}$ to a clinical setting continues to be a challenge and will be monitored closely by companies as more NMEs, with involvement of non-CYP enzymes emerge in coming years.

Knowledge of $f_{CL,metabolism}$ as it grows with the progression of a potential drug candidate through various development stages, is used in concert with the f_m data and allows better informed estimates of the overall victim DDI potential of a NME. Next section will discuss common approach how information gathered at various stages of drug development is used to estimate the contribution of metabolism to overall NME clearance.

APPROACHES TO DETERMINE CLEARANCE PATHWAYS

Regulatory guidances from FDA and EMA (FDA, 2012; EMA, 2012) indicate that a clearance pathway which constitutes ≥ 25% of the total clearance, is a reasonable starting point for further clinical assessment to mitigate a potential victim DDI risk. Additionally, this information may be used to assess the need for evaluating pharmacokinetics in special populations, such as hepatic and renal impairment or pediatrics depending on the expected patient population. It is important to have a conceptual understanding as to why we are interested in determining whether a clearance pathway is major or minor. Two fundamental questions aid in providing a basis: 1) what are the intrinsic (e.g. genetics, disease state, age) and extrinsic (e.g. co-administered drugs, herbal supplements, smoking, diet) factors that may influence the pharmacokinetics of a NME and 2) are these intrinsic or extrinsic factors likely to result in dose adjustment based on pharmacological response or safety issues. Once these factors have been considered, a development program with appropriate drug interaction studies and/or co-medication exclusions can be implemented. The process to understand the clearance pathways starts early in pre-FIH stages and is refined during the course of clinical development. Knowledge gained to better understand clearance pathways at various stages is summarized in this section.

Prediction from Preclinical Species and *In vitro* Systems - Learning from an *In vitro*: *In vivo* Correlation Approach (Pre-FIH).

Early qualitative assessment of clearance routes in human starts in preclinical stage via assessment of IVIVC in preclinical species. A typical IVIVC approach is outlined in Figure 8. Assessments of the contribution of metabolism, renal, and biliary elimination ($f_{CL,\ metabolism/renal/biliary}$) towards total NME clearance are commonly made in at least two preclinical species. Preclinical PK studies, conducted with un-labeled or radiolabeled NME using intact or bile duct-cannulated (BDC) animals can provide information as to the route(s) of clearance based on quantification of unchanged drug in urine, feces, and bile (in case of BDC). Assessment of f_{CL} quantitatively can be best done with the use of a radiolabeled NME. However, useful information can also be obtained from studies using un-labeled NMEs under careful considerations such as the potential lack of mass balance and the assumption that all un-recovered NME was metabolized (i.e. major route of clearance was via metabolism) which may not be correct.

The route of clearance derived from *in vivo* preclinical animal studies may be used to provide some qualitative insight into a NME’s route of clearance in humans. Experience amongst different pharmaceutical companies suggests that if the NME is predominantly metabolized in animals and there is a good IVIVC in multiple preclinical species, metabolism is likely the major clearance pathway in humans. Similarly, if a NME is primarily cleared unchanged renally in animals, a substantial proportion of the NME may be expected to be cleared unchanged renally in humans. Thus, insight into the qualitative involvement of metabolic or renal clearance in humans may be obtained based on results from multiple preclinical species.

Prediction of $f_{CL,renal}$ in humans from preclinical species has been done with reasonable success when the NME is cleared via passive filtration (Paine et al., 2011), but knowledge is still emerging for NMEs cleared via active secretion and re-absorption. Since these latter processes usually involve transporters, inter-species differences can be expected and extrapolation from animals to humans may not be straight forward. Based on analysis of a large dataset of renally excreted compounds (Varma et al., 2009), physicochemical properties such as ionization state, lipophilicity, and polar descriptors have been proposed as important determinants of human renal clearance, taking into consideration both net reabsorption as well as net secretion. Such early assessments may be valuable in identifying compounds with potentially high $f_{CL,\ renal}$, and aid to refine overall contribution of metabolism towards compound’s clearance. The BDC rat is a routinely relied upon model for the prediction of biliary excretion in humans and to provide qualitative insight into hepato-biliary disposition in humans. However, due to significant species differences in the function, substrate specificity, and regulation of transporter proteins, it is difficult to directly extrapolate animal hepato-biliary data quantitatively to humans (Ghibellini et al., 2006; Swift et al., 2010; Wang and LeCluyse 2003; Ishizuka 1999). *In vitro* models have begun to emerge to characterize hepato-biliary elimination (Pfeifer et al., 2013) and predict the extent of biliary excretion of drugs in humans. Establishing IVIVC of biliary excretion in humans remains a challenge in part due to the lack of high quality and quantitative biliary excretion data in humans. Hence, the predictive power of *in vitro* models for estimation of $f_{CL,biliary}$ in humans remains debatable.

During preclinical PK studies metabolite profiles from *in vivo* animal studies are usually compared to those from *in vitro* animal studies in subcellular fraction or hepatocytes. A good IVIVC of metabolic pathways in multiple preclinical species can offer some confidence in predicting major metabolic pathways in humans from *in vitro* human data. An early estimate of *in vitro* contribution of phase 1 (CYP vs non-CYP oxidation) and phase 2 (conjugation) metabolic pathways towards metabolic clearance of a NME also gives a preliminary estimate of any potential victim DDI risk associated with a pathway (such as those involving CYP).

Availability of current robust *in vitro* human models including human-derived sub-cellular fractions, recombinant enzymes or hepatocytes, have allowed researchers to gain better confidence from successful prediction of *in vivo* human profiles prior to Phase I studies.

This IVIVC exercise allows early qualitative assessment of the role of metabolism and potential metabolic pathways in humans and raises any alert of potential victim DDI risks. Researchers should recognize that the metabolic pathways *in vitro* may not reflect primary metabolic pathways *in vivo*. Also, reliance on animal data to predict human metabolic pathways is not recommended since the extent of metabolism and metabolic pathways in humans can be very distinct from that in animals. This is especially true when non-CYP metabolizing enzymes and transporters are involved in the disposition of a NME.

Learning from First-in-Human Studies

In early clinical development information of metabolic clearance pathways is gained from metabolite profiling studies using plasma and urine samples routinely collected from the FIH single and/or multiple dose (SAD and/or MAD) studies. Innovative advances in high efficiency sample preparation and response normalization-based bioanalytical techniques (^{14}C- calibrant approach, use of quantitative NMR; Yu et al., 2007; Ramanathan et al., 2010; Dear et al., 2011; Vishwanathan et al., 2009) have made it possible for investigators to gain reliable quantitative estimates of metabolic pathways in plasma and urine in the absence of authentic metabolite reference standards. Quantitation of unchanged drug in urine enables preliminary estimation of $f_{CL,renal}$.

Assessment of metabolic clearance pathways in feces in cold FIH studies is not a common practice due to the lack of established quantitative methods to assess drug/metabolite recovery in cold fecal samples. It is therefore recommended to exercise caution for victim DDI risk assessments with estimates of metabolic clearance pathways in plasma and urine only, as the metabolism information based on these two matrices will be incomplete. A common and often erroneous assumption at this stage is that metabolites detected in plasma and urine truly represents all the major metabolic pathways of a NME. This assumption may hold in cases where a NME and its metabolites are predominantly cleared renally, where $f_{CL,metabolism}$ and $f_{CL,renal}$ may be obtained with reasonable confidence. However, elimination of unchanged NME and/or metabolites via biliary excretion into feces may also contribute substantially towards overall clearance in which case, clearance pathway information derived from fecal samples will be an important determinant of f_{CL} and f_m. Recent advances in clinical devices as Entero-Test® (Guiney et al., 2011; Bloomer et al., 2013) have enabled direct assessment of drugs and metabolites excreted into the bile of humans. This method can be applied in early clinical development to provide qualitative information on the risk of interactions for drugs that are metabolized and eliminated in bile. Increased conduct of ^{14}C-micro-tracer studies (trace ^{14}C-NME administered as part of SAD and MAD studies), coupled with accelerator mass spectrometry (AMS) detection (Lappin et al., 2008) have also enabled investigators to gain quantitative information of metabolic clearance pathways in circulation and in feces early inclinical development.

Information gathered from early metabolite profiling in FIH studies is valuable to get a first look into the major metabolic clearance pathways in plasma and urine and in certain cases bile (quantitative information when ^{14}C-microtracer studies have been incorporated into FIH study designs or qualitative when techniques such as Entero-Test® have been used). In addition, metabolite profiling in FIH studies can prove valuable in identifying primary metabolic pathway *in vivo* that may not have been previously identified in *in vitro* human models pre-FIH although such cases are of low incidence. With certain caveats as discussed above, the overall learning from FIH study is helpful in refining victim DDI risk assessments made at pre-FIH stages.

Route of Clearance Determination in Human ^{14}C -ADME Study

Human ^{14}C-ADME study is deemed as the gold standard *in vivo* study to quantitatively determine routes of clearance and metabolic pathways of a NME (ensuring good mass balance is observed), where unchanged drug and drug-derived metabolites are measured in plasma, urine and feces. Data from the radiolabeled metabolite profiling in human are informative in the planning and design of definitive *in vitro* reaction phenotyping and clinical DDI studies required in support of a NME's registration. When formation of secondary or tertiary metabolites is observed, it is assumed that the enzyme involved in the formation of the primary metabolite is of the most interest for DDIs (assuming a worst-case scenario).

A caveat to consider in these studies is the difficulty sometimes in interpretation of clearance pathways in feces based on metabolite profiles observed in fecal samples. Gut microflora can potentially convert certain metabolites back to their parent (e.g. known for glucuronides and N-oxides). Following an oral dose (dosing route for majority of drugs), unchanged NME detected in feces could be due to 1) either unabsorbed drug or hepatically formed metabolites excreted in bile and further metabolized to parent drug by gut microflora or 2) absorbed drug excreted unchanged in bile via liver or 3) intestinal secretion of unchanged drug. Metabolites in feces especially if observed at substantially higher levels in feces compared to lower/non-existing levels in circulation may also indicate substantial metabolism via intestinal enzymes. Potential contribution of intestinal and hepatic metabolizing enzymes (and transporters) towards NME metabolism and excretion into feces vs unabsorbed drug component is important to understand for victim DDI risk evaluation.

Ideally the best estimate of $f_{CL,biliary}$ can be made after IV dosing of a NME (radiolabeled or radiotracer) followed by determination of unchanged NME in feces but IV ^{14}C-ADME studies are not routinely done (approximately 20% of ^{14}C-ADME studies done across IQ companies were via IV route). Enhanced appreciation of information gathered from such studies, has resulted in increased conduct of microtracer IV ^{14}C-ADME in recent years with primary goals of providing better understanding absolute bioavailability/PK, mass balance, and routes of clearance. It should be noted that intestinal secretion cannot be confidently determined even after an IV dose due to the fact that quantitative, uninterrupted bile collection from healthy humans is not possible.

Investigators need to comprehensively understand the contribution of metabolism towards the overall routes of clearance of a NME via careful quantitative evaluation of metabolite profiles in plasma, urine, and feces. Understanding the site of formation of primary metabolites (e.g. intestine, liver, or kidney) is crucial in evaluating metabolizing enzymes involved in the primary metabolic pathways and responsible for precipitation of metabolic victim DDI.

Incorporating robust *in vitro* f_m data and $f_{CL,metabolism}$ information with appropriate modeling and simulation may also provide better confidence in prediction of victim DDI liability in the clinic. Modeling and simulation allows investigators to better assess dependence/sensitivity (i.e. the uncertainties in measurement of these parameters) of these key victim DDI parameters and their impact on clinical DDI timing and study design at different stages of drug development. The following section will briefly discuss various commonly used modeling approaches along with an explanation of their suitability in specific development phases based on available data and specific questions of interest.

APPLICATION OF MODELING AND SIMULATION TOOLS IN VICTIM NME DDI PREDICTIONS

Over the past ten years, modeling and simulation approaches have increasingly been evaluated for its utility in predicting the magnitude of DDIs particularly those mediated by CYPs, by pharmaceutical companies and regulatory agencies. Results from these evaluations have led to increased application of quantitative modeling and simulation approaches, which has been attributed to an improved understanding of the factors that affect prediction accuracy and leading to increased confidence in DDI prediction. Pharmaceutical companies have used quantitative DDI predictions to impact decision-making in drug development and support interactions with regulatory agencies. Regulatory agencies recognize the value of DDI prediction using modeling and simulation tools as highlighted by the recent FDA and EMA draft guidance documents (FDA 2012; EMA 2012).

The most commonly used approaches for quantitative DDI prediction include static concentration predictions and dynamic concentration prediction by a more mechanistic physiologically based pharmacokinetic (PBPK) modeling approaches. Numerous methodologies based on both simplified and PBPK-based approaches have been well-defined previously in the literature (Fahmi and Ripp 2010; Peters et al., 2012; Brown et al,). In all cases model selection should be driven by specific development questions and the extent of data available at the time of model development. Briefly, available predictive models for victim DDI prediction can be generally organized into three categories: simple static, mechanistic static and mechanistic dynamic models (Supplemental Table 3S):

Simple Static Models: Static models are based on a single static perpetrator concentration. In the most simplified versions f_{mCYP} for victim drug is assumed to be 100% (worst case scenario). Numerous reference concentrations for perpetrator (total or unbound) have been proposed previously (Ito et al., 2004; Obach et al., 2007). Generally for the perpetrator, the total average concentration at steady state, maximum concentration at steady state, or hepatic inlet concentration are recommended and used to predict victim drug interactions in the liver using the static model (FDA 2012). Therefore, this approach often leads to over-predictions of victim

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DDIs (Ito et al., 2003). The static model can be a useful tool for early DDI risk assessment when supporting data are limited (e.g. during early discovery phase).

Mechanistic Static Models: Mechanistic static models incorporate more victim drug information such as estimated f_{mCYP} and impact of the DDI on both hepatic and gut metabolism (Obach et al., 2006) or the integrated impact of competitive or mechanism-based inhibition and induction (Fahmi et al., 2008). The Mechanistic static model is often applied once *in vivo* victim drug data are available although this is not required (Table 4, Example 6).

Mechanistic Dynamic Models: Mechanistic dynamic models including Physiologically-Based Pharmacokinetic (PBPK) simulations incorporate the time-dependent change of both perpetrator and victim drug concentrations over the time course of the DDI. Therefore, this approach permits investigation of non-linear kinetics and time-dependent phenomena which include drug accumulation, additional impact due to the formation of inhibitory metabolite(s) and effect of dosing regimen. Commercial software packages are available which allow incorporation of literature data on human physiology, changes in special populations and disease states, and drug effects on physiological rates (e.g. Simcyp (Certara), Phoenix (Certara), GastroPlus (Simulations Plus), PKSim (Bayer)). PBPK modeling requires the greatest degree of parameterization based on both *in vitro* and *in vivo* data. Key victim drug parameters required for PBPK model development include volume of distribution (V_{dss}) and clearance (CL) (Supplemental Table 4S). Although PBPK approaches may be applied at the stage of discovery or development, it is most powerful in later stages when more data become available.

Model Application: “When and what”

Model selection must always be issue driven and based on particular development stage and available data. During early clinical development (i.e. clinical candidate selection) predictions are primarily used to improve mechanistic understanding and support internal decision-making. At this stage the understanding of victim drug disposition is mainly based on preclinical and/or *in vitro* data, which are generally limited. Applications of modeling at this stage include support of prediction of clinical DDI risk, DDI study design, and victim drug dose selection (especially critical for a victim drug that has narrow safety window). In these situations a simplified model may be more suitable. In situations where more definitive *in vitro* ADME data and Phase I clinical PK data are available PBPK simulation is applied. Retrospective model simulation of *in vivo* data can be used to estimate $f_{m,CYP}$ and the impact of alternative trial design on outcomes. In later stages of development when data from human mass-balance and DDI studies with a specific inhibitors (e.g. itraconazole for the inhibition of CYP3A4 for drugs metabolized by this enzymatic pathway) becomes available the model is further refined and used for simulation of victim DDIs with other inhibitor(s) or inducer(s) of the same pathway, or inhibitors of a minor metabolic pathway, victim drug PK in special populations (e.g. hepatic or renal impaired), dose selection, influence labeling, or justify of waivers for additional DDI studies.

Building Confidence in Model Predictions

Several approaches have been developed to build confidence in and ensure mechanistic relevance of the simulation results, which will lead to increased impact of modeling and simulation efforts. These approaches include: verification of assumptions, sensitivity analysis, and assessment of variability. The level of model verification or validation will depend upon the stage of development and its impact on critical decision-making.

Verification of Assumptions Although many model parameters may be experimentally determined, assumptions must often be made about the translatability of *in vitro* data to *in vivo* DDI. Assumptions must always be evaluated particularly in scenarios when complicated disposition mechanisms are expected. Investigation of assumptions to which the model is particularly sensitive, may help explain disconnects between *in vitro* data and *in vivo* DDI (e.g. complex DDI, poor IVIVE, lack of quantitative prediction of non-CYP and transporter pathways, non-linear PK, inhibitory metabolites, altered PK in disease state).

Sensitivity Analysis. Sensitivity analysis is often employed to assess the mechanistic relevance of the model and to identify gaps in current mechanistic understanding. In this approach model parameters which have the greatest impact on the simulation output are identified as requiring increased scrutiny. Sensitivity analyses can lead to the design and execution of additional supporting *in vitro* or *in vivo* studies. For victim drug characterization, $f_{m,CYP}$ will be especially important and sensitivity analysis on this parameter is strongly recommended. Consideration of $F_{G,inhibited}/F_G$ for NMEs which undergo substantial intestinal metabolism (e.g. CYP3A4-metabolized drugs such as midazolam, cyclosporine, tacrolimus, felodipine, simvastatin, atorvastatin, verapamil, and nifedipine), is important in DDI risk assessments. F_G values are usually not determined but predicted via various available gut models incorporated in commercial softwares such as Gastroplus and Simcyp. The Q_{gut} model (Yang et al., 2007) is a simple and more commonly used model where the parameter $f_{u,gut}$ (fraction unbound in gut) is one of the key determinants of F_G. Since $f_{u,gut}$ cannot be experimentally measured *in vitro* or *in vivo*, sensitivity analysis of estimated $f_{u,gut}$ on exposure change of a victim drug, is often assessed and is strongly recommended. The sensitivity analysis may inform the impact of variability of these parameters on predicted victim DDI with different perpetrators.

Assessment of Variability. Variability in both observed and simulated data is required to ensure the relevance of model outputs. An essential component of model verification is a comparison of simulated and observed variability around pharmacokinetic parameters of interest. Variability associated with $f_{m,CYP}$ of a victim drug could result in higher predicted PK and DDI variability between individuals, especially in the case of DDI prediction. Sources of variability include inter-subject differences in relative CYP abundance, and in the case of some isoforms, polymorphic gene expression.

Case Examples

The utility of modeling and simulation in victim DDI prediction is best illustrated through the presentation of case examples which highlight approaches used at various phases of drug development. Several case examples have been summarized (Table 4) to illustrate some of the questions typically addressed by modeling in the course of drug development. A typical case example has been described in greater detail to demonstrate how to use and optimize predictive models. The data required to support model development, appropriate assumptions, and the manner in which these are combined to impact development programs are also discussed.

Example 1: What is the DDI risk for Compound which is predominantly metabolized by CYP3A4 *in vitro*? An early development compound was found to be predominately metabolized by CYP3A4 *in vitro*. Based on preliminary preclinical metabolite identification, a significant contribution of non-CYP (phase 2) metabolism was suspected. This compound was highly cleared both *in vitro* and in preclinical animal models. Prior to initiation of modeling, additional data were requested including *in vitro* phase 2 metabolism, *in vitro* permeability, human mass-balance information (fraction absorbed, elimination pathways) and *in vitro* phenotyping in human liver microsomes. Utilizing both *in vitro* and *in vivo* data a PBPK model was developed to predict the DDI risk. The PBPK model incorporated a mechanistic absorption model and tissue distribution based on physiochemical data. Compound elimination was linked to enzyme abundance and based on *in vitro* intrinsic clearance measured in human hepatocytes and $f_{m,CYP3A4}$ predicted from *in vitro* phenotyping in human liver microsomes. A parallel tube model was selected to predict *in vivo* clearance because of the high intrinsic clearance observed *in vitro* and in preclinical species. The model simulations were subsequently verified using clinical data in healthy subjects and in a ketoconazole DDI trial. In addition, a sensitivity analysis was completed to investigate the effect of $f_{u,gut}$, , on baseline and inhibited compound PK profiles.

The simulated DDI with the potent CYP3A4 inhibitor ketoconazole was consistent with the observed clinical outcome. Based on the confidence building approaches of model verification and sensitivity analysis the model was used to simulate additional potential DDIs with other inhibitors. The accurate prediction of the observed inhibition DDI provided high confidence in the ability of the model to accurately predict DDI with potent CYP3A4 inducers.

Recommended Strategy In Modeling and Simulation

Modeling and simulation is a valuable tool in predicting victim DDI throughout every stage of drug development. In each stage fit-for-purpose strategies should be based on the issue to be addressed. During early development stages modeling and simulation will support internal decision-making by leveraging mechanistic understanding when only preclinical and *in vitro* data are available. When data are limited a more simplified modeling approach is advised. At later stages modeling aids study design, dose selection, labeling and justification of delay/waiver of clinical victim DDI studies. As more data become available more complex models may be developed. For initial metabolic victim DDI risk assessments a qualitative understanding of contribution of metabolism towards overall clearance of a NME ($f_{Cl,metabolism}$) and fractional contribution of a DME towards overall metabolism (f_m) is necessary. Typically some human PK data (after single dose with reasonable expectation of linear PK or after multiple dose if not) will be necessary for more quantitative prediction of *in vivo* victim DDI.

PBPK modeling requires the greatest amount of data. However, a PBPK approach allows the greatest potential for “what-if” scenario analysis and simulations leading to improved mechanistic understanding. Specifically PBPK-based predictions allows analyses of how $f_{m,CYP}$ characterization may impact victim DDI prediction. This information may then be used to inform exclusion criteria (i.e. if a polymorphic enzyme involved), victim drug dose selection, sampling schedule and optimal chemical inhibitor.

Sensitivity analysis is strongly recommended in all situations but especially when data are incomplete or uncertain (Jones et al., 2015; Zhao et al., 2012). This assessment can help identify to what extent the model can tolerate uncertainty and the range of uncertainties, as well as indicate additional experimental data to be generated and/or confirmed. For victim drug DDI prediction the fraction metabolized (f_m) and fraction escaping first pass metabolism in the gut (F_G) are common parameters which need to be considered for sensitivity analysis. The worst case scenario from sensitivity analysis may be included in the risk assessment. It is essential that confidence and acceptance/cut-off criteria be well defined at the beginning of model development to ensure a well-established metric for comparing model performance.

GENERAL PERSPECTIVES AND STRATEGIC RECOMMENDATIONS

Since metabolism remains to be the predominant clearance pathway for the majority of small molecule drugs it is important to accurately evaluate the metabolic victim DDI liability of a NME. Two key determinants of a metabolic victim DDI magnitude are $f_{CL,metabolism}$ and f_m, where high values of $f_{CL,metabolism}$ x f_m can have a drastic effect on a victim NME exposure, resulting in undesirable clinical outcomes. In the majority of the cases a good understanding of $f_{CL,metabolism}$ and f_m may be obtained from quantitative human ADME and DDI studies, respectively. The determination of the routes of clearance, including $f_{CL,metabolism,}$ is usually conducted in the ^{14}C-radiolabeled human ADME study, while the determination of f_m is usually conducted in clinical DDI studies with standardized inhibitors or studies in genotyped populations. These definitive studies are most commonly done in the later stages of clinical development (Phase II or Phase III), however, this information is valuable earlier in clinical development, such as when studies are done in patients on various concomitant medications. It is particularly challenging to accurately predict contribution of metabolism towards human clearance pathways ($f_{CL,metabolism}$ x f_m) quantitatively before data from the definitive ^{14}C-ADME and clinical DDI study is available, and this area remains a focus of continued research. There is a common set of *in vitro* and preclinical *in vivo* studies that pharmaceutical companies rely on through various stages of drug development (Table 5) to obtain sufficient data to provide the best estimate of $f_{CL,metabolism}$ x f_m prior to running large clinical trials.

At early stages when no human data are available, reliance is made on the knowledge gained in preclinical models and *in vitro* systems to get preliminary estimates of $f_{CL,metabolism}$ and

f_m. Assessments are made to understand whether a NME is cleared primarily by metabolism or excretion in animals since it has been commonly observed that if a NME is primarily cleared by metabolism in animals or renally via passive filtration, it follows a similar qualitative trend in humans. In parallel establishment of IVIVC of metabolic pathways in multiple preclinical species also gives researchers confidence in predicting metabolic pathways in humans from *in vitro* metabolism studies conducted with human-derived matrices. Metabolism studies in *in vitro* human-derived systems (usually HLM and/or hepatocytes) are conducted to understand the primary metabolic pathways as well as primary DMEs involved in these pathways.

Complementing the $f_{CL,metabolism}$ information obtained in animals with f_m assessments in *in vitro* human systems, may provide an early estimate of the importance of metabolism and various metabolic clearance pathways (e.g. oxidative or conjugation, CYP vs non-CYP-mediated DDI risk) in humans. This preliminary information although qualitative, is informative for victim DDI risk assessment and setting of inclusion/exclusion criteria prior to first dose in humans, especially when the FIH studies are conducted in patients. During these pre-FIH assessments, researchers should be cognizant of the limitations of assumptions made at this stage such as 1) possibility of differences in $f_{CL,metabolism}$ in humans compared to animals; 2) *in vitro* metabolic pathways in human-derived matrices may not be reflective of those *in vivo*. It is also not recommended to extrapolate animal metabolic pathways to quantitatively predict human metabolic pathways since the extent of metabolism, metabolic enzymes, and pathways in humans have been observed to be very distinct from those in animals (Martignoni et al., 2006).

Confidence in *in vitro* determination of f_m of CYP and non-CYP DMEs has grown tremendously with the advancement of research in the area. In the majority of cases researchers are able to accurately identify the enzyme family primarily involved in NME metabolism, although a quantitative measure of f_m for isoforms of several non-CYP DMEs is still absent, as summarized in Table 3. Primary reasons include a combination of factors such as the lack of specific probe substrates, selective chemical inhibitors, and knowledge of extra-hepatic tissue abundance and activity for majority of the non-CYP enzymes. However, considering the reported incidence and magnitude of clinical DDIs in recent years the vast majority of which are CYP-mediated, the inability to quantitatively determine f_m via non-CYP pathways may have lower impact. On the other hand, it is still crucial to be able to accurately identify the overall class of non-CYP enzyme family since involvement of non-CYP enzymes suggests a lower risk of victim

DDI potential via a CYP-mediated pathway; i.e. reduces the victim DDI risk due to involvement of non-CYP enzymes. As more drugs that are primarily metabolized by non-CYP enzymes are developed, understanding the clinical risk of DDI due to these enzymes may also emerge.

Information gathered from metabolite profiling, routinely conducted in plasma and urine in FIH studies allows investigators to assess the presence of any human-specific routes of metabolism not previously identified in preclinical studies or *in vitro* human matrices. It is also possible to get qualitative information into metabolic pathways of orally administered drugs in bile with the use of recent techniques such as Entero-Test® (Guiney et al., 2011; Bloomer et al., 2013), although this has not been widely adopted yet. Identification of human-specific metabolic pathways along with any saturable pathways (as indicated by non-linear PK) guides further refinement of victim DDI potential at FIH stage. Since typically no mass balance is accounted for in FIH studies (fecal samples are typically not collected for analyses), assessment of metabolic pathways to overall NME clearance ($f_{CL,metabolism}$ x f_m), is still qualitative. Availability of advanced techniques such as quantitative NMR has made it possible to reliably obtain quantitative estimates of metabolic pathways in plasma and urine. In such cases it may possible to estimate minimum f_m from the assessment of metabolites in plasma and urine. Conduct of microtracer IV ^{14}C-ADME has also risen in recent years with the primary goal of obtaining more accurate insight into the absolute bioavailability/PK, mass balance, and routes of clearance earlier in clinical development.

Information from a definitive human ^{14}C-ADME study is used to confirm and refine f_{CL} (and $f_{CL,metabolism}$) and metabolic pathway data derived from earlier *in vitro* and *in vivo* studies. Designated clinical DDI studies such as those with a potent CYP inhibitor or those conducted in genotyped population is valuable in further refining the contributions of CYP and non-CYP enzymes ($f_{m,CYP}$ and $f_{m,non\text{-}CYP}$). Information from these definitive human studies can then be incorporated into planning additional victim DDI trials and drug labeling of a NME. Uncertainties may be encountered even in these definitive studies due to the following: 1) challenge in estimating $f_{CL,biliary}$ when substantial unchanged NME is recovered in feces, 2) poor mass balance resulting in inability to estimate f_{CL} (and $f_{CL,metabolism}$) pathways, 3) wide range in f_m values in clinical victim DDI studies due to variability of PK of a victim NME in humans. Inaccurate or variable f_m may also result from PK studies conducted in genotyped population, if a polymorphic enzyme has differential residual activity in poor metabolizers as opposed to the assumption of null phenotype. Case by case consideration of issues as high first pass intestinal extraction (≤ 50% F_G) and inhibition of parallel metabolic pathways of the victim NME is recommended since magnitude of DDI may be very sensitive to inaccuracies in estimating the $F_{G,inhibited}/F_G$ ratio and parallel multiple metabolic pathways, respectively. It is crucial to understand the underlying reason, effect, and extent of variability on the magnitude of a victim drug's DDI. Modeling and simulation can provide significant impact in terms of improving our understanding of inter- and intra-individual PK variability, supporting optimal victim DDI study design, and leading hypothesis-testing exercises. When properly applied modeling and simulation, coupled with relevant sensitivity analyses and model validation helps to build confidence to support the overall clinical strategy, including justification of delay or waiver of additional DDI studies.

A thorough understanding of the contribution of various metabolic pathways to overall drug clearance in humans allows investigators to make informed decisions for metabolism-based victim DDI risk assessment with the goal to ensure the safety of healthy volunteers and/or patients throughout all stages of clinical development. Whether leveraging metabolism-based victim DDI predictions for internal decision-making or impacting clinical DDI study planning and regulatory responses, factors such as : (1) whether a NME has a narrow TI that may compromise safety in either healthy volunteers or patient populations; (2) common co-medications used by the population and whether the major clearance pathway of the NME can be substantially affected by such co-medications; (3) whether a NME will be administered to special populations such as those with hepatic impairment, renal impairment or pediatrics; and (4) experience with an earlier drug candidate of the same chemical series, should be adequately considered.

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Figure Legends

Figure 1: A - Percent of small molecule drugs approved between 2010-2014 where metabolism contributed to at least 25% of total clearance. B - Percent of metabolically cleared small molecule drugs approved between 2010-2014, which had involvement of CYPs in their metabolism as either the primary enzyme or one of the primary enzymes (at least 25% contribution via CYP towards overall metabolism).

Figure 2: Overall enzyme mapping strategy to identify class of DME involved in metabolism of NME.

Figure 3: Scheme A: Distinguishing CYP and FMO mediated oxidation towards overall NME metabolism.

Figure 4: Scheme B: Distinguishing AO, XO and MAO mediated oxidation towards overall NME metabolism.

Figure 5: Overall strategy to confirm role of FMO 1, 3, and 5 in NME metabolism.

Figure 6: Confirming role of MAO-A or MAO-B towards NME metabolism.

Figure 7: Confirming role of NAT1- and NAT2 -mediated metabolism of an NME.

Figure 8: Estimating fractional metabolic clearance of an NME using information from preclinical studies and in vitro information using human liver matrices- IVIVC approach.

Table 1. Cytochrome P450 (CYP)-selective inhibitors and recommended concentration range to use *in vitro* phenotyping studies

CYP Enzyme	Probe Substrate (median reported Km value, µM)[a]	Selective inhibitor	Inhibitor median reported K_i or IC_{50} value (µM)[a]	Recommended experimental inhibitor concentration range (µM)
CYP1A2	Phenacetin (28)	Furafylline[b]	1.9	0.01-10
CYP2A6	Coumarin (2.5)	Methoxsalen[c] Tranylcypromine	0.48 0.30	0.005-5 0.005-5
CYP2B6	Bupropion (68)	Ticlopidine[b,d] PPP[e] thio-TEPA[f]	0.17 5.5 5.7	0.005-5 0.025-25 0.025-25
CYP2C8	Amodiaquine (3)	Montelukast[g]	0.019	0.002-2
CYP2C9	Tolbutamide (125) Diclofenac (5)	Sulfaphenazole	0.50	0.005-5
CYP2C19	*S*-mephenytoin (40)	Ticlopidine[b,d] (+)-N-3-benzyl-nirvanol	1.2 0.24	0.01-10 0.005-5
CYP2D6	Bufuralol (9)	Quinidine	0.058	0.002-2
CYP2E1	Chlorzoxazone (74)	Diethythiocarbamate	5.3	0.025-25
CYP3A4/5	Midazolam (3.5) Testosterone (54) Nifedipine (12)	Ketoconazole Azamulin[b]	0.10 0.15	0.001-1 0.005-5

[a] The University of Washington Metabolism & Transport Drug Interaction Database (http://www.druginteractioninfo.org) was queried in the category of "*In vitro* Parameters" for studies providing K_m values for the indicated substrates or K_i or IC_{50} values for the indicated precipitants. If more than one CYP was inhibited by the selected precipitant for a particular substrate metabolism, the corresponding K_i or IC_{50} values were excluded from the analysis. If individual K_i or IC_{50} values were listed for one study, the average was taken. If a range of values were given, the lowest value was taken. All queries were of data reported in the Database as of Jan 2013 or Nov 2015.

[b] Time-dependent inhibitor, pre-incubation recommended (e.g. 15 min at 37°C prior to substrate addition)

[c] Methoxsalen also inhibits CYP1A2 (median IC50 or Ki value of 0.70 µM)

[d] Ticlopidine inhibits both CYP2B6 and CYP2C19

[e] 2-phenyl-2-(1-piperdinyl)propane

[f] N,N,N-triethylene thiophosphoramide

[g] IC_{50} dependent upon microsomal protein concentration due to substantial microsomal protein binding of montelukast [Walsky, R. L., Obach, R. S., Gaman, E. A., Gleeson, J. P., and Proctor, W. R. (2005). Selective inhibition of human cytochrome P4502C8 by montelukast. Drug Metabolism and Disposition, 33, 413-418.]

(+)-N-3-benzyl-nirvanol

Table 2. Inhibitors and recommended concentration range for non-CYP enzymes to use *in vitro* phenotyping studies

Enzyme	**Selective Substrates (K_m, µM)**	**Selective Inhibitor**	**Recommended Inhibitor Concentration Range, µM (reported IC_{50} value , µM)**	**References**
AO	DACA[a] (8) Phthalazine (5)	Hydralazine Raloxifene[c] Menadione[c]	0.05-50 (IC_{50} = 0.5-5[b]) 0.0001-1 (IC_{50}= 0.0029) 0.01-100 (IC_{50}= 0.2)	Johnsonet al., 1985; Chen et al., 2002; Obach et al., 2004; Strelevitz et al., 2012; Barr et al., 2011 & 2013
XO	Pterin (34) 6-mercaptopurine(6)	Allopurinol	0.01-100 (IC_{50}= 2)	Obach et al., 2004; Panoutspoulos et al., 2004; Tapner et al., 2004; Pacher et al., 2006
MAO-A	Serotonin(100)	Clorgylline[d] ***	0.0002-2 (IC_{50}= 0.002)	Kalgutkar et al., 1995; Herraiz et al., 2009 &2012; Geha et al., 2001; Leonardi et al., 1994;
MAO-B	β – phenylethylamine(2)	(-) Deprenyl[d] ***	0.0002-2 (IC_{50}= 0.001)	Herraiz et al., 2009 &2012; Geha et al., 2001
FMO (1, 3, and 5)	Benzydamine (FMO 1= 24; FMO3 = 40)	Methimazole	10-500 (IC_{50}= 120)	Stromer et al.,2000;

[a] DACA = N-[(2-dimethylamino)ethyl]acridine-4-carboxamide

[b] Demonstrates substrate-dependent IC50

[c] Suitable in liver cytosol, but are less suitable for inhibition studies in liver S9 fractions or hepatocytes due to their potential to inhibit CYP enzymes &/or instability

[d] Pargylline can also be used as non-specific inhibitor of MAO (Recommended concentration for phenotyping studies 0.5-2 µM; IC50 for MAO-A is 0.01152 µM and for MAO-B is 0.00820 µM) (Fisar et al., 2010; Murphy et al., 1998)

*** Irreversible inhibitors

Recommended *In vitro* Substrates and Inhibitors of UGTs

UGT	Selective Substrates Km (µM)	Selective Inhibitor	Recommended Inhibitor Concentration Range, µM (reported IC_{50} value, µM)	References
1A1	UGT1A1: 17β-Estradiol (3-glucuronide formation) (11, 170[b])	Atazanavir	0.01-20 (UGT1A1 IC_{50} = 0.31[a])	Zhang et al., 2005; Walsky et al., 2012; Itäaho et al., 2008; Lepine et al., 2004; Seo et al., 2014 ;
1A3	UGT1A3: 17β-Estradiol (3-glucuronide formation) (250) (17-glucuronide formation) (67)	Lithocholic acid	0.01-20 (UGT1A3 IC_{50} = 7.9)	Zhang et al., 2005; Walsky et al., 2012; Itäaho et al., 2008; Lepine et al., 2004; Seo et al., 2014 ;
1A4	Trifluoperazine (42, 67[b])	Hecogenin	0.02-20 (IC_{50} = 1.5[a])	Uchaipichat et al., 2006; Walsky et al., 2012;
1A6	5-Hydroxytryptophol (420, 330[b])	Troglitazone	0.1-100 (IC_{50} = 28)	Walsky et al., 2012; Krishnaswamy et al., 2004; Ito et al., 2001;
1A9	Propofol (98, 46[b])	Digoxin Niflumic acid Transilast	0.1-100 (IC_{50} = 1.7) [b] 0.01-100 (IC_{50} = 0.3[a]) 0.1-100 (IC_{50} = 0.7)[b]	Lapham et al., 2012;Vietri et al., 2002; Bernard et al., 2004; Court et al., 2005; Mano et al., 2006;
2B7	Zidovudine (586, 150[b], 77[c])	β-Phenyllongifolol-2	0.001-1 (IC_{50} = 0.006)	Lapham et al., 2012; Bichlmaier et al., 2007; Manevski et al., 2011; Walsky et al., 2012;

[a] The University of Washington Metabolism & Transport Drug Interaction Database (http://www.druginteractioninfo.org) was queried in the category of "*In vitro* Parameters" for studies providing K_m values for the indicated substrates or K_i or IC_{50} values for the indicated precipitants. If individual K_i or IC_{50} values were listed for one study, the average was taken. If a range of values were given, the lowest value was taken. Median value was derived across all reported values. All queries were of data reported in the Database as of Dec 2015.

[b] In the presence of 2% BSA

[c] In the presence of 1% BSA

Recommended *In vitro* Substrates and Inhibitors of other non-CYP Phase II enzymes

Enzyme	Selective Substrates Km (µM)	Selective Inhibitor	Recommended Inhibitor Concentration Range, µM (reported IC_{50} value, µM)	References
GST	CDNB[a] 450 (A1-1) 580 (A2-2) 650 (M1a-1a) 910 (P1-1)	Ethacrynic acid[b]	0.1-1 mM [IC_{50} =6 (α), 0.3 (µ), 3.3 (π)]	Phoemen et al., 1990; Polidoro et al., 1981; Warholm et al., 1983;
NAT1	p-Amino benzoic Acid (7.5)	Caffeic Acid	0.1-1 mM (K_i = 479)	Kukongviriyapan et al., 2006
NAT2	Sulfamethazine (40)	Curcumin	1-100 (K_i = 15)	
CES	Temocapril (CES1) (NA) Trandolapril (CES1) (1734) Imidapril (CES1) (4.3)	Digitonin BNPP[c]	0.1-100 (IC_{50} = 9.2) 0.01-10 (IC_{50} = 0.1)	Nishimuta et al., 2014; Fukami et al., 2010;
	Mycophenolate mofetil (CES1/2) (225- CES1; 22.3- CES2) Irinotecan (CES2) (14.4)	Telmisartan Loperamide BNPP[c]	0.01-10 (IC_{50} = 0.5) 0.01-10 (IC_{50} = 0.1) 0.01-10 (IC_{50} = 0.1)	Fukami et al., 2010;

[a] CDNB: 1-chloro-2,4-dinitrobenzene. Non-selective

[b] Partially selective

[c] Bis(4-nitrophenyl)phosphate (non-specific)

Reported *In vitro* Substrates and Inhibitors of SULT isoforms				
SULT	**Selective Substrates Km (µM)**	**Non-Selective SULT Inhibitor**	**Reported IC50 (µM)**	**References**
1A1	4-nitrophenol (4)	2,6-Dichloro-4-Nitrophenol	1.44	Wang et al., 2006; Riches et al., 2009
1A3	4-nitrophenol (3000)		86.9	
2A1	Dehydroepiandrosterone (5)		40	
1B1	3,3', 5-Triiodothyronine (60)		400	
1E1	3,3', 5-Triiodothyronine (75)		30	
2,6-Dichloro-4-Nitrophenol is a potent SULT1A-selective inhibitor, with IC50 for inhibition of EE 3-O-sulfation of ~6.3 nM, which is more than 2 order magnitude over other SULTs (DMD 2004, 32: 1299-1303)				

FMO= Flavin-containing Monooxygenase; AO/XO=Aldehyde Oxidase/Xanthine Oxidase; MAO= Monoamine Oxidase; UGT = Uridine Diphosphoglucuronosyltransferase; SULT= Sulfotransferase; NAT= N-acetyl Transferase; GST= Glutathione S-transferase; CES= Carboxylesterase

Table 3: Currently available tools for in vitro prediction of f_m for CYP and non-CYP enzymes

Enzyme	Tools available for RAF or ISEF			Chemical Inhibitors	f_m Measurement(from in vitro studies)	Clinical Victim DDI Risk*
	Recombinant Enzyme	Probe metabolic pathway	Tissue abundance			(Reported example of victim drug)
CYP	Yes (various)	Yes[a]	Yes[a]	Yes[a]	Quantitative	High (terfenadine, astemizole, cisapride)
FMO	Yes (1, 3, 5)	Yes[b]	Yes[c]	Yes[b]	Qualitative	Low
AO/XO	Limited	Yes[d]	Emerging[e]	Yes[d]	Qualitative	Moderate (allopurinol-XO)
MAO	Yes (A and B)	Yes[d]	No	Yes[d]	Qualitative	Low
UGT	Yes (various)	Yes[f]	Emerging[g]	Yes[h]	Quantitative[f]	Moderate (morphine, zidovudine, lorazepam, mycophenolate mofetil)
SULT	Yes (various)	Limited[i]	Emerging[j]	Yes[b]	Qualitative	Low
NAT	Yes (1 and 2)	Yes[k]	No	Yes[k]	Qualitative	Moderate (isoniazid)
GST	Yes (various)	No	Emerging[l]	Yes[b]	Qualitative	Low
CES	Yes (1 and 2)	Yes[n]	Emerging[n]	Yes[n]	Qualitative	Low

RAF= Relative Activity Factor; ISEF: Intersystem Extrapolation Factor; DDI= Drug Drug Interaction; CYP= Cytochrome P450; FMO= Flavin-containing Monooxygenase; AO/XO=Aldehyde Oxidase/Xanthine Oxidase; MAO= Monoamine Oxidase; UGT = Uridine Diphosphoglucuronosyltransferase; SULT= Sulfotransferase; NAT= N-acetyl Transferase; GST= Glutathione S-transferase; CES= Carboxylesterase

*High: Several Reported; Moderate: Rare/Occasional Reported; Low: None Reported
a: For major isoforms [Table 1]
b: Non-isoform selective [Table 1]
c: mRNA –based abundance reported [FMO section]
d: Table 1
e: In liver [AO/XO section]
f: For some isoforms e.g 1A1, 1A4, 1A6, 1A9, 2B7 [UGT Section & Table 1]
g: For some isoforms e.g 1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17 in liver, intestine, and kidney [UGT section]
h: For some isoforms e.g.1A1/3, 1A4, 1A6, 1A9, 2B7 [UGT section & Table 1]
i: Limited [Table 1]
j: mRNA-based for 1A1, 1A3/4, 1B1, 1E1, 2A1 in liver, intestine, kidney, lung [SULT section]
k: Limited Reports [NAT section & Table 1]
l: Limited report -GSTA1, A2, M1, M2, M3 and P1[GST section]

m: Table 1
n: Limited Reports [CES section & Table 1]

Table 4: Case Examples Illustrating The Predictive Utility of Modeling and Simulation Approaches

	Development Stage & Question	Data Available	Modeling Approach	Outcome and impact	comments
Compound-1	Clinical development. *Can PBPK modeling inform the optimal ketoconazole[1] drug interaction study design for a drug with an extended t1/2?*	• In vitro metabolic phenotyping • Human mass-balance • Phase I human PK • DDI study with ketoconazole	•PBPK model •CL based on in vitro CL_{int} •F_a, k_a and V_{dss} based on in vivo data •Retrospective simulation of Comp-1 –ketoconazole DDI was used to build confidence in the model •Simulations were used to compare alternative dosing regimens	•Refined mechanistic understanding of disposition •Supported improved clinical study design for subsequent studies	Shardlow et al., 2013
Compound - 2	Clinical development. *What is the DDI risk for Compound which is predominantly metabolized by CYP3A4 in vitro?*	• Metabolism in recombinant system • In vitro metabolic phenotyping • Metabolism in human hepatocytes • Human mass balance • DDI study with ketoconazole	•PBPK •CL based on IVIVE and in vitro phenotyping •Clinical PK data in healthy subjects and in ketoconazole DDI trial used to verify model relevance •Sensitivity analysis conducted on $f_{u,gut}$	•Provided high confidence in ability of model to accurately predict DDI with potent CYP3A4 inducers •Supported additional simulations of DDI with moderate CYP3A4 inhibitors	In-house example. J & J (Templeton, 2014)
Compound - 3	Discovery to early development *Can DDI of CYP3A victim drug with strong and moderate CYP3A inhibitor be predicted using in vitro data and static model?*	• human mass balance • Reaction phenotyping • P450 activity remaining (fA_{CYP}) in the presence of ketoconazole or fluconazole (human hepatocyte suspended in human plasma)	•Static model • Measured in vitro fA_{CYP} and fm_{CYP} linked to represent the factor of $(1/(1+ I/K_i)$ •$f_{A,CYP}$ corrected by comparing extracellular inhibitor concentration (determined in vitro) with in vivo C_{max} •Calculated steady-state DDIs compared with clinical observations	•Allowed DDI prediction when PBPK model and extensive in vitro and in vivo data were not available	Lu et al, 2007, 2010
Compound - 4	Clinical development *What is the risk of DDI for a compound primarily metabolized by CYP3A4 in vitro? Can PBPK model explain the observed clinical data and make predictions of the outcome of novel scenarios (DDI and pediatrics)?*	• Metabolic phenotyping (HLM) • Rat QWBA • human mass balance study data available • Phase I human PK • DDI studies (with three inhibitors)	•PBPK model •CL based on in vivo CL_{IV} and retrograde extrapolation of in vivo CL_{int} •Predicted V_{ss} consistent with IV dose data and rat QWBA •Model was verified using observed clinical PK data from single, multiple-dose and three clinical DDI studies	•Improved mechanistic understanding of the observed DDIs •Suggested previously unexpected role of efflux transport in fraction absorbed – which was subsequently verified by an in vitro study •Supported design of clinical pediatric study	In-house example. J & J (Templeton)

Compound - 5	Clinical Development *What is the DDI effect of ketoconazole and rifampin on comp-4 after a single dose and at steady state, respectively in clinical trials?*	• in vitro metabolism (HLM and hepatocytes) • in vitro phenotyping (HLM and rhCYP) • Rat ADME data • Clinical DDI with rifampin or ketoconazole	• PBPK model • CL based on clinical data • V_{dss} predicted from physiochemical data • Sensitivity analysis for $f_{u,gut}$ conducted • Model verified with clinical DDI data	• Guided selection of single-dose over proposed multiple-dose study to maximize DDI potential	Novartis in-house example
Compound-6	Prior to FIH *Should PMs of CYP2C9 be excluded from FIH trials?*	• In vitro metabolism (HLM and hepatocytes – across species) • In vitro phenotyping (HLM and rhCYP) • rhCYP2C9*1, *2 *3 kinetics • Rat ADME data	• PBPK model • CL based on rhCYP2C9*1, *2, *3 kinetic data	• Exclusion of CYP2C9*3 genotype in FIH trials due to safety risk for the compound in this population	Novartis in-house example

[1] Historically, ketoconazole was preferred as a clinically administered potent inhibitor of CYP3A4. However, a recent FDA memo FDA Drug Safety Communication: FDA limits usage of Nizoral(ketoconazole) oral tablets due to potentially fatal liver injury andrisk of drug interactions and adrenal gland problems. http://www.fda.gov/Drugs/DrugSafety/ucm362415.htm. () and industry white paper (PMID: 26044116) have proposed that alternative inhibitors be administered in clinical DDI studies

PBPK - Physiologically-Based Pharmacokinetic; PK – Pharmacokinetics; CYP – Cytochrome P450; F_A – Fraction Absorbed; k_a – Absorption Rate Constant; CL – Clearance; V_{dss} – Steady-state Volume of Distribution; CL_{int} – Intrinsic Clearance; IVIVE – In vitro In vivo Extrapolation; f_m – fraction metabolized; f_A – fraction activity remaining ; HLM – Human Liver Microsomes; $f_{u,gut}$ – Fraction Unbound Enterocytes; rh – Recombinant Human; QWBA – Quantitative Whole Body Autoradiography; FIH – First in Human; ADME - Absorption Distribution Metabolism Excretion; PM – Poor Metabolizer

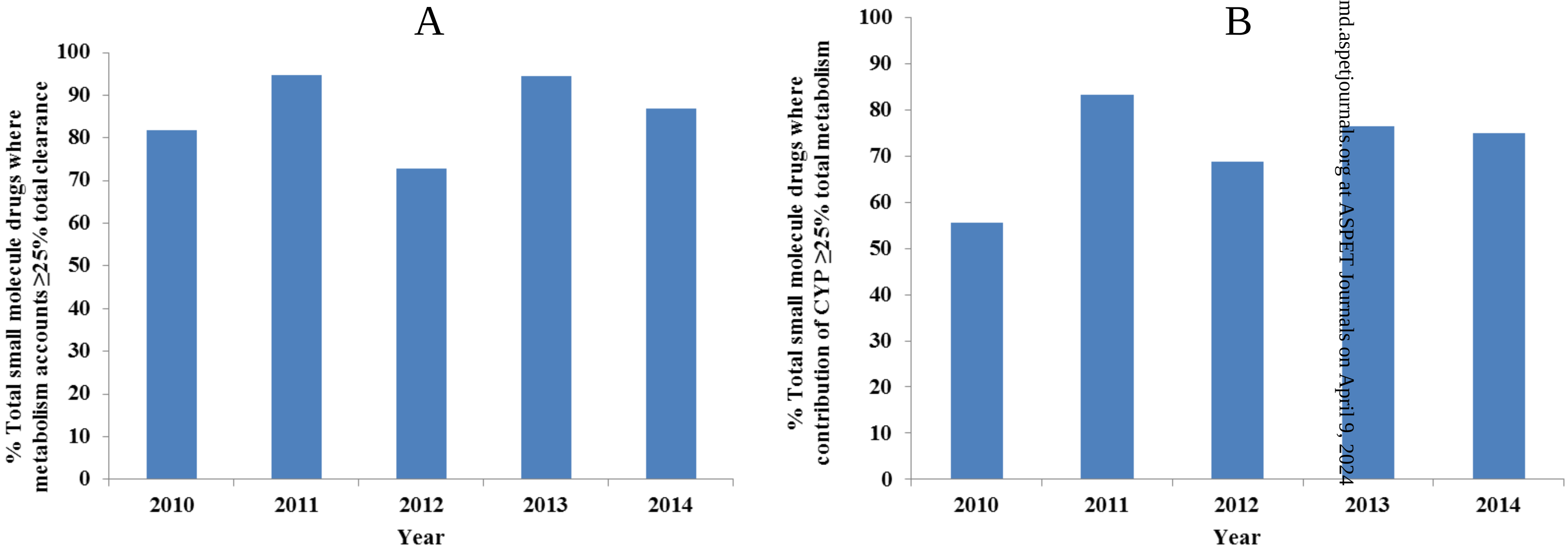
Table 5: Studies commonly done through the course of drug development to Estimate f_{CL} and f_m

Stage	Study Type	Information Gathered	Pros & Cons
Pre-FIH/ Preclinical Development	• Preclinical Species PK/ADME (in vivo) • Preclinical Species CL_{int} + Metabolite Profiling (in vitro)	• NME cleared via Metabolism or Excretion in animals • Major metabolic pathways in animals • Metabolic pathway in vitro similar to that in vivo in animals	Pros: •Confidence from IVIVC in animals Cons: • Human routes of metabolism maybe quite different from animals
	• Human CL_{int}+Metabolite Profiling (in vitro) • CYP and other DME Identification (in vitro human matrices)	• Major metabolic pathways in humans in vitro • Relative contribution of Oxidation vs Conjugation • Relative contribution of CYP enzymes • Metabolism involve single or multiple enzymes	Pros: • Preliminary estimates of CYP-mediated DDI risk Cons: • In vitro pathways may not be major pathways in vivo
FIH (SAD /MAD)	• Detailed Reaction Phenotyping (in vitro) • Metabolite Profiling in plasma and urine in human usually available; in certain cases in bile (Entero-Test®)	• f_m of CYP or other major enzyme involved (in vitro) • First look into major metabolic pathways in humans(commonly in plasma and urine; sometimes in bile) • Crude estimate of $f_{CL,renal}$ (if NME substantially cleared renally); quantitative metabolite information in plasma and urine in certain cases (e.g. with quantitative NMR) • PK linearity (understand saturable processes)	Pros: • Usually metabolite monitoring in in vitro phenotyping studies • First look in humans– is metabolic pathways similar in vivo vs in vitro? Any human unique pathway not captured in vitro Cons: • All qualitative estimates; quantitative estimate in plasma and urine possible if using quantitative NMR which allows minimum estimate of f_m • Missing metabolites info in feces
Definitive Human Studies	Human Radiolabel ADME Study	• Route of CL in humans (f_{CL}) • Quantitative metabolite profiling	Pros: • $f_{CL,metabolism}$ + $f_{CL,renal}$ + $f_{CL,biliary}$ quantitatively determined • Metabolites determined in feces • Learn if f_{CL} pathways predicted earlier is consistent with observed human f_{CL} pathways Cons: • $f_{CL,biliary}$ challenging after PO dose when substantial unchanged NME in feces • In case of poor mass balance f_{CL} pathways still not well-defined

	DDI with potent and selective enzyme inhibitor	• Contribution of a DME towards overall NME metabolic clearance (f_m) (assumption that inhibitor completely inhibits only enzyme of interest and for orally administered drugs, maximal intestinal inhibition is achieved)	Pros: • f_m quantitatively determined Cons: • Wide range of f_m values when variability of PK which can have significant impact on DDI magnitude
	PK in Genotyped population	• Contribution of a polymorphic drug metabolizing enzyme towards overall NME metabolic clearance (f_m) (assumption that in null phenotype (PM), polymorphic enzyme pathway is completely absent)	Pros: • f_m quantitatively determined Cons: • Inaccurate f_m if residual activity of polymorphic enzyme in PM

FIH – First in Human; PK – Pharmacokinetics; NME – New Molecular Entity; ADME - Absorption Distribution Metabolism Excretion; CL_{int} – Intrinsic Clearance; CL – Clearance; IVIVC – In vitro In vivo Correlation; CYP – Cytochrome P450; DDI – Drug-drug Interactions; f_{CL} – Fraction of Clearance; f_m – Fraction Metabolized; NMR – Nuclear Magnetic Resonance; PM – Poor Metabolizer

Figure 1



Year	Total number of NDAs[a]	Number of typical SMD[b]	Number of drugs cleared via metabolism[c]	Number of drugs cleared by CYP[d]
2010	15	11	9	5
2011	24	19	18	15
2012	33	22	16	11
2013	25	18	17	13
2014	29	23	20	15

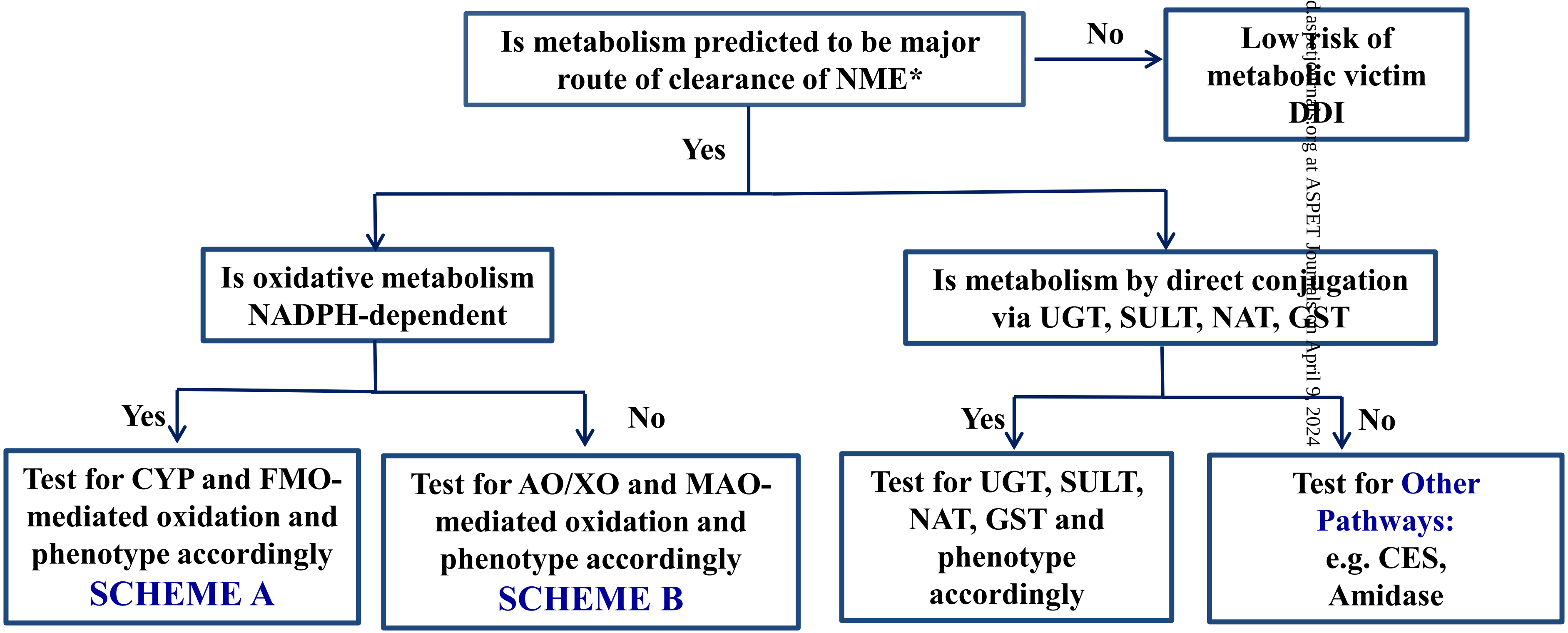
[a] NDA = New Drug Application; Assessments made on drugs cleared via intravenous, oral, or inhaled routes and excluding imaging agents, enzyme replacement therapies, sclerosing agents, and topical applications.

[b] SMD = Small Molecule Drugs.

[c] Contribution of metabolism towards total clearance was ≥ 25% as reported or inferred from exposure changes in reported clinical DDI studies.

[d] Contribution of CYP towards total metabolism was ≥ 25% as reported or inferred from exposure changes in reported clinical DDI studies.

Figure 2

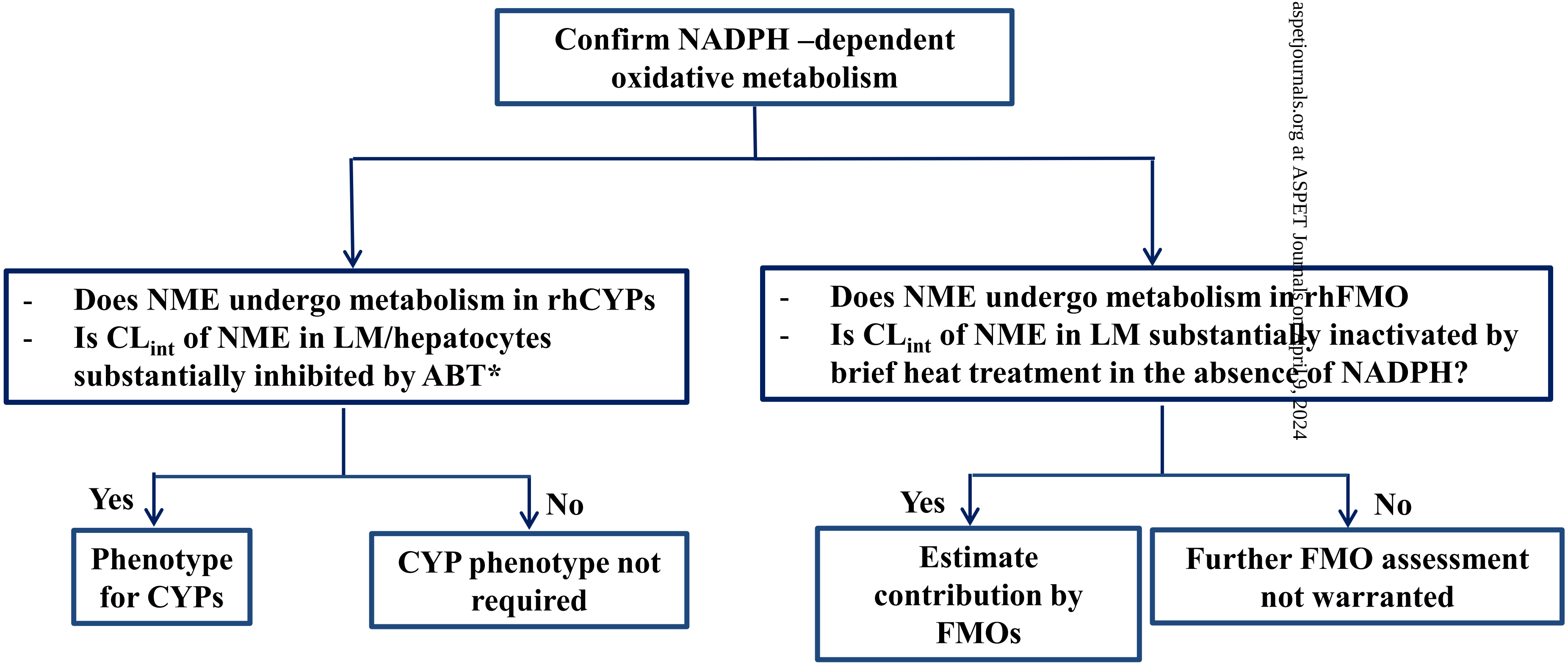

Is metabolism predicted to be major route of clearance of NME*
No
Low risk of metabolic victim DDI
Yes
Is oxidative metabolism NADPH-dependent
Is metabolism by direct conjugation via UGT, SULT, NAT, GST
Yes
No
Yes
No
Test for CYP and FMO-mediated oxidation and phenotype accordingly
SCHEME A
Test for AO/XO and MAO-mediated oxidation and phenotype accordingly
SCHEME B
Test for UGT, SULT, NAT, GST and phenotype accordingly
Test for Other Pathways:
e.g. CES, Amidase

*Predicted from preclinical studies or confirmed from ^{14}C-ADME human studies

NME= New Molecular Entity; DDI= Drug Drug Interaction; NADPH= Nicotinamide Adenine Dinucleotide Phosphate reduced; CYP= Cytochrome P450; FMO= Flavin-containing Monooxygenase; AO/XO=Aldehyde Oxidase/Xanthine Oxidase; MAO= Monoamine Oxidase; UGT = Uridine Diphosphoglucuronosyltransferase; SULT= Sulfotransferase; NAT= N-acetyl Transferase; GST= Glutathione S-transferase; CES= Carboxylesterase

Figure 3

SCHEME A

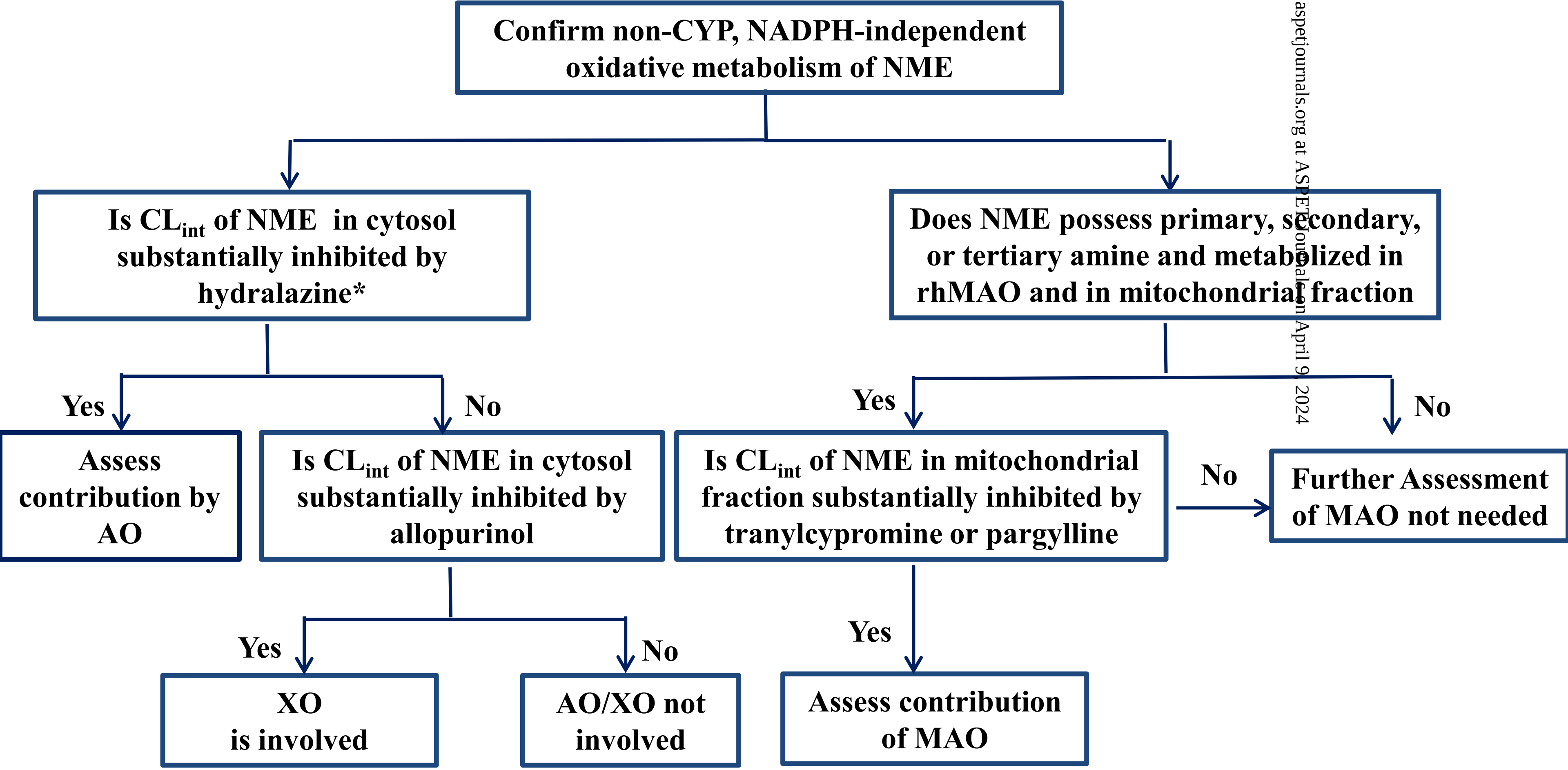

Confirm NADPH –dependent oxidative metabolism
- Does NME undergo metabolism in rhCYPs
- Is CL_{int} of NME in LM/hepatocytes substantially inhibited by ABT*
- Does NME undergo metabolism in rhFMO
- Is CL_{int} of NME in LM substantially inactivated by brief heat treatment in the absence of NADPH?
Yes
No
Phenotype for CYPs
CYP phenotype not required
Yes
No
Estimate contribution by FMOs
Further FMO assessment not warranted

*Caution using ABT: Demonstrated to not inhibit certain CYP such as 2C9 (Linder et al., 2009)

NADPH= Nicotinamide Adenine Dinucleotide Phosphate reduced; NME= New Molecular Entity; rh= recombinant; CYP= Cytochrome P450; FMO= Flavin-containing Monooxygenase; LM = Liver Microsomes; ABT = 1-Aminobenzotriazole; CL_{int} = Intrinsic learance

Figure 4

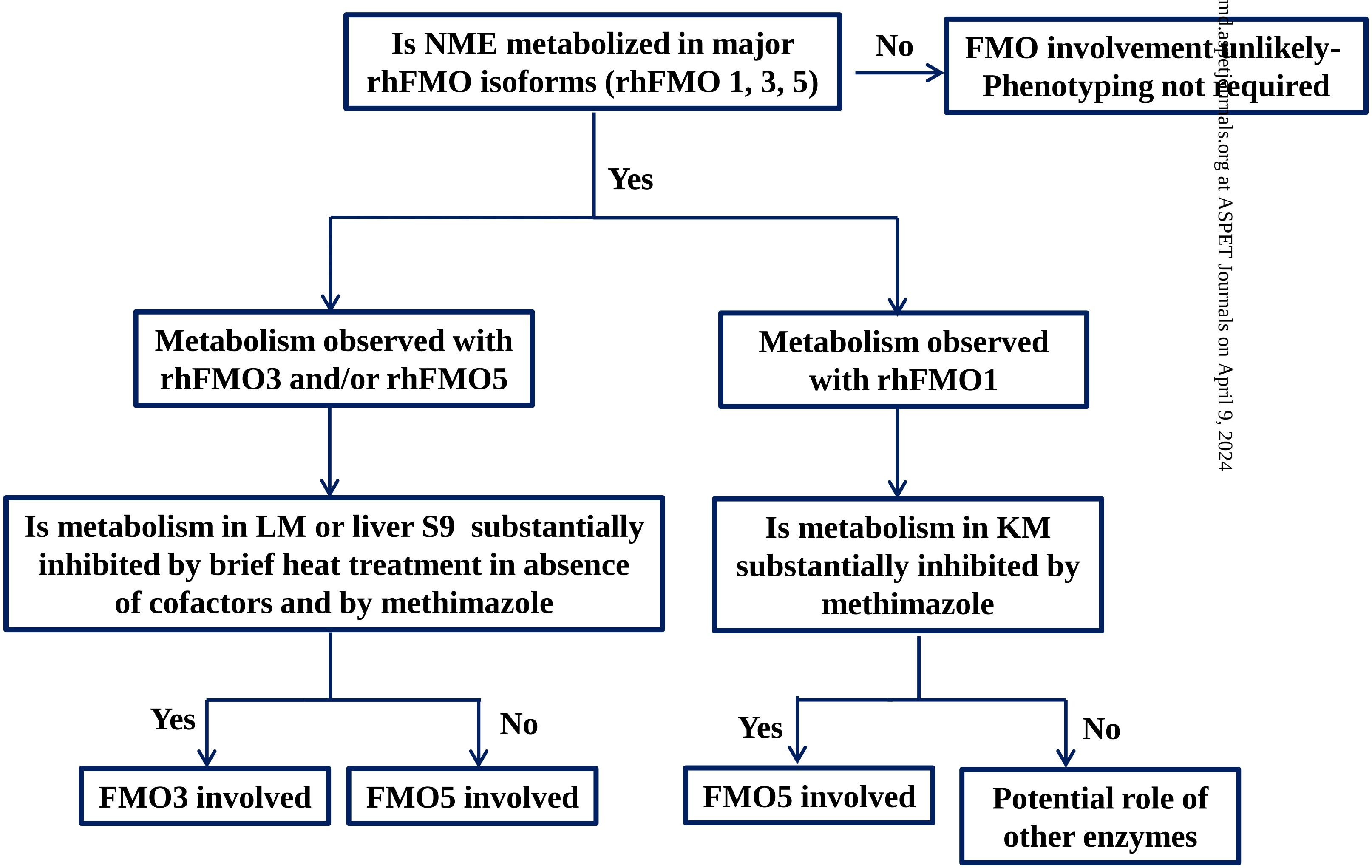
SCHEME B


Confirm non-CYP, NADPH-independent oxidative metabolism of NME
Is CL_{int} of NME in cytosol substantially inhibited by hydralazine*
Does NME possess primary, secondary, or tertiary amine and metabolized in rhMAO and in mitochondrial fraction
Yes
No
Yes
No
Assess contribution by AO
Is CL_{int} of NME in cytosol substantially inhibited by allopurinol
Is CL_{int} of NME in mitochondrial fraction substantially inhibited by tranylcypromine or pargylline
No
Further Assessment of MAO not needed
Yes
No
Yes
XO is involved
AO/XO not involved
Assess contribution of MAO

*Raloxifene is also suitable AO inhibitor for use in cytosol

NME = New Moelcular Entity; CL_{int} = Intrinsic clearance; NME= New Molecular Entity; NADPH= Nicotinamide Adenine Dinucleotide Phosphate reduced; CYP= Cytochrome P450; AO=Aldehyde Oxidase; XO= Xanthine Oxidase; MAO= Monoamine Oxidase; rh = recombinant; CL_{int} = Intrinsic Clearance

Figure 5


Is NME metabolized in major rhFMO isoforms (rhFMO 1, 3, 5)

No

FMO involvement unlikely- Phenotyping not required

Yes

Metabolism observed with rhFMO3 and/or rhFMO5

Metabolism observed with rhFMO1

Is metabolism in LM or liver S9 substantially inhibited by brief heat treatment in absence of cofactors and by methimazole

Is metabolism in KM substantially inhibited by methimazole

Yes

No

FMO3 involved

FMO5 involved

Yes

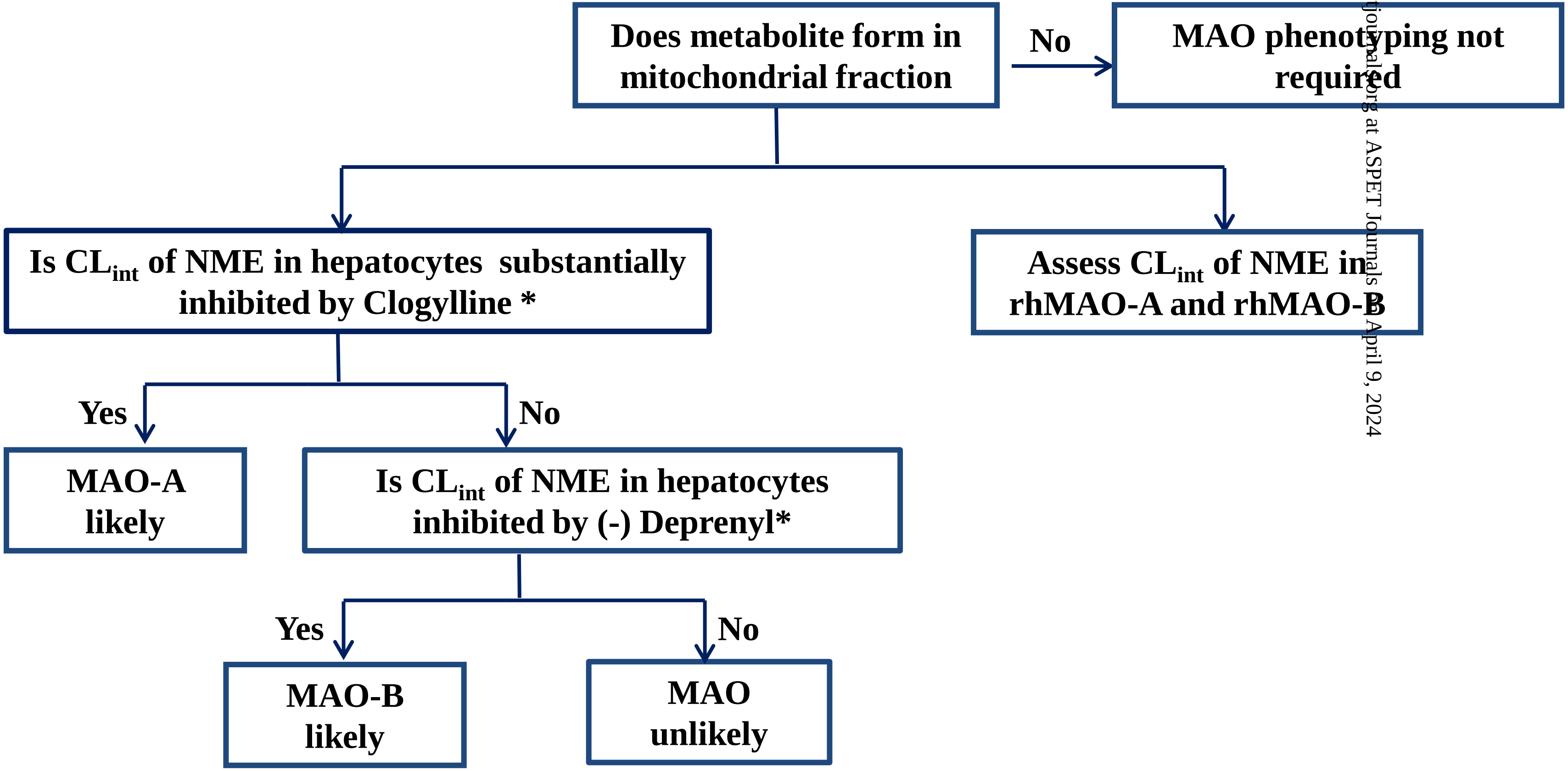
No

FMO5 involved

Potential role of other enzymes

NME = New Molecular Entity; FMO= Flavin-containing Monooxygenase; rh = recombinant; LM = Liver Microsomes; KM = Kidney Microsomes

Figure 6



Does metabolite form in mitochondrial fraction

No

MAO phenotyping not required

Is CL_{int} of NME in hepatocytes substantially inhibited by Clogylline *

Assess CL_{int} of NME in rhMAO-A and rhMAO-B

Yes

MAO-A likely

No

Is CL_{int} of NME in hepatocytes inhibited by (-) Deprenyl*

Yes

MAO-B likely

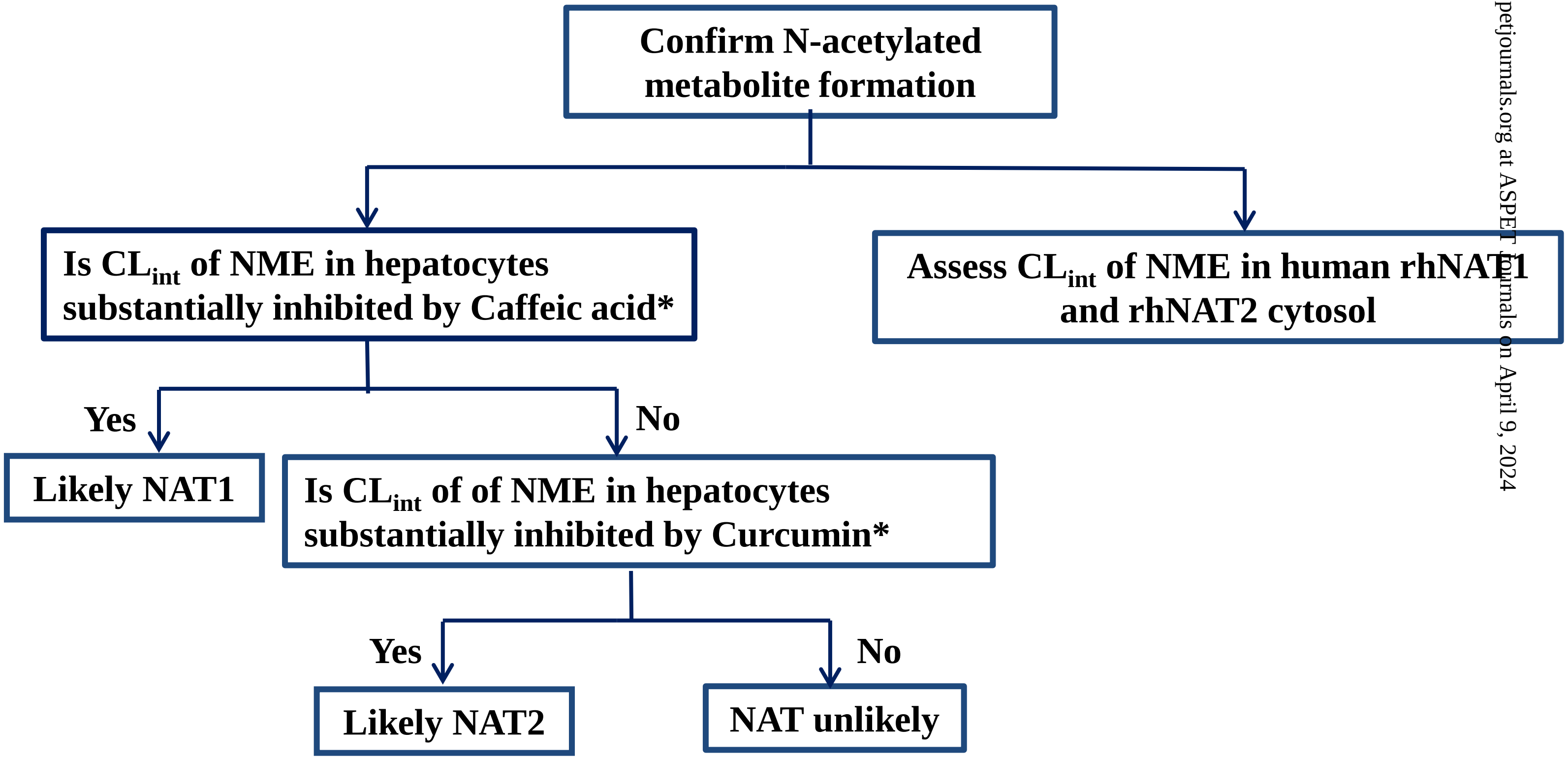
No

MAO unlikely

* % Contribution= $(CL_{int} – CL_{int(+inhibitor)]})/ CL_{int}$

NME= New Molecular Entity; MAO= Monoamine Oxidase; CL_{int} = Intrinsic Clearance; rh = recombinant

Figure 7



Confirm N-acetylated metabolite formation

Is CL_{int} of NME in hepatocytes substantially inhibited by Caffeic acid*

Assess CL_{int} of NME in human rhNAT1 and rhNAT2 cytosol

Yes

Likely NAT1

No

Is CL_{int} of of NME in hepatocytes substantially inhibited by Curcumin*

Yes

Likely NAT2

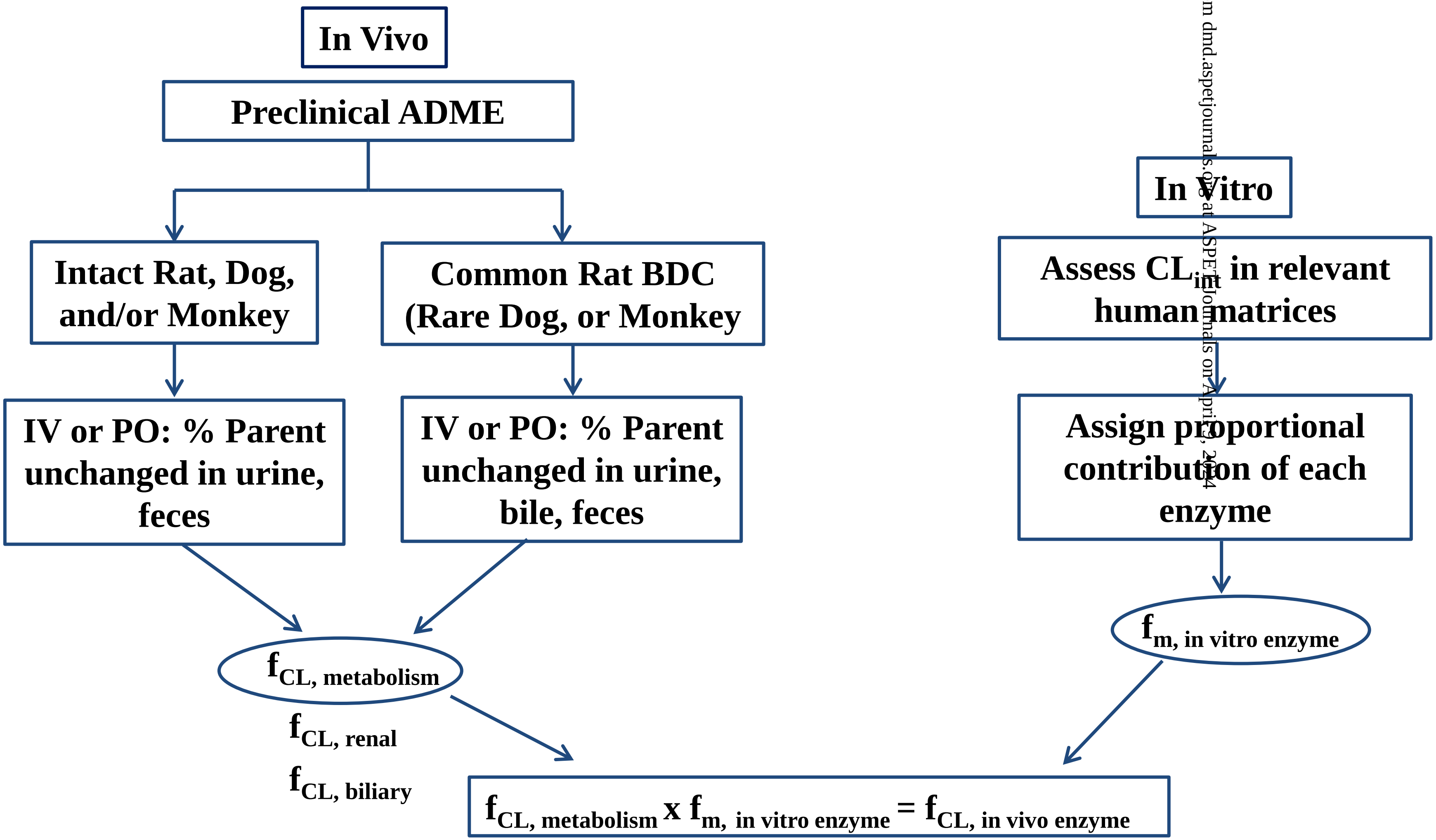
No

NAT unlikely

* % Contribution= $(CL_{int} – CL_{int\ (+inhibitor)]})$/ CL_{int}

CL_{int} = Intrinsic Clearance; NME= New Molecular Entity; NAT= N-acetyl Transferase; rh = recombinant

Figure 8



In Vivo

Preclinical ADME

Intact Rat, Dog, and/or Monkey

Common Rat BDC (Rare Dog, or Monkey

IV or PO: % Parent unchanged in urine, feces

IV or PO: % Parent unchanged in urine, bile, feces

$f_{CL,\ metabolism}$

$f_{CL,\ renal}$

$f_{CL,\ biliary}$

In Vitro

Assess CL_{int} in relevant human matrices

Assign proportional contribution of each enzyme

$f_{m,\ in\ vitro\ enzyme}$

$f_{CL,\ metabolism} \times f_{m,\ in\ vitro\ enzyme} = f_{CL,\ in\ vivo\ enzyme}$

ADME=Absorption Distribution Metabolism Excretion; BDC = Bile Duct Cannulated; CL_{int} = Intrinsic Clearance ; IV= Intravenous; PO= Oral; f_m= Fraction Metabolized; f_{CL}= Fraction Cleared