Chapter

Drug Metabolism in Drug Discovery and Preclinical Development

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Abstract

Drug metabolism or more generally, xenobiotic metabolism, is the biotransformation of exogenous compounds by living organisms, usually through specialized enzymatic systems. The metabolism of experimental therapeutics is an important aspect of pharmacology and translational medicine as the rate and the interindividual variability of drug metabolism can determine the duration and/or efficacy of a drug's pharmacologic action. Since the introduction of metabolites in safety testing guidance by the Food and Drug Administration, major changes have occurred in the experimental methods for the identification and quantification of metabolites, evaluation of metabolites, and the timing of critical nonclinical studies to generate this information.

Keywords: Drug Metabolism, Drug Discovery, Preclinical Development, Lead Selection, Lead Optimization, Drug Candidate Selection, Metabolite Safety Testing, CYP450 Inhibition, CYP450 Induction, Soft Spot Analysis, Metabolite Profiling, Membrane Drug Transporters

1. Introduction

Drug metabolism is a drug-clearing event from systemic circulation influencing efficacy and toxicity in humans and preclinical species. The primary endpoints of enzymatic metabolism studies in both the drug discovery and preclinical stages are to resolve metabolic stability, identify and quantify primary metabolites, identify metabolic routes, and measure the potential for drug–drug interactions (DDI) [1]. The majority of *in vivo* and *in vitro* drug metabolism assessments center around hepatic models as the liver is the main metabolizing organ; however, drug metabolism does occur in other organs and tissues (e.g., lungs, kidneys, and intestine). As a result, drug discovery and preclinical development investigations include evaluation of drug metabolism in both hepatic and extra-hepatic models.

Over the past several decades *in vitro* and *in vivo* methodologies to quantitatively measure the absorption, distribution, metabolism, and excretion (ADME) properties of a chemical entity have matured to the point to where these properties can be reliably modeled and simulated in order to predict the general disposition of the chemical entity (or class of molecules) across multiple species and into humans [2]. As a result, approximations of oral bioavailability (F), total systemic clearance (CL), volume of distribution (Vd), and half-life ($t_{1/2}$) can be predicted and subsequently evaluated. These intrinsic properties of the chemical entity with

consideration of the physiological processes can be used to assess the disposition of single and multiple dose escalation studies, identify potentially saturable hepatic pathways, and assess the formation of metabolites. From this information, potential DDIs can be predicted and investigated.

In order to effectively utilize the drug metabolism and pharmacokinetic (DMPK) information generated from *in vitro* and *in vivo* studies, it is important to have a proper understanding of when in the development timeline the data should be generated. Additionally, a thorough understanding of the clearance mechanism of a chemical entity can help lessen the need for some downstream *in vivo* studies potentially saving the pharmaceutical organization hundreds of thousands of dollars. While the timing of specific investigations may change due to the nature of the chemical entity being tested, the following general DMPK chronology and endpoints should be considered during discovery and development [3, 4]. The bullets relating to drug metabolism are bolded for emphasis and will be expanded in greater detail in the following pages.

1. Lead Selection (Hit-to-Lead; 100s to 1000s of compounds across multiple chemical series)

   - Plasma protein binding, red blood cell partitioning, and intestinal permeability (Caco-2) assessments
   - ***In vitro* metabolic stability screening using hepatocytes, microsomes, and S9 fractions**
   - *In vivo* rodent PK studies
   - Investigate the *in vitro*:*in vivo* correlation (IVIVC) of CL and $t_{1/2}$ and identify the series with problematic kinetics (e.g., high CL or low $t_{1/2}$)
   - Identification of the relevant tissues where the chemical entity and metabolites may be sequestered

2. Optimization (Lead series identified and evaluated; <100 compounds)

   - Pharmacokinetic studies in rodent and nonrodent species
   - **Identification of clinically relevant DDIs (*In vitro* assessment of P450 induction and P450 inhibition)**
   - **Screening Cocktail DDI Study**
   - ***In vitro* soft spot analysis/metabolic identification to identify metabolically liable sites on investigated chemical entities**
   - **Drug transporter and tissue sequestration assessments**
   - Pharmacokinetic-Pharmacodynamic (PK-PD; exposure-effect) modeling

3. Drug Candidate Selection (< 5 compounds evaluated)

   - **Mass balance study in rodents and nonrodents in order to support nonrodent toxicology species selection**

- Allometric scaling to predict human $t_{1/2}$ and exposures in order to identify the starting dosages to be evaluated in the Phase 1 first-in-human (FIH) trials.

- **Evaluation of DDIs**

- **Metabolite profiling of high dose blood samples near the peak whole blood/plasma concentrations from the repeat-dose toxicity studies.**

4. Metabolite Safety Testing (Post FIH and concurrent with Phase 2 trials)

- **Human specific metabolites or metabolites with exposures at higher levels in humans than in any of the animal test species are assessed in preclinical species.**

2. Lead selection

During the Lead Selection stage of drug discovery, candidate chemical series and potential lead compounds are screened for preferential physiochemical properties and metabolic stability. Promising chemical entities are then investigated *in vivo* in rodents (N = 3-5) via cassette dosing [5] in order to generate PK data. With recent advances in microsampling techniques [6] and automated blood sampling systems [7], robust PK data can be generated in a limited number of rodents resulting in reliable IVIVC models. Pharmacokinetic data can then be predicted across multiple species to evaluate and identify the chemical series and chemical entities that are ideal candidates for optimization.

Most chemical entities are substrates of *in vivo* enzymatic metabolic reactions that modify the chemical structure to clear the exogenous compound over time. Metabolizing enzymes are primarily expressed in the liver but are also found in the intestines, lungs, and other various organs. Traditionally, there are two phases of drug metabolism that exist to transfrom lipophilic compounds into hydrophilic products that are more readily eliminated from systemic circulation. Phase 1 biotransformations are primarily oxidative; however, reduction, hydrolysis, and hydration reactions are also observed. Phase 1 reactions are primarily carried out by microsomal expressed cytochrome P450s (CYP450), flavin monooxygenases, aldehyde oxidase, and monoamine oxidase in the hepatocytes and microsome preparations from hepatocytes (a fragment of endoplasmic reticulum and attached ribosomes obtained by the centrifugation of homogenized cells). Phase 2 biotransformations are conjugative and occur within the S9 fractions (harvested from a mixture of unfractionated microsomes and cytosol by the centrifugation [9000 g] of homogenized cells) and hepatocytes. The primary conjugative reactions are glucuronidation, sulfation, methylation, acetylation, glutathione conjugation, and amino acid conjugation.

2.1 Metabolic stability

The industry standard for screening the metabolic stability of a compound or compound-series is via the substrate depletion approach and the determination of half-life in rodent, nonrodent and human. Briefly, the metabolic stability, or intrinsic clearance (Cl_{int}) is assessed by incubating the compound at a concentration assumed to be below the K_m for P450 metabolism. The *in vitro* Cl_{int} can be assessed in multiple species hepatocytes, hepatic S9 fractions and/or microsomes [8]. A series of samples are collected and analyzed over the time course in order to

determine the percentage of compound remaining. The resulting half-life ($t_{1/2}$) is then appropriately converted to an activity (Cl_{int}, mg/mL/Kg), taking into account the ratio of protein content to liver mass (e.g., mammalian microsomal protein, 45 mg protein/gm liver), and the ratio of liver mass to total animal or body mass (e.g., human, 20 gm/Kg body weight); the principles of allometry apply in this context of scaling protein content and P450 activity (physiological parameter) versus organ-to-body mass ratio (anatomical parameter) [9]. The intrinsic clearance *in vitro* PK parameter may be generated from a number of subcellular fractions, including microsomes, S9 fraction, cytosol and mitochondria (e.g., MAO metabolism), as well as from whole cell incubations employing freshly isolated or cryopreserved hepatocytes. The high throughput nature of the aforementioned metabolic stability assay enables the rapid generation of *in vitro* PK parameters ($t_{1/2}$ and Cl_{int}) with each cycle of medicinal chemistry. With the continued innovation of mass spectroscopy and the liquid chromatogram coupled mass spectrometry (LC/MS) bioanalytical techniques, the contemporary biotransformation laboratory and scientist can now identify metabolites in the same intrinsic clearance assay, thus elucidating the relevant pathway of metabolism under kinetically controlled conditions. Alternatively, the same subcellular fraction and whole cell metabolism experiment may be employed to determine all relevant pathways and under conditions where compound(s) concentrations have been elevated appreciably above the anticipated K_M for a particular drug metabolizing enzyme (DME). The merits of either approach will be discussed in a subsequent section.

2.2 Soft spot analysis/metabolite identification

The medicinal chemist utilizes the metabolic stability data generated during the Lead Selection and Lead Optimization stages of discovery as a tool to understand the impact of structural modifications within one or more chemical series or a lead series, respectively. Depending on the subcellular fraction employed, the Cl_{int} informs the chemist as to ensuing stabilization of the compound or series to oxidative (or reductive) metabolism via P450 (e.g., microsomes or S9 fractions). In addition to the $t_{1.2}$ and Cl_{int} data produced from *in vitro* screening, the incubations also provide an opportunity to determine the site of metabolism on the molecule, or elucidate the so-called metabolic "soft-spot". From P450 mediated oxidation, to direct glucuronidation and sulfation, to ester and amide hydrolysis, small molecule drug candidates possess physico-chemical properties that are perfectly suited to drug metabolism-mediated, hepatic clearance. Typically, the soft-spot analysis is performed in the same subcellular fraction or hepatocyte system employed in the intrinsic clearance assessment. With recent improvements in electrospray ionization liquid chromatography tandem mass spectrometry (ESI-LC–MS/MS) bioanalysis, the DMPK scientist is able to associate one or more predominate sites of metabolism (e.g., oxidation, reduction, hydrolysis, conjugation) with the clearance of a compound or series *in vitro*, and under the same kinetically controlled conditions of the metabolic stability assessment (i.e., ≤ 1 μM). The described soft-spot analysis construct enables a concerted execution of both the Cl_{int} assessment (substrate depletion) as well as the identification of the principal metabolites produced *in vitro* in "one plate". Whether in concert with the Cl_{int} assessment or as a discrete stand-alone in vitro experiment, innovation in mass spectroscopy hardware and software has enabled significant means of metabolite detection and structural elucidation, the results of which commonly augment the nonclinical pharmacology reports and summaries within an Investigational New Drug application (IND).

Still, the DMPK scientist may seize on the opportunity to determine the soft-spot(s) of metabolism to simultaneously assess the extent and range of

biotransformation likely produced in multiple species during preclinical and clinical development. Access to study samples (e.g., plasma, urine, bile, feces, organ tissue) presents the opportunity to survey the metabolites produced in the rodent and nonrodent species selected for development, comparing to that observed in human hepatocytes and hepatic (e.g.) subcellular fractions. In order to provide a comparative analysis to the *in vivo* metabolism picture, the DMPK scientist will produce in parallel a set of *in vitro* experiments in rodent and nonrodent, where the compound or preclinical candidate is incubated at concentrations assumed to exceed the K_m for most drug metabolizing enzymes (e.g., ≥25 μM ≤50 μM). Importantly, *in vitro* incubations of subcellular fractions should be fortified with appropriate cofactors (or co-enzymes) to "fuel" the relevant catalytic activities of select enzymes and at excess (1-2 mM): P450 (NADPH, or NADPH-regenerating system), FMO (NADPH), UGT (UDPGA), SULT (PAPS), NAT (acetyl-CoA), GST (GSH).

2.3 *In vivo-in vitro* correlation

The *in vitro* hepatic clearance of a compound (within a series) is a valuable PK parameter for the medicinal chemist and the DMPK scientist. Because the intrinsic clearance (Cl_{int}, mL/min/kg) describes the unrestricted, unscaled clearance of a compound, the medicinal chemist may utilize Cl_{int} to gauge the impact of structural alterations in the series on P450 metabolism (oxidation or reduction in microsomes); the primary goal of which to stabilize a compound or chemical series towards hepatic clearance, thus increasing the *in vitro* half-life. The value of the Cl_{int} PK parameter is in its correlation to a plasma clearance (CL_p), as typically determined in a rodent (e.g., Sprague–Dawley rat) or nonrodent (e.g., beagle dog) species during the hit-to-lead and later in the lead optimization stages of discovery. Establishing an in *vitro:in vivo* correlation (IVIVC) between predicted hepatic clearance (CL_H) and CL_p serves two purposes: (1) validation of the CL_{int} screening approach for the ensuing lead optimization stage of discovery, and (2) establishes the nonclinical species for *in vivo* PK screening and the species predictive of human hepatic clearance. The selection of appropriate *in vitro* and *in vivo* PK screening approaches during early discovery provides a mechanism for an iterative medicinal chemistry optimization of one or more chemical series, with the goal of predicting human PK parameters.

3. Lead optimization

Once a lead series is selected, further *in vitro* and *in vivo* testing is conducted on a fewer number of compounds, illuminating metabolism and PK (and pharmacologic) attributes for select compounds. This stage of drug discovery is known as Lead Optimization. From an *in vitro* perspective, potential DDIs are identified with CYP450 inhibition and induction assays, and reaction phenotyping assays. Soft spot analysis is performed to identify areas liable to biotransformation, metabolic identification of potential *in vivo* metabolites, and drug transporters are identified for which the candidate entities are a substrate. Rodent and nonrodent PK studies are conducted in order to optimize the exposure and disposition (PK) of the lead series while determining the nonclinical pharmacologic effects of the lead series in select rodent and/or nonrodent disease models. These data are then used to establish preliminary exposure-effect relationships (PK-PD). The exposure data collected from rodent (e.g.) models of efficacy are particularly valuable and provide a critical assessment of dose-exposure relationships of the lead series in anticipation of advancing into the single- and repeat-dose tolerability assessments prior to

candidate selection. At minimum this exposure information guides the discovery team to the dose range required for a tolerability screening assessment; ideally, the efficacy model exposure assessments guide the selection of the dosing frequency required to maintain exposure during the repeat-dose tolerability assessment.

The Food and Drug Administration's (FDA) January 2020 guidance on clinical drug interaction studies states that "clinically relevant DDIs between an investigational drug and other drugs should therefore be: (1) defined during drug development as part of the sponsor's assessment of the investigational drug's benefits and risks; (2) understood via nonclinical and clinical assessment at the time of the investigational drug's approval; (3) monitored after approval; and (4) communicated in the labeling." Furthermore, the FDA defines the goals of studies that evaluate P450 enzyme- and transporter-mediated DDIs to be: (1) determine whether the investigational drug alters the pharmacokinetics of other drugs; (2) determine whether other drugs alter the pharmacokinetics of the investigational drug; (3) determine the magnitude of changes in pharmacokinetic parameters; (4) determine the clinical significance of the observed or expected DDIs; and (5) inform the appropriate management and prevention strategies for clinically significant DDIs [10].

Additionally, the FDA also provided guidance for *in vitro* drug interaction studies (P450 and transporter) in January 2020. This guidance provides the framework for designing and conducting *in vitro* experiments in order to assess potential clinical DDIs. The CYP450 experiments are to: (1) determine which CYP450 enzyme the drug entity is a substrate of (reaction phenotyping); (2) determine if the drug entity is a CYP450 inhibitor; and (3) determine if the drug entity is a CYP450 inducer. Metabolite investigations may be warranted on a case-by-case basis. If the metabolite is pharmacologically active and contributes ≥50% of the overall activity, then reaction phenotyping analyses should be conducted. Inhibition studies are to be conducted if the total exposure/area under the curve (AUC) of the metabolite is ≥25% of the parent or if the metabolite is more polar than the parent entity and the AUC of the metabolite is greater than or equal to the parent. Transporter studies are to investigate if the drug entity is a substrate of efflux pumps (P-glycoprotein [P-gp] and breast cancer resistance protein [BCRP]), hepatic transporters (OATP1B1 and OATP1B3), and renal transporters (OAT, OCT, and MATE) [11].

3.1 Reaction phenotyping

Having assembled relative *in vitro* pharmacokinetic data (e.g., Cl_{int}) and elucidated metabolism pathways for a compound or preclinical candidate, it's incumbent upon the DMPK scientist to identify the particular human drug metabolizing enzymes that are contributing to the *in vitro* clearance in an effort to identify and/or manage latent drug–drug interaction potential that exist. To identify such victim drug–drug interaction (DDI) potential, [12] the DMPK scientist employs a variety of recombinantly expressed drug metabolizing enzymes, notably P450 enzymes to determine the extent any one enzyme contributes to the clearance of a compound [13]. The fraction-metabolized (f_m) term is often employed within the context of P450 mediated metabolism, but more recently applied to the growing number preclinical candidates observed to be non P450 substrates (e.g., UGT). Correlating the *in vitro* microsomal (e.g.) clearance to contributions from any one or more P450 enzymes, most notably P450 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4, is the goal of so-called reaction phenotyping. Importantly, this reaction phenotyping of drug clearance involves the comparative metabolism (and intrinsic clearance thereof) of the preclinical compound by the recombinant enzyme to what is observed in the subcelluar fraction; in the case of P450 or UGT, that can be accomplished in hepatic microsomes or S9 fractions. Two industrial standard approaches involve the

generation of relative activity factors (RAF method) and intersystem extrapolation factors (ISEF) to adequately relate a single recombinant enzyme activity to human liver microsomes bearing a full complement of expressed P450 enzymes. Each of these methods, while instrumental in arriving at the f_m value for a particular drug candidate, fall short in estimating the impact of polymorphically expressed enzymes (e.g., P450 2D6, 2C9, 2C19, UGT1A1) in the metabolism-mediated clearance of a compound. More recently, and as a result of pharmacogenomics and pharmacogenetics clinical research and impact, the DMPK scientist can gain access to subcellular fractions obtained from sparse or densely genotyped individual liver donors.

The importance of linking a particular biotransformation reaction to one or more metabolites is of particular interest during preclinical development. Whether in terms of pharmacology (e.g., P450 mediated production of an active metabolite), drug safety (e.g., UGT mediated production of an acyl glucuronide) or confirming multiple enzymes producing the same pathway (e.g., risk mitigation of a clinically relevant DDI), the use of recombinantly expressed enzymes are critical in mapping the range of metabolites observed in human hepatocytes or subcellular fractions to specific drug metabolizing enzymes. At the elevated concentrations employed in the generation of metabolite(s) *in vitro*, there is limited kinetic value to these experiments and should be viewed as informative in nature and restricted to the metabolite ID and structure elucidation exercise previously described.

3.2 CYP450 inhibition studies

Once the metabolic pathways are identified, *in vitro* P450 inhibition and induction studies are conducted in order to predict clinically significant DDIs. Per the FDA's January 2020 guidance, "The sponsor should evaluate an investigational drug's potential to inhibit CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A in both a reversible manner (i.e., reversible inhibition) and time-dependent manner (i.e., time-dependent inhibition (TDI))" [14].

In general, each microsomal assay includes the preparation of both test compound and positive control samples in order to assess the metabolite formation specific to each CYP450 isoform. Experiments are typically prepared with both the test compound at increasing concentrations and a selective P450 probe substrate prepared at a predetermined concentration that will produce first-order kinetics. Following an incubation under physiologic conditions (pH and temperature), the experiment is quenched and resulting incubations are prepared for analysis employing LC–MS/MS. The data is then analyzed by comparing the metabolite formation of the test samples relative to the control samples and dose–response plots are created to determine the specific endpoints of the assay.

3.3 Screening DDI cocktail assay

Concurrent with the early optimization stage P450 assessments, a cocktail of the test compound and substrates that exclusively bind to specific P450 isoforms is incubated with human liver microsomes across multiple concentrations of the test compound. Following incubation, P450 isoform specific metabolites are measured in each sample and compared to a control sample. Based on the metabolite abundance of the test samples relative to the control sample, as measured by liquid chromatography and tandem mass spectrometry (LC–MS/MS), a potential DDI liability can be identified early to inform future *in vitro* and potentially future *in vivo* investigations. Based on the results of this screening assay and the P450 inhibition and induction assays, definitive *in vitro* DDI screening may be warranted during the Drug Candidate Selection stage.

3.4 CYP450 induction studies

CYP induction is typically measured *in vitro* using three separate lots of human hepatocytes. A metabolically active human hepatocyte cell line (e.g. HepaRG) can sometimes be substituted for one of the human hepatocyte lots. Three P450s are commonly measured for induction – CYP1A2, 2B6 and 3A4. If CYP3A4 induction is observed, it is recommended to assess CYP2C8, 2C9 and 2C19 induction in a separate experiment. For the induction experiment, human hepatocytes are incubated in a sandwich culture format prior to the experiment. The media is changed to serum-free prior to the start of the experiment in order to lower the basal expression of CYPs. On day one, test compounds are added and incubated with the cells for 48-72 hours, with daily replenishment of media and test compound. Induction can be assessed by either measuring mRNA levels or activity levels. For the mRNA method, the cells are harvested after 48 hours, mRNA is extracted and the expression level of CYPs is measured by qPCR. For the activity assay, specific substrates for each of the CYPs are added following 72 hours of incubation and the rate of metabolism is measured over a defined time point, e.g. 2 hours. A positive result is considered to be ≥2-fold increase over the vehicle control.

3.5 Membrane drug transporters

Membrane drug transporters play an important role in the uptake, distribution and elimination of both endogenous substances and drugs in the body. Because they help regulate the flux of many substances across cell membranes they are often implicated in detoxification mechanisms, multidrug resistance and clinical DDIs [15]. Drug transporters control the concentration of drug substrates available for P450 reactions by regulating drug disposition within the cell for both the parent drug and metabolites. Drug metabolizing enzymes are often coupled with transporters to efficiently modify the level of drug present in a specific tissue. A well-known example of this is the efficient removal of bilirubin from plasma by OATP1B1-mediated uptake into the liver, UGT1A1-mediated formation of mono- and diglucuronide metabolites and subsequent elimination into the bile via the MRP2 transporter. In addition, the expression levels of drug metabolism enzymes are closely tied to transporters. For example, the nuclear receptor PXR regulates the expression of both drug metabolism enzymes such as CYP3A4 and CYP2C9 as well as several efflux drug transporters including P-gp and MRP2.

Membrane transporters relevant to drug development include two major superfamilies – ATB-binding cassette (ABC) and solute carrier (SLC). Members of the ABC superfamily utilize ATP hydrolysis to actively transport a solute across a cell membrane against a concentration gradient. The most relevant ABC transporters include P-glycoprotein (P-gp, MDR1), breast cancer resistance protein (BCRP) and multidrug resistance-associated protein 2 (MRP2). One of the primary functions of these transporters is to efflux small molecule substrates out of the cell in order to reduce cellular exposure and protect cells and organs against potentially harmful drugs or toxins. They are widely expressed in the epithelia of the intestine, liver and kidney, and in the endothelium of the blood–brain barrier and other blood-tissue barriers where they are localized on apical membranes.

In contrast, members of the SLC superfamily utilize solute exchange mechanisms to drive drug transport, including endogenous anions/cations or electrogenic mechanisms. SLC transporters include the major uptake transporters such as organic anion (OAT, OATP), organic cation (OCT) and the multidrug and toxin extrusion transporters (MATE1, MATE2-K). OATP1B1 and OATP1B3 are located

primarily on the hepatocyte plasma-facing apical membrane, while OAT1, OAT3 and OCT2 are located on the basolateral membrane of the kidney proximal tubule. MATE1 and MATE2-K are located both in the proximal tubule and in the liver (canalicular membrane).

Several of the ATP and SLC transporters have been implicated in clinical DDIs and the FDA has focused on the following list as relevant for *in vitro* screening: P-gp, BCRP, OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1, and MATE2-K. *In vitro* evaluation of specific transporter interactions can employ a variety of tools including human and animal monolayer systems, transfected cell lines with single or multiple transporters over-expressed and membrane vesicles, along with a panel of substrates and inhibitors as control probes (**Table 1**).

3.5.1 Monolayer systems

The standard assay format for transporter function involves measuring the permeability of a test article through a confluent monolayer of cells grown on a permeable membrane. The cells and membrane are part of a transwell insert which fits into a normal 24-well plate, thus creating two media chambers. The upper reservoir is referred to as the apical (or A) chamber while the lower is the basolateral (or B) chamber. Addition of test article to each reservoir in separate wells, allows measurement of the apparent permeability in both directions (A to B, and B to A). The cell line used, therefore, must have the ability to form tight junctions between cells to prevent leakage of test article through the monolayer, and must express the transporters in a polarized fashion, enabling measurement of transport of substrates in two directions. Two cell lines are commonly used in this format – Caco-2 and MDCKII. The Transwell format is used not only to measure the permeability of the test article in a single direction, but also determine the efflux ratio, calculated as the ratio of permeability in both directions (B to A/A to B). If the efflux ratio is greater than 2, an interaction with a transporter is probable and needs further study.

Caco-2 cells were originally derived from a human colorectal adenocarcinoma. Although the cells originated from the human colon, they are widely used as a model of intestinal absorption and transporter activity [16]. When placed in culture, these cells undergo differentiation to an intestinal phenotype. The cells are characterized by a well-defined apical brush border, formation of tight junctions, and the endogenous expression of the majority of uptake and efflux transporters normally present in intestinal enterocytes [17]. Caco-2 cells are regarded as the most sophisticated *in vitro* tool for medium to high throughput modeling of drug transport across human plasma/tissue barriers.

A non-human alternative cell line that is also widely used in monolayer studies is MDCKII (Madin-Darby canine kidney strain II). A number of MDCKII cell lines

Assay	Cell system	Endpoint
Permeability Assessment	Caco-2, MDCK	Apparent permeability (P_{app}), efflux ratio
ATP Transporters (efflux)	Caco-2, MDCK, membrane vesicles, genetically modified cell lines	Substrate/Inhibitor
SLC Transporters (uptake)	Hepatocytes, transfected cells (MDCK, HEK293, CHO)	Substrate/Inhibitor

Table 1.
Examples of in vitro *systems to investigate transporter-mediated drug interactions.*

have been generated with single or double human transporters transfected into the cells. These modified animal cell lines enable the study of individual human transporters in the absence of competition from other human transporters. One caveat with MDCK cells is that there is an active form of canine P-gp present that is functionally similar to the human form and needs to be accounted for when transporter data is analyzed.

Other animal cell lines used for transporter studies include LLC-PK1 (porcine) and Chinese hamster ovary (CHO) cells.

3.5.2 Membrane vesicle assays

An alternative to cell-based transporter assays involves membrane vesicles, in which the assay is conducted with membrane preparations from baculovirus-infected insect cells or mammalian cells that have been transfected with the transporter of interest. When isolated, a small portion of the membrane vesicles end up in an inverted configuration ("inside-out") and are particularly useful for studying efflux transporters. Transporter activity (activation or inhibition) can be assessed by either measuring uptake of a substrate into the vesicles over time, or by measuring ATPase activity.

3.5.3 Genetically modified cell lines

Transporters recognize and interact with of a broad range of compounds, and each transporter has been characterized for their physicochemical preferences in substrates [18]; however, there remains a large overlapping area of substrate recognition between transporters. Some of the current ambiguity in assigning specificity toward a single transporter is due to the use of different cellular systems to define the interaction. Small molecule inhibitors are commonly used to define transporter interactions, but inhibitors often show overlapping interactions between transporters [19]. Novel test systems that avoid the use of small molecule inhibitors may be able to improve our ability to unambiguously identify specific substrate interactions Two approaches have recently been used to address this situation. Knockdown of transporter gene expression can be accomplished using small inhibitory RNA (siRNA). Alternatively, complete gene knockout can be accomplished using zinc finger nucleases (ZFNs) or Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). For example, several individual or dual knockout cell lines were recently developed in the C2BBe1 cell line (a subclone of Caco-2) and are commercially available [20]. In addition, the canine P-gp transporter in MDCK cells has recently been knocked out.

4. Drug candidate selection

Following the investigations of the Lead Optimization stage, the pharmaceutical organization should have weaned their list of promising drug candidates to less than five entities. From a drug metabolism perspective, there are three major investigations that occur: (1) ADME profiling in rodents and nonrodent species; (2) definitive *in vitro* DDI screening and potentially *in vivo* confirmatory investigations; and (3) metabolite profiling in the identified toxicology species. By the end of the Drug Candidate Selection stage, the pharmaceutical organization should have all the information they need to support their Investigational New Drug application (IND) or Exploratory IND application.

4.1 Mass balance study

Due to the high expense, complete ADME profiling (non-radiolabeled) does not typically occur until a strong candidate for IND submission has been identified. The *in vitro* metabolism, tissue distribution analyses, and *in vivo* PK studies performed in the prior development stages are used to inform the investigators regarding the potential route of clearance, the tissues to be collected and quantitated, and if a major metabolite should be investigated as part of the study. The goals of these studies are to definitively identify the route of elimination (including biliary excretion), assess tissue distribution, and if applicable, characterize metabolite exposure relative to parent exposures. The animals in these studies are typically split into two groups where one group has their bile duct cannulated in order to assess biliary excretion from the liver. The animals are individually housed in metabolism cages that collect the urine and feces excreted by each animal. Depending on the study design, each animal may be sacrificed at various time points in order to assess critical tissues for exposure to the drug entity.

4.2 DDI evaluation

Based on the results of the P450 inhibition/induction assays and the screening DDI studies, specific P450 isoforms are further investigated *in vitro* for the DDI potential instead of a general cocktail approach as employed in the Lead Optimization stage. If an *in vitro* DDI is observed, an *in vivo* study is warranted to confirm if the DDI persists.

4.3 Metabolite profiling

At dose levels greater than 100 mg/kg, the primary clearance mechanism is typically saturated resulting in greater interaction between the drug entity and other P450 enzymes. Metabolite profiling analyses typically use blood samples collected at tox level doses greater than 100 mg/kg; ideally samples at or near the C_{max}. The metabolites are typically identified early during the lead optimization stage in the soft spot/metabolite identification studies. The parent and metabolites are typically quantitated via LC–MS/MS methods. The end goal is to obtain the percent abundance of each metabolite relative to the parent.

5. Metabolite safety testing

Following Phase 1 FIH studies, the blood samples at or near the C_{max} from the high dose cohort of the multiple ascending dose study undergo metabolite profiling. The resulting metabolite abundancies are then compared to the preclinical metabolite profiling studies in order to identify human only metabolites or metabolites that are present at higher plasma concentrations in humans than in the animals used in the preclinical studies. These metabolites are referred to as disproportionate drug metabolites. Per the FDA's March 2020 guidance, "In general, these metabolites are of interest if they account for plasma levels greater than 10 percent of total drug-related exposure, measured as area under the curve at steady state." In the instance that a disproportionate meets these criteria, futher *in vitro* (genotoxicity) and *in vivo* (general toxicity and embryo-fetal development toxicity) studies are required to assess the safety of the metabolite. Typically, these studies can be conducted concurrently with Phase 2 studies prior to the large-scale Phase 3 trials.

6. Summary

Drug metabolism is a drug-clearing event from systemic circulation influencing efficacy and toxicity in humans and preclinical species. The primary endpoints of enzymatic metabolism studies in both the drug discovery and preclinical stages are to resolve metabolic stability, identify and quantify primary metabolites, identify metabolic routes, and measure the potential for DDIs and are used to predict human PK. The intrinsic kinetic properties of the chemical entity with consideration of the physiological processes can be used to assess the disposition of single and multiple dose escalation studies, identify potentially saturable hepatic and transporter pathways, and assess the formation of metabolites. From this information, potential DDIs are predicted and subsequently investigated.

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