# 2 *In Vitro* and *In Vivo* Models of Drug Metabolism

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## 2.1 SUMMARY

Drug metabolism is the body's inherent ability to remove foreign compounds (xenobiotics) that have either intentionally or unintentionally made their way to the systemic circulation or organs within the body. Understanding the metabolism of newly developed drugs is paramount because of the central role metabolism plays in unraveling the possible toxicity or developability of new drug candidates. For example, the rate of drug metabolism in part determines the bioavailability, clearance, and half-life of a drug, and together, these parameters define how much drug (dose) and how often the drug needs to be dosed. Both these factors (dose size and frequency) can be significant hurdles to new drugs especially if the dose or dosing frequency is too high, making the drug unmanageable to develop or market. Also, metabolism is often considered a detoxification pathway by which the molecules are chemically modified to make them more polar and easily excreted from the body, eventually rendering them pharmacologically inactive. However, there are numerous examples where metabolism renders a compound less soluble (N-methylation reactions), pharmacologically active, or toxic. Therefore, it is always important to understand the full set of metabolic reactions and enzyme involved in the metabolism of a compound in both preclinical models (pharmacokinetic, pharmacological, and toxicological) as well as in human to avoid any unnecessary complications or failures in drug development.

Drug metabolism occurs everywhere within the body to differing extents; however, metabolism occurs predominately in those organs involved in elimination (e.g., kidneys and liver) and those organs to which the drug is first exposed [e.g., gastrointestinal

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tract (GI) and liver]. The most common drug-metabolizing organs or compartments are liver, GI tract, kidney, plasma, brain, skin, lung, and nasal mucosa with the liver and GI tract being the most abundant in enzyme level and capacity [1]. Often overlooked are another set of drug-metabolizing enzymes found in the GI tract, which being the microflora that can metabolize foreign compounds as they travel along the lower GI [2–4]. Typically, these microorganisms act through hydrolysis and reductive metabolic pathways, given the environment in the lower GI tract. Therefore, metabolites found in feces often need to be considered as being derived from metabolic reactions of the host organism as well as the microflora.

Clearly, metabolism can occur in many sites and organs within the body, but often specific cell types within the organ are responsible for the majority of metabolic reactions, such as the hepatocyte in the liver and the enterocytes within the intestine.

Focusing even further down, there are numerous subcellular organelles within each organ that contain significant quantities of drug-metabolizing enzymes that participate in the metabolism of xenobiotics, such as the microsomal fraction (derived from the endoplasmic reticulum), cytosol, and mitochondria. The following section describes various *in vitro* and *in vivo* models that are often employed throughout drug discovery and development, as well as the purpose each model system serves to provide valuable information to elucidate the metabolic disposition of new and existing drug molecules. The information contained within will be based on the authors own experiences and numerous seminal publications in the area of *in vitro* and *in vivo* drug metabolism [1,5,6].

## 2.2 *IN VITRO* MODELS OF DRUG METABOLISM

*In vitro* models of drug-metabolism range from single enzymes to whole organs. The models can be categorized into two distinct groups: (i) models that contain intact cells, such as whole organ, slices, or cells and (ii) subcellular fractions. Each model system has its own utility in the study of drug metabolism in either drug discovery and/or development along with a set of advantages and disadvantages, all of which are highlighted in the following sections. General procedures are provided for the isolation and characterization of the most commonly employed *in vitro* model systems, such as microsomes, cytosol, and hepatocytes, which are described in detail by Guengerich and LeCluyse, respectively [7,8].

### 2.2.1 Individual Enzymes

The single or individual enzyme system is a useful tool to study enzymatic activity in a controlled environment of enzyme content, buffer, and cofactors all of which can be easily manipulated. Generally, the total protein content of such incubations is extremely low and reduces the effects of nonspecific binding, which can be a benefit as opposed to using large quantities of enzyme mixtures to obtain the same amount of enzyme activity. Often, the specific content or quantity of enzyme is known and as such the enzyme activity or turnover can be directly measured as a function of "nanomoles of enzyme" as opposed to the generic measure of "milligrams of protein" or "number of cells."

Enzymes were initially isolated from a tissue, which is a labor-intensive procedure, leading to significant loss of enzyme content (activity) with each successive isolation

**TABLE 2.1 Applications of *In Vitro* Drug Metabolism Models**

| | Single Enzymes | Subcellular Fractions | | Hepatocytes | |
|---|---|---|---|---|---|
| | | Microsomes | Cytosol | Suspension | Culture |
| Metabolic stability | — | XXX | X | XX | — |
| Clearance prediction | X | XXX | — | XXX | X |
| Metabolic profiling/species comparisons | X | XXX | XX | XXX | X |
| Metabolite identification/bioactivation | XX | XXX | X | XX | — |
| Inhibition studies | XXX | XXX | — | X | — |
| Induction studies | — | — | — | — | XXX |
| Reaction phenotyping | XXX | XXX | XX | XX | — |
| Bioreactor | XXX | XXX | XX | X | X |

XXX, commonly used; XX, sometimes used; X, rarely used.

step. Few enzymes today are isolated by this method, however, some are still easy and cost effective to isolate in this manner, such as β-glucuronidase from snails and alkaline phosphatase from bovine liver or intestine. More commonly, enzymes are individually expressed as recombinant enzymes through cDNA-directed expression systems in a variety of host cell lines (mammalian, bacterial, insect, and yeast) and are commercially available from multiple vendors [9–12].

The most commonly employed single-enzyme family is the cytochrome P450 (CYP) family of isozymes, which have been used to evaluate metabolic stability, human hepatic clearance, drug–drug interaction potential (inhibition), reaction phenotyping (i.e., identification of enzymes involved in metabolism of a drug candidate), the effect of allelic variants of drug-metabolizing enzymes on metabolism and elimination, and the potential to undergo bioactivation or covalent binding; serve as bioreactors to generate significant quantities of metabolites for identification purposes; and as a tool to directly study enzyme structure and function [10,12–16]. Table 2.1 illustrates the utility of single-enzyme systems across a range of drug-metabolism studies conducted throughout drug discovery and development. Many of the other individually expressed drug-metabolizing enzymes (e.g., glucuronyltransferases, sulfotransferases (STs), epoxide hydrolases, flavin monooxygenases (FMOs), glutathione *S*-transferases, and *N*-acetyltransferases [11,17]) can also be used for the aforementioned studies; however, they are used less often than the CYP enzymes because of the propensity of most drugs to be substrates of one or more of the CYP isoforms.

In some situations, the results from single-enzyme systems may not always mimic other *in vitro* models or *in vivo*. For example, if the overall metabolism is derived from multiple individual enzymes then the activity from a single enzyme will not properly reflect the overall metabolic clearance. Also, there are numerous examples where mechanism-based inactivation has been observed in recombinant enzyme preparations but not in other *in vitro* models such as liver microsomes likely because of more efficient formation of reactive metabolites in the focused individual enzyme system [18]. In general, the single-enzyme system will tend to overemphasize the importance or potency of metabolic reactions, nonetheless, they are extremely useful in breaking down complex metabolic pathways into individual pathways or enzymes.

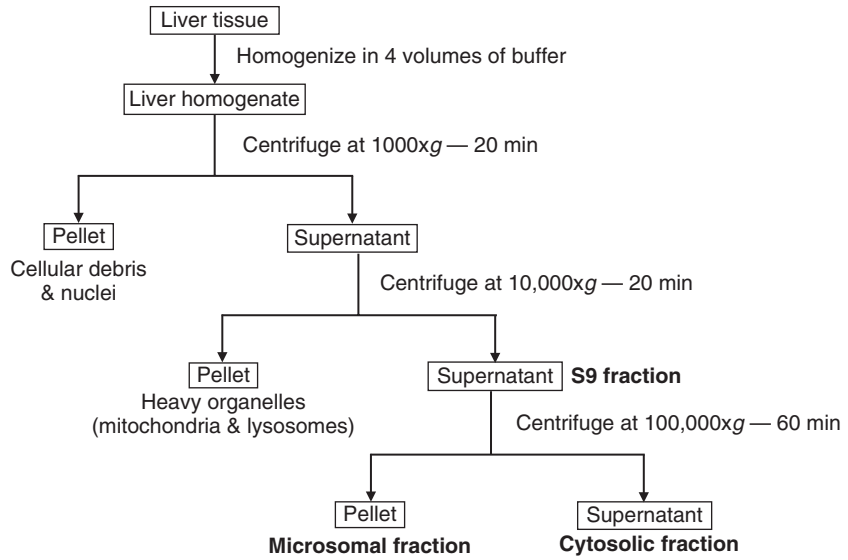


**Figure 2.1** Isolation procedure for liver subcellular fractions: S9 fraction, cytosol, and microsomes.

### 2.2.2 Subcellular Fractions

By use of tissue homogenization and differential centrifugation, it has become possible to obtain various subcellular fractions such as S9, cytosol, and microsomes. Depending on the tissue, either the entire organ or portions of the organ are more appropriate for subcellular isolations. For example, when preparing liver subcellular fractions, the entire liver is used; however, for intestinal or kidney microsomes, generally only certain cell types or tissue layers containing the enzyme(s) of interest are used to prepare subcellular fractions (e.g., enterocytes from the intestine).

Figure 2.1 outlines the general procedure for the isolation of liver subcellular fractions and the procedures for the most common drug-metabolizing organs, liver, and intestine are described in detail in the Refs 8 and 19. Briefly, the procedure begins with cooling the excised liver as quickly as possible to 4°C with buffer and all remaining buffers and steps are conducted at 4°C to ensure the best possible enzyme stability. The liver is then homogenized in four volumes of ice cold buffer and centrifuged at low speed ($1000 \times g$) to remove unbroken cells, cellular debris, and nuclei. The supernatant from this step is then centrifuged at $10,000 \times g$ to further pellet unwanted organelles such as mitochondria and lysosomes (note this pellet may be used for other drug-metabolism-related experiments). The supernatant from this step is the S9 fraction and is centrifuged at $100,000 \times g$ to yield the supernatant (cytosolic fraction) and the microsomes (pellet of endoplasmic reticulum). These subcellular fractions can be stored frozen at −80°C for long periods of time (years) and as such they serve as a convenient source of enzyme for drug-metabolism studies and have become widely available commercially. Owing to their ready availability, subcellular fractions are used in a variety of drug-metabolism- and toxicity-related studies [11–13,20–22].

Most drug-metabolizing enzymes are located in the cytosolic and microsomal fractions as outlined in Table 2.2, although others can be found in lysosomes, peroxisomes,

and mitochondria. The predominate subcellular fraction for evaluating drug metabolism is the microsomal fraction. The microsomal fraction containing the smooth endoplasmic reticulum is the most commonly employed *in vitro* model because most drugs are metabolized by the cytochrome P450s or by glucuronidation, both of which are found in the microsomal fraction. Table 2.1 illustrates how the microsomal model is used in nearly every evaluation except enzyme induction. The cytosolic fraction is used less frequently, however, when the major enzyme involved in metabolism or inhibition (e.g., sulfotransferase or aldehyde oxidase (AO)) of a particular drug is located in the cytosol then this model becomes extremely useful. The S9 fraction also has utility when complex metabolic pathway need to be studied, for example, when multiple different metabolic pathways are involved, such as hydroxylation by CYP followed by sulfation or carbonyl reduction followed by CYP-mediated oxidation and then some form of conjugation. Often when the metabolic pathways become too complex, it is better to use a more appropriate and relevant system such as the hepatocyte model, which is discussed in the following section.

### 2.2.3 Cellular Systems

Cellular systems can be categorized into two general groups: primary cells isolated from tissues and immortalized cell lines. Both have utility in assessing a variety of drug-metabolism functions; however, the primary cells tend to be employed more often in routine characterization, whereas immortalized cell lines are often used in early drug discovery and in higher throughput assays [23,24]. Primary animal or human hepatocytes isolated from liver tissue is the predominate cell-based model to evaluate drug metabolism (Table 2.1) [23,25]. Like the aforementioned subcellular fractions, hepatocytes can also be cryopreserved and stored for long periods of time, which makes them highly useful for routine or even high throughput assays to assess metabolic stability, metabolite identification, induction, or cytotoxicity. The most common method of isolating hepatocytes from all species is the two-step perfusion method that utilizes a calcium-free buffer in the first step followed by a buffer containing the enzyme collagenase [7,26]. The isolation of hepatocytes from liver tissue is not extremely challenging; however, time and practice are necessary to obtain high quality cells especially from animals other than the rat. Depending on the study type, the hepatocytes can be used as a cell suspension (generally only for 2–3 h) or in cell culture (for several days) [25]. Hepatocytes in suspension represent a fully functional unit of drug metabolism from the liver containing normal concentrations of enzymes and cofactors with the ability to perform complex and multistep metabolic reactions. Therefore, hepatocytes in suspension are commonly used to determine metabolic stability, predict human metabolic clearance, and compare metabolic pathways or metabolite identification across multiple species [22,25,27–30]. Less often, but still of value are studies using hepatocyte suspensions for assessment of drug inhibition, reaction phenotyping, bioreactors for metabolite formation, and cytotoxicity measurements. Unfortunately, when in suspension, the hepatocytes are no longer attached to an extracellular matrix or to one another, which is an inappropriate environment for the cells. Hence, the viability of the cells will begin to decline somewhere around 2–3 h in suspension limiting the length of incubation time and, therefore, the degree or extent of metabolism. Hepatocytes in culture will survive for several days or even weeks because of the artificial environment created in culture with an extracellular matrix and cell-to-cell contact. Hepatocytes in culture

are predominately used to assess enzyme induction (transcriptional activation) and the potential for drug–drug interactions or cytotoxicity [22,26,31,32]. Unfortunately, even in culture, the hepatocytes will begin to dedifferentiate and the phenotypic properties of the cells may start to change within 24–48 h in culture. Most important of these changes are those of the CYP enzymes, which in general will decrease with time in culture; however, some CYPs have been shown to increase and others to decrease and then increase with culture time. Owing to these ever changing levels of the major drug-metabolizing enzymes, many of the other drug-metabolism-related studies are not suited for this model. However, modern advances in cell culturing techniques such as microfluidic flow systems, micropatterned hepatocyte coculture systems, and different three-dimensional cultures such as spheroids have made significant advances in improving the stability and longevity of drug-metabolizing enzymes in culture [33–35].

Immortalized cells are cells that can grow and divide indefinitely under optimal conditions, hence they are attractive as a cellular model, which can provide an inexpensive supply of cells that are phenotypically stable. A variety of cell lines are currently available and used in the assessment of drug metabolism, such as HepG2, Fa2N4, BC2, and HepaRG [23,24,36,37]. Each cell line has a particular or general utility in drug-metabolism studies because each cell line is unique in the expression of drug-metabolizing enzymes (oxidative enzymes such as CYPs or conjugation enzymes), transcription factors (e.g., pregnane X receptor or constitutive androstane receptor), or drug transporters. Therefore, when deciding which cell line to employ, one must first consider the type of evaluation or question is being asked, for example, is the experiment designed to assess metabolite formation/identification or enzyme induction. Although immortalized cell lines are readily available and useful for many applications, there is no single cell line that recapitulates the genotype and phenotype of a primary human hepatocyte and therefore cannot be used as a replacement for primary hepatocytes but can be used in higher throughput models as primary or secondary screens.

### 2.2.4 Organ Slices

Tissue slices have been prepared from multiple organs, predominately the liver and also intestine, kidney, brain, and lung [11,38,39]. Tissues such as the liver and heart are rigid enough to be sliced directly; however, soft tissues such as the intestine and lung are generally imbedded in agarose and then sliced [39]. Tissues slices, in contrast to the aforementioned *in vitro* models, represent a higher level of structural integrity and architecture containing all of the organ cell types, cellular attachments to basement membranes, and cell-to-cell contacts [40,41]. Liver slices, first noted in the literature in the 1920s and 1930s, are one of the earliest *in vitro* models to evaluate metabolism, pharmacology, and toxicology [42,43]. The early slices were extremely crude and slice thickness was not reproducible, however, they did demonstrate metabolic activity. With the advent of specialized slicing, such as the Krumdieck and Brendel tissue slicers, tissue slice thickness became more reproducible and slices were prepared with minimal tissue damage. Typical liver slices range in thickness from about 200 to 300 μm and generally maintain metabolic function for 24–96 h under appropriate culture conditions. Today, the technology of tissue slicers continues to improve [44], as do the culture conditions, which have moved in the direction of fluidic perfusion techniques [45] as

compared to the earlier roller incubators or multiwell plates, which bathed the tissue slice in media in an atmosphere of oxygen and carbon dioxide.

Tissue slices have many of the same applications as hepatocytes or other cell-based models; however, the advantage of intercellular connections between different cell types within the slice can provide a greater level of cellular communication and function especially in cases where multiple cell types are necessary for complex metabolic or toxicological pathways. As with the cellular models, the enzymatic activity (e.g., CYPs) within the slice begins to decline shortly after being placed in culture. Early applications of tissue slices were the assessment of toxicology and pharmacology, however, drug-metabolism studies such as metabolite identification, species comparisons, and enzyme induction have been prominently noted in the literature [39,41,46–48]. A somewhat controversial application of liver slices is that to predicting metabolic clearance. Concerns have been noted that the slice thickness acts as a diffusional barrier for access of drug and oxygen to the interior of the slice. With some drugs, the rate of metabolism may not be entirely based on metabolic reactions but on the rate of drug diffusion throughout the slice, hence the rate of determination may be compromised by diffusion or transport. Owing to this uncertainty, slices have not been used routinely as a model to assess clearance, however, they do recapitulate the rank order of metabolic rates, just not the values calculated from *in vivo* or other *in vitro* methods such as hepatocytes [40,41].

Tissue slices are not amenable to cryopreservation, therefore, experiments must be conducted on freshly isolated and sliced organs that makes tissue slices not as useful for routine studies, especially when slices are required from species other than the rat. Many of the same drug-metabolism studies that are conducted in liver slices can also be performed using hepatocytes, which can be cryopreserved making the hepatocyte model a more routine model for most applications. An advantage of tissue slices, similar to hepatocytes, is the ability to reproduce complex metabolic processes where more than one metabolic reaction occurs on the molecule (oxidation in combination with conjugation) or when more than a single cell type is required for a reaction or biochemical process.

### 2.2.5 Organ Perfusion

Organ perfusion experiments can be conducted either *ex vivo* or *in situ*, but it is not technically an *in vitro* method; however, it is covered in this section to distinguish the technique from live intact animal studies. Perfusions have been conducted on many organs such as the liver, heart, lung, kidney, brain, intestine, intestine–liver preparations, and pancreas [49–51]. There are several key references that outline the procedures for organ isolation and perfusion for both liver and intestine [50,52,53]. Owing to the size of these organs, most organ perfusions are generally limited to smaller animals such as rats and rabbits. Organ perfusions, such as the liver or intestine, offer several advantages over other techniques in that the delivery and sampling of the drug under investigation can be by physiological routes, such as the portal vein, bile duct, or intestine. In addition, the concentration of drug, buffer, and rate of drug delivery can all be easily manipulated during the experiment and tailored to the needs of the investigator. A significant disadvantage of the isolated perfused organ method is that only a single organ (i.e., single experiment) can be conducted per animal, which is in contrast to all of the previously described methods.

Liver perfusions or the combination of intestine–liver perfusions provide a good model to assess first-pass metabolism as well as the combination of drug-metabolizing enzymes in conjunction with transporter-mediated efflux (both are mechanisms of drug clearance from the liver). For example, Xu *et al*. [49] used the intestine–liver dual perfusion model to evaluate the first-pass metabolism of salicylamide, while other researchers have used the liver perfusion model as a means to study the interplay between metabolism and transport-mediated elimination or inhibition of transport-mediated elimination [54,55]. There is no doubt that the organ perfusion model replicates many of the delivery, sampling, and connectivities between enzymes and transporters that other *in vitro* models cannot duplicate, making it a powerful tool to study these combinatorial drug disposition and metabolism effects.

## 2.3 *IN VIVO* MODELS OF DRUG METABOLISM

*In vitro* models of drug metabolism are extremely useful to study specific reactions, mechanisms, or structure–activity relationships, however, *in vivo* systems are required to understand additional factors that may contribute to the type of metabolism, rate of metabolism, or other elimination pathways in the overall disposition/exposure of a drug or metabolites. For example, extrahepatic metabolism, protein-binding effects, first-pass metabolism, or the effects of a disease state on drug-metabolizing enzymes [56]. In addition, when metabolites are present in high concentrations, they may need to be quantitated as they may contribute to target pharmacology or off-target liabities [57,58]. *in vivo* drug-metabolism studies are categorized as preclinical (animal) and clinical (human) studies. In broad terms, the purpose of these studies is to assess the metabolic profile of a drug across animal species and eventually humans. By identifying the *in vivo* metabolites, correlations can be made to the *in vitro* metabolic profiles, assess the possibility of active or toxic metabolites, and determine the elimination pathways of parent drug and metabolites.

### 2.3.1 Preclinical Animal Studies

The most commonly sampled biological matrices from *in vivo* drug-metabolism studies include plasma, urine, and bile, as well as feces in some situations. Plasma and urine are readily sampled matrices, however, bile collection requires surgically altered animals that have been bile duct cannulated [56]. In such studies, plasma, urine, bile, and feces can be examined for parent drug and metabolites [59,60]. Many drugs are eliminated in bile as parent drug and especially as metabolites. Without the use of bile duct cannulated animals (e.g., intact animals), it would be difficult to assess the overall routes of drug or metabolite elimination, metabolic pathways, or amount of metabolism because of the elimination of bile into the GI tract, which eventually appears in feces. As mentioned previously, identification of parent drug or metabolites is challenging from feces because of the microflora in the GI tract, which can further metabolize compounds or alter metabolic profiles.

In early drug discovery, metabolite identification is conducted with “cold” or non-radiolabeled drug, and it can often be difficult to obtain a full metabolic profile along with metabolite concentrations. However, in late drug discovery or drug development,

radiolabeled compounds (carbon-14 or tritium) are employed as substrates and metabolite identification is more straightforward as even minor metabolites can be observed and quantitated with respect to one another and parent drug. In rarer instances, stable labeled drugs can aid in elucidating the pharmacokinetics, disposition, or metabolism of a drug from *in vivo* studies as well as address mechanistic questions from *in vitro* models [61].

An essential *in vivo* animal study is the mass balance study conducted in rodent and nonrodent species. In this study, animals are dosed either orally or intravenously (or both) with a radiolabeled drug and radioactivity is measured from bile, urine, feces, exhaled $^{14}CO_2$, animal carcasses, and cage washings [62]. The purpose of this study is to (i) determine the major elimination pathways of drug and drug-related material; (ii) determine the total recovery of radiolabeled material (what goes in should come out); and (iii) conduct metabolic profiling from the various matrices collected to verify that the human metabolites generated are the same as those formed in sufficient quantities in the toxicology models. Sometimes, specific tissues may be collected at various time points for determination of total radioactivity or metabolites to determine tissue distribution (e.g., brain, liver, adrenal). In general, good recovery of radioactivity after a sufficiently long collection period would be 90%, 85%, and 80% for rat, dog, and human studies, respectively [63]. Generally, monkey studies are more challenging and lead to somewhat less recovery of total radioactivity due to issues with incomplete dosing and sample collection.

Additional studies related to drug metabolism in preclinical species can include regulation of drug-metabolizing enzymes, understanding the mechanism of low systemic exposure, and determination of hepatic extraction. Enzyme induction in animals can be assessed by dosing animals for multiple days (generally one to two weeks) after which the liver is removed and the RNA expression or enzyme activity of various drug-metabolizing enzymes (typically CYP1A, 2B, and 3A) are measured. In some situations, the administration of specific or global enzyme inhibitors can help elucidate the enzymes involved in metabolism or determine the mechanism of low exposure. For example, the global CYP450 inhibitor 1-aminobenzotriazole (ABT) can be administered to rats to address (i) gut versus liver contributions to first-pass metabolism [64] or (ii) low systemic exposure due to poor absorption or high first-pass metabolism [65]. Determination of hepatic extraction by can be accomplished by infusing the drug via the portal vein and measuring systemic parent drug and/or metabolites [56]. This is a reasonable model when conducted in rats; however, in higher animal species such as the dog or monkey, it generally requires implantation of vascular access ports that lead to the portal vein [65].

### 2.3.2 Species Differences in Drug Metabolism

Species differences in drug metabolism are common and frequently complicate the extrapolation of metabolite data from preclinical animal models (in particular, pharmacology and toxicology species where active or toxic metabolites, respectively, can have dramatic effects) to the human metabolic profile [66]. Species differences in drug metabolism are generally related to (i) lack of a specific enzyme either in humans or animals; (ii) similar enzyme orthologs between species, which form different metabolite products; or (iii) greatly reduced activity of particular enzymes in preclinical species

that may predominate in humans, for example, AO [66]. Many common species differences in drug metabolism is described in the chapter titled ***Sex Differences in Drug Metabolism*** of this volume. Some of the most common species difference relate to the CYP family of enzymes [66,67], metabolites formed by N-acetylation where dogs lack this particular enzyme [68,69], or the formation of quaternary *N*-glucuronides where this particular conjugate is typically only formed in humans and monkeys but not in standard preclinical species [70]. For example, the anticonvulsant lamotrigine is formed in humans and monkeys as a major metabolite; however, this same metabolite is not found in standard preclinical species (rat and dog). Interestingly, the quaternary *N*-glucuronide of lamotrigine is the major metabolite formed in guinea pigs [71,72]. Finally, a number of drugs have the ability to induce various sets of drug-metabolizing enzymes in the liver and various other tissues such as the intestine. The major mechanism by which drug-metabolizing enzymes are induced are via transcriptional activation through nuclear hormone receptors or transcription factors. These receptors have been well characterized and shown to have significant species differences with regard to ligand binding and ultimately induction of drug-metabolizing enzymes. Therefore, observation of enzyme induction in any single species has little association to induction in other species, in particular, human enzyme induction [31,32,67,73].

### 2.3.3 Transgenic and Chimeric Mouse Models

The involvement of a single enzyme, transporter, transcription factor, or a small set of these proteins that are involved in metabolism, bioactivation, disposition, gene expression, or toxicity of a drug can be evaluated through the use of genetically modified knockout or humanized mouse models [74–78]. A large number of knockout mouse models (drug-metabolizing enzymes, drug transporters, and transcription factors involved in the regulation of enzymes and transporters) have been generated through genetic manipulation, Tables 2.3–2.5 [78–84]. The knockout mouse model is extremely useful in understanding the function of a single gene (protein) or the function of multiple enzymes all of which are controlled from a single gene. For example, cytochrome P450 reductase is an obligatory enzyme involved in all cytochrome P450 oxidations, and a cytochrome P450 reductase knockout mouse would be devoid of significant P450-mediated oxidation. The P450 reductase mouse (also known as POR KO or Cpr KO) was useful in distinguishing between cytochrome P450-mediated and FMO-mediated N-oxidation of the antitumor agent C-1311 [85]. When C-1311 was dosed to reductase knockout mice, there were only limited changes to the metabolism of the parent drug and formation of the *N*-oxide was unchanged. These data helped support the presumption that drug–drug interactions due to changes in cytochrome P450 enzyme activity would be greatly reduced when employing this antitumor agent because the drug interacted with FMO and not P450.

Humanized mouse models are created by insertion of a human gene(s) into the mouse genome, or more often, by deletion of the mouse orthologous gene and insertion of the human gene (Tables 2.3–2.5). This second method is generally preferred as it eliminates the confounding factors involved with having both human and animal orthologous genes present and functional at the same time. For example, the transgenic mice expressing the human UGT1A (1–9) locus demonstrated expression of the nine

**TABLE 2.2 Subcellular Location of Drug-Metabolizing Enzymes**

| Reaction | Enzyme | Location |
|---|---|---|
| Oxidation | Cytochrome P450 (CYP) | Microsomes |
| | Flavin monooxygenase (FMO) | Microsomes |
| | Aldehyde oxidase (AO) | Cytosol |
| | Xanthine oxidase (XO) | Cytosol |
| | Alcohol dehydrogenase | Cytosol |
| | Aldehyde dehydrogenase | Mitochondria and cytosol |
| | Monoamine oxidase (MAO) | Mitochondria |
| | Dihydrodiol dehydrogenase | Cytosol |
| | β-Oxidation | Mitochondria and peroxisomes |
| Hydrolysis | Carboxylesterases (esterase/amidase) | Microsomes, cytosol, lysosomes, plasma, blood |
| | Epoxide hydrolase | Microsomes and cytosol |
| | Peptidase | Blood and lysosomes |
| Reduction | Sulfoxide/disulfide reductase | Cytosol |
| | Quinone reductase | Microsomes and cytosol |
| | Reductive dehalogenation | Microsomes |
| | Azo and nitro reductase | Microsomes, cytosol, microflora |
| | Carbonyl reductase | Microsomes, cytosol, blood |
| Conjugation | Glucuronide conjugation | Microsomes |
| | Sulfate conjugation | Cytosol |
| | Methyltransferase | Microsomes, cytosol, blood |
| | *N*-Acetyltransferase | Cytosol and mitochondria |
| | Glutathione conjugation | Microsomes and cytosol |
| | Amino acid conjugation | Microsomes and mitochondria |

human UGT1A genes in a similar pattern as observed in human tissue. Senekeo-Effenberger *et al*. [86] used this transgenic model to demonstrate that human UGT1A1, 3, 4, and 6 were targets of the peroxisome proliferator-activated receptor alpha (PPAR) and that with an appropriate PPAR ligand (WY-14643) dosed to transgenic mice, and these human genes were induced in the liver, GI, or kidney.

Along with the advantages of employing an *in vivo* knockout or humanized model, come a few precautionary notes. There are genetic manipulations that can precipitate inappropriate expression of the human transgene or endogenous mouse genes. For example, the expression of a human gene to evaluate metabolism should be expressed at levels similar to those found *in vivo* in humans. Often the expression of human transgenes can be lower than those found *in vivo* or may be expressed in the wrong tissues. This can be due to a lack of necessary regulatory sites in promoter regions or a lack of cellular components such as coactivators/cofactors from the mouse that do not interact properly with human promoter/gene complexes. In addition, the deletion of a mouse gene (knockout) or insertion of a human gene (humanized) can lead to changes (up or down) in the expression of other mouse genes because of changes in endogenous

**TABLE 2.3 Knockout and Humanized Mouse Models of Phase I and II Drug-Metabolizing Enzymes**

| Gene | Knockout and/or Humanized |
|---|---|
| Cyp1a1 | Knockout |
| Cyp1a2 | Knockout |
| Cyp1a1/2 with CYP1A1/2 | Knockout/humanized |
| Cyp2a5 with CYP2A6 | Knockout/humanized |
| Cyp2c | Knockout |
| CYP2C18/19 | Humanized |
| Cyp2d | Knockout |
| CYP2D6 | Humanized |
| Cyp2e1 with CYP2E1 | Knockout/humanized |
| Cyp3a | Knockout |
| CYP3A4 | Humanized |
| Cyp3a with CYP3A4 (liver and/or gut expression) | Knockout/humanized |
| Gstp (α, ω, π, σ, θ, ζ) | Knockout |
| Sult1e1 | Knockout |
| Ugt1 with UGT1A | Knockout/humanized |
| UGT2B7 | Humanized |
| Nat1 | Knockout |
| Nat2 | Knockout |
| Nat1 with NAT1 | Knockout/humanized |
| NAT2 | Humanized |
| mEh (microsomal) | Knockout |
| sEh (soluble) | Knockout |
| P450 Reductase | Knockout |

*Source:* Taken from Refs 76,78,80,82–84.

metabolic pathways caused by the gene deletion/insertion. Nonetheless, these models offer the advantage of studying a human drug-metabolizing gene (function) or transcription factor in an *in vivo* setting (mouse), thereby avoiding many of the limitations imposed by *in vitro* systems that cannot appropriately recapitulate such affects as protein binding, liver uptake, or the time course of drug/metabolite exposures. The use of these mouse models have significantly increased our knowledge of the expression and function of many enzymes and proteins involved in drug metabolism. The generation of additional knockout or humanized mouse models beyond those currently available will provide tools, much like the plethora of *in vitro* tools, to routinely study drug metabolism in greater detail using an *in vivo* approach.

The chimeric mouse model is a tissue-modified (humanized) mouse model of human ADME (absorption, distribution, metabolism, and excretion) toxicology. In brief, the mouse hepatocytes are destroyed *in vivo* and the liver is repopulated with human hepatocytes that function normally [87]. In this model, mice are genetically altered in such a way that leads to destruction of the native mouse hepatocytes. Two methods for destruction of the existing mouse hepatocytes have been reported: (i) expression of an albumin promoted urokinase-type plasminogen activator (uPA) or (ii) knockout of the fumarylacetoacetate hydrolase genes (Fah). In order for the human hepatocytes to repopulate the mouse liver, the mouse must be immunodeficient. This is accomplished

**TABLE 2.4 Knockout and Humanized Mouse Models of Drug Transporters**

| Gene | Knockout and/or Humanized |
|---|---|
| Mdr1a | Knockout |
| Mdr1a/1b | Double knockout |
| Mdr1a/1b/Bcrp1 | Triple knockout |
| Bsep | Knockout |
| Mrp1 | Knockout |
| Mrp2 | Knockout |
| Mrp2 with MRP2 | Knockout/humanized |
| Mrp3 | Knockout |
| Mrp4 | Knockout |
| Bcrp1 | Knockout |
| Ost (α) | Knockout |
| Pept2 | Knockout |
| Mate1 | Knockout |
| Oct1 | Knockout |
| Oct1/2 | Double knockout |
| Oct3 | Knockout |
| Oat1 | Knockout |
| Oat3 | Knockout |
| OATP1B1 | Humanized |
| Oatp1b2 | Knockout |

*Source:* Taken from Refs 78 and 83.

**TABLE 2.5 Knockout and Humanized Mouse Models of Transcription Factors**

| Gene | Knockout and/or Humanized |
|---|---|
| Ahr | Knockout |
| Ahr with AHR | Knockout/humanized |
| Car | Knockout |
| Car with CAR | Knockout/humanized |
| Pxr | Knockout |
| Pxr with PXR (SXR) | Knockout/humanized |
| Car and Pxr | Double knockout |
| Car and Pxr with CAR and PXR | Double knockout/double humanized |
| Car and Pxr and Cyp3a with CAR and PXR and CYP3A4 | Triple knockout/triple humanized |
| Ppar (α) | Knockout |
| PPAR (α) | Humanized |
| Rxr (α) | Knockout |

*Source:* Taken from Refs 78,81 and 83.

by using existing immunodeficient mice, for example, severe combined immunodeficiency (SCID) mice or genetic manipulation, such as knocking out the recombinant activation gene-2 (RAG-2). After injection of human hepatocytes into the mouse spleen, over time, the mouse liver will become repopulated with human hepatocytes replacing >70–90% of the native mouse hepatocytes.

The chimeric mouse model has been shown to be useful in evaluating the metabolism, disposition, pharmacokinetics, pharmacology, and toxicity of many xenobiotics [87,88]. Using chimeric mice, several authors have been able to demonstrate metabolic profiles that were specific to humans and different from wildtype mice, for example, dexamethasone (CYP3A4), diclofenac (CYP2C9), paclitaxel (CYP2C8), and debrisoquine (CYP2D6) [88]. The model has also been shown to be a predictor of circulating human metabolites especially metabolites derived from multiple metabolic reactions [89]. The chimeric mouse model was also able to predict the human disposition of the drug cefmetazole. In mice and rats, cefmatazole is eliminated in feces (bile), whereas in humans, urinary elimination of cefmatazole is found. When dosed to chimeric mice, cefmatazole was found to be eliminated in urine, similar to the human elimination pathway and different than the wildtype mouse [88]. Drug–drug interactions have also been evaluated in the humanized mouse liver model. For example, the human-specific CYP3A4 inducers rifampicin and rifabutin were shown to induce human CYP3A4 in the chimeric mice but not in wildtype mice. Also, inhibition of human CYP2D6 was demonstrated by using quinidine as an inhibitor and debrisoquine as the probe substrate. The AUC and $C_{max}$ of 4-hydroxy debrisoquine decreased significantly in the uPA$^{-/-}$/SCID humanized mice when codosed with debrisoquine and quinidine but did not change in the control uPA$^{-/-}$/SCID mice [88].

### 2.3.4 Human

A great deal of metabolism-related information can be obtained through preclinical animal studies and *in vitro* human studies, such as metabolite identification and disposition of metabolites. However, none of these systems can act as a substitute for metabolism studies in healthy volunteers or patients. As quickly as the first human pharmacokinetic study in phase 1, information can be obtained on the identity of metabolites in human plasma and their concentrations relative to the parent drug either from single- or multiple-dose studies. Although these early phase 1 studies provide information on plasma metabolites and exposure, they often do not represent the full metabolic profile or the routes of parent drug and metabolite clearances. The human mass balance/AME (absorption, metabolism, elimination) study provides definitive information on metabolic and clearance pathways of metabolites and parent drug. This experiment, required by regulatory agencies, has several key outcomes: (i) an accounting of the mass balance of a drug and drug-related material through excretion into feces and urine; (ii) identification of rates and routes of elimination and clearance pathways (urine, bile, metabolism); (iii) quantitative identification of metabolites and metabolic pathways; and (iv) qualitatively and quantitatively supporting the use of preclinical toxicology species regarding exposure of metabolites between the preclinical species and humans (i.e., no unique human metabolites not found in preclinical toxicology species or human metabolites with much greater exposures than those found in preclinical species) [90].

In order to achieve these goals, a radioactive dose of the drug is given, generally carbon-14 ($^{14}$C, C-14) or hydrogen-3 (tritium, $^{3}$H, H-3) and urine and feces are collected for 7–10 days. If significant amounts of parent drug or metabolites are expected to be eliminated in bile (based on animal data) then bile can also be collected and quantitated for parent drug and metabolites, as well as metabolite identification [91]. The study is conducted in healthy volunteers or patients ($\sim N = 4–6$) as a single oral therapeutic dose or as an intravenous dose if the intended route of clinical use will be intravenous [92,93]. The amount of radioactivity is "as low as feasible" without compromising the ability to identify metabolites and measure elimination routes. The typical amounts of radioactivity for C-14 and H-3 studies are ~50–100 μCi and 100–300 μCi, respectively [94]. Also important is the location of the radiolabeled element in the parent drug molecule. The site of radiolabel should be chemically and metabolically stable so that the radiolabel remains with the parent drug and parent drug/major metabolites and is not lost into the endogenous pool of biochemicals (e.g., release as carbon dioxide). In some situations, typically with compounds with a central amide or ester functionality, the drug molecule can be metabolically "split" into two components. In this case, the radiolabel will be retained in only a portion of the drug and the nonradiolabeled fragment will be difficult to follow and quantitate. When this occurs, two radiolabels are placed in the drug molecule so that both portions of the drug can be traced and a full accounting can be made of metabolic pathways and routes of elimination.

In humans, a successful mass balance study will account for 80–85% of the radioactive dose in urine and feces, which is generally achievable for a compound with a reasonable half-life (<50 h). Roffey *et al*. have shown that when the human half-life of the drug is longer than 75 h, the ability to recover >80% of the radioactive dose decreases dramatically. They found that 80% of drugs with a short half-life achieved 85% or greater recovery, whereas only 28% of drugs with a half-life of >75 h achieved 85% recovery [63]. In this situation, the collection period can be extended to collect more of the radioactive dose, but in some situations, the amount of radioactivity being eliminated at later times becomes too low to accurately measure, and it is no longer practical to continue collecting urine or feces. Fortunately, complete recovery of radioactivity from this study is not a requirement as long as the other objectives of the study are met, such as an understanding of routes of elimination and metabolic pathways, as well as exposure to metabolites similar to preclinical toxicology species.

The human radiolabeled mass balance/AME study provides a great deal of information about human *in vivo* metabolic pathways and the identification of metabolites and their exposure. This study provides a definitive and quantitative profile of metabolites found in human plasma. These can be inactive metabolites, pharmacologically active metabolites, or toxic metabolites. The FDA in 2008 published a guidance entitled "Safety Testing of Drug Metabolites" that describes how and when to identify and characterize drug metabolites whose nonclinical toxicity needs to be evaluated [95]. Since its publication in 2008, many other publications by the pharmaceutical industry have described how to practically address the situation of human metabolites that may need to be evaluated for toxicity [96]. Throughout the literature, these are known as *MIST* publications (Metabolites in Safety Testing). Although human *in vitro* systems do a reasonably good job of identifying major metabolic pathways, they cannot recapitulate the phenomenon of absorption, drug or metabolite disposition, elimination

(plasma, urine, bile, feces), or the combined effects of hepatic and extrahepatic (e.g., gut or kidney) metabolism.

## 2.4 METABOLISM STUDIES IN DRUG DISCOVERY AND DEVELOPMENT

### 2.4.1 Predicting Human Hepatic Clearance

*In vitro* metabolism studies using human liver microsomes or human hepatocytes to predict human metabolic clearance from the liver is routinely performed as early as drug discovery screening and throughout drug development. The ability to predict the metabolic clearance is important because the clearance is related to the exposure and half-life of the compound, two components that are essential in predicting the dosing regimen [20,97]. One of the major objectives of the pharmaceutical industry is to develop low dose drugs that are taken once a day thereby improving patient compliance and efficacy. In order to achieve this goal, drugs are often tested in human *in vitro* systems that assess metabolic stability. Compounds that are very rapidly metabolized are indicative of drugs that will have a high metabolic clearance and are therefore undesirable because they will require twice daily or three times a day dosing to achieve efficacy. Compounds that demonstrate no turnover or metabolism *in vitro* may indicate that the drug may have a long half-life in patients. Compounds with extremely long half-lives may be a benefit for some indications; however, they are also a concern should undesirable side effects or toxicity be uncovered for which the effects are prolonged because of the longer exposure time. Therefore, understanding the metabolic stability of both unstable and stable drug candidates is important to our understanding of dosing regimen, efficacy, and safety.

Liver microsomes and hepatocytes are two *in vitro* systems commonly employed to assess metabolic stability by measuring the disappearance of substrate over time [29,98,99]. The simplest method of assessing metabolic clearance is by incubating the compound of interest at a low substrate concentration (typically $<1\,\mu M$) and measuring the disappearance of substrate. This "half-life" method uses the assumption that the substrate concentration is much lower than the $K_m$ of the metabolic reaction(s), hence the low substrate concentration. This half-life method is often utilized in drug discovery during lead optimization because it has the potential to be high throughput when paired with human liver microsomes and provides a quick estimation of human metabolic clearance. The *in vitro* intrinsic clearance is determined from the following equation:

$$CL'_{int} = \frac{0.693}{\textit{In vitro}\ \text{half-life} \times \text{microsomal protein concentration}}$$

For more sophisticated assessments of hepatic clearance, measurements of Michealis–Menten kinetic parameters ($K_m$ and $V_{max}$) are determined. From the rate of metabolism, the microsomal or hepatocyte intrinsic clearance (measure of enzyme activity) is determined. The *in vitro* intrinsic clearance ($CL'_{int}$) is then scaled to whole liver intrinsic clearance ($CL_{int}$) and incorporated into the following equation (well-stirred model of hepatic extraction) to estimate hepatic clearance ($CL_H$).

$$CL_H = \frac{Q \times f_u \times CL'_{int}}{Q + f_u \times CL'_{int}}$$

where $Q$ = liver blood flow, $f_u$ = fraction unbound in plasma, and intrinsic clearance is defined as $CL'_{int} = V_{max}/K_m$. See the chapter titled Enzyme Kinetics of Drug Metabolizing Reactions and Drug–Drug Interactions of this volume for additional information. This method is much more labor intensive and therefore not often used in drug discovery, however it does eliminate the assumption inherent in the half-life method and the need to use a low substrate concentration.

For drugs that undergo oxidative metabolism (e.g., CYP450 mediated) or glucuronidation, liver microsomes are a simple and effective *in vitro* system to predict hepatic clearance [98,100]. For compounds that undergo extensive phase II metabolism or metabolism via cytosolic enzymes, hepatocyte incubations are the more appropriate *in vitro* system. The same equations as illustrated above are also employed, however, the scaling factors will be different. In order to build confidence in the predicted human hepatic clearance determination from either microsomes or hepatocytes, *in vitro–in vivo* correlations of predicted and actual clearance values with several animal species should be performed. Assuming that the metabolism and clearance pathways are similar between the animal species and humans, this *in vitro–in vivo* correlation can support or refute the predictability of human *in vitro* hepatic clearance determinations.

### 2.4.2 Reaction Phenotyping

It is now widely accepted that the fraction of the dose metabolized ($f_m$) by a given drug-metabolizing enzyme is one of the major factors governing the magnitude of a drug interaction and the impact of a polymorphism on drug clearance. Therefore, pharmaceutical companies routinely determine the enzymes involved in the metabolism of potential new drugs *in vitro* and relate this information to human data on ADME [13,101,102]. This so-called reaction phenotyping or isozyme mapping, usually involves the use of multiple reagents, such as recombinant enzymes, liver microsomes, human hepatocytes, and clinical studies with polymorphic populations [103]. Through an understanding of the enzymes involved in the disposition of a new drug, investigators can predict potential drug interactions, such as those due to metabolism by polymorphic drug-metabolizing enzymes [104,105] (e.g., CYP2D6 or CYP2C9/19) or coadministration with potent inhibitors or inducers of CYP450 enzymes (e.g., a CYP3A4 substrate with ketoconazole or rifampin, respectively).

There are three *in vitro* techniques that can address reaction phenotyping and many researchers will utilize at least two of them if not all three [106]. The first technique utilizes recombinantly expressed single enzymes. In this situation, the drug of interest can be incubated with individual enzymes and turnover of parent or formation of metabolite(s) can be measured. Those enzymes that demonstrate turnover are considered to be involved in the metabolism of the compound. By scaling the amount of turnover with the amount of each particular enzyme in the human liver, the quantitative relative order (or ranking) of importance of each enzyme to the total metabolism of the compound can be calculated [6,13,101]. A second common approach utilizes human liver microsomes and selective chemical inhibitors or inhibitory antibodies for individual CYP450 enzymes. These chemical inhibitors or inhibitory antibodies selectively knockout or eliminate the activity of a single CYP450 enzyme and when compared to uninhibited incubations, the relative contribution of each CYP450 can be determined. For example, furafylline is a selective inhibitor of only CYP1A2 at a concentration of 20 μ and is routinely used to assess the contribution of CYP1A2 to the microsomal

**TABLE 2.6 Chemical Inhibitors and Recommended Concentrations for CYP Reaction Phenotyping in Human Liver Microsomes**

| CYP Form(s) | Inhibitor | Inhibitor Concentrations |
|---|---|---|
| CYP1A2 | Furafylline α-naphthoflavone | 10–30 μM[a] 1 μM |
| CYP2A6 | Methoxsalen | 1 μM |
| CYP2B6 | ThioTEPA | 50 μM |
| CYP2C8 | Montelukast | 0.1 μM |
| CYP2C9 | Sulfaphenazole | 10 μM |
| CYP2C19 | Benzylnirvanol | 1 μM |
| CYP2D6 CYP2D6 & CYP3A4/5 | Quinidine | <2 μM and 10 μM |
| CYP2E1 | Diethyldithiocarbamate | 50 μM |
| CYP3A4/5 & others | Ketoconazole | 1 μM and 10 μM |
| CYP3A4/5 | Troleandomycin | 50 μM[a] |

[a]Time-dependent inhibitors (inhibition potency increases with preincubation in the presence of NADPH).

metabolism during reaction phenotyping studies [13,101]. Table 2.6 lists the common inhibitors used for CYP450 reaction phenotyping studies in human liver microsomes, many of which can also be used for inhibition studies in human hepatocytes. The third *in vitro* technique is termed *correlation analysis* whereby the rate of turnover of the substrate is compared to the rate of turnover of an isoform-selective probe substrate. A good correlation indicates that the enzyme is involved in the metabolism. For example, correlating the rate of metabolism of a drug candidate with the CYP3A4-mediated metabolism of midazolam (formation of 1-hydroxymidazolam is specific to CYP3A4) with preparations of human liver microsomes from 8 to 10 different donors. If the rates of metabolism demonstrate a good correlation, then it is presumed that CYP3A4 is involved in the metabolism of the compound.

It is important that the data are consistent between the three techniques to develop an integrated *in vitro* reaction phenotype for the substrate. In addition, these data are integrated with the overall elimination (metabolism, renal, biliary) of the parent drug so that the impact of a drug interaction can be ascertained. For example, consider two compounds both metabolized only by CYP2D6; however, compound A also undergoes significant renal elimination as parent drug but compound B does not undergo significant renal or biliary elimination. In this situation, compound A will not show a significant drug interaction (increase in parent drug exposure) in poor CYP2D6 metabolizers because renal elimination will provide a secondary elimination pathway. Compound B, however, would be expected to show significant increases in plasma exposure in CYP2D6 poor metabolizers because the drug has no elimination pathway besides CYP2D6-mediated metabolism.

For the human cytochrome P450s, reagents for reaction phenotyping are readily available, such as recombinant enzymes, specific chemical inhibitors/antibodies, and probe substrates for correlation analysis. Therefore, CYP450 reaction phenotyping is routinely performed and required for regulatory submissions. Recombinant enzymes are also available for most of the remaining drug-metabolizing enzymes (UGTs (UDP-glucuronosyltransferase), STs, FMOs), however, specific inhibitors, scaling factors, or probe substrates are not as readily available for many of these enzymes, which

severely limits the amount of information that can be extrapolated to human metabolism or potential drug interactions. Phase II glucuronidation is the second most common metabolic reaction after the CYP450 oxidations and reaction phenotyping is gaining momentum with these enzymes as well [107]. In recent years, new selective substrates and inhibitors have been identified for many of the UGT isoforms [100,108].

### 2.4.3 Drug–Drug Interactions

***2.4.3.1 Enzyme Inhibition.*** Inhibition of drug-metabolizing enzymes will have a profound effect on any other drugs that are predominately eliminated by the same enzyme. In today's environment of poly pharmacy, many patients take more than one medication at a time and the risk of a drug–drug interaction also increases with the number of comedications. While CYP450 enzymes are the most significant drug-metabolizing enzyme family, inhibition of these enzymes has a major effect on drug concentration or exposure of coadministered drugs. Inhibitory drug interactions often cause exaggerated pharmacology or off-target toxicities because of increased plasma exposures and can result in serious side effects or death in extreme cases. Therefore, pharmaceutical companies and regulatory agencies are keen to reduce potential liabilities associated with inhibition of drug-metabolizing enzymes and eliminate such drugs being developed or marketed.

Two types of inhibition are routinely evaluated in drug discovery and development: reversible inhibition and irreversible inhibition (see Chapter 2 for kinetic details on each type of inhibition). Reversible inhibition is the easiest to determine and often included in high throughput screening in early drug discovery. Reversible inhibition can take the form of multiple mechanisms; however, the most common are competitive inhibition and noncompetitive inhibition (or a combination of the two referred to as *mixed inhibition*). Commonly, drugs that are substrates for P450 enzymes will also be inhibitors of the same enzyme (competitive inhibition), however, drugs can also inhibit CYP450 enzymes but not be substrates (noncompetitive inhibition). A common example is quinidine, which is a potent inhibitor of CYP2D6 but is not a substrate of CYP2D6 (quinidine is a substrate for CYP3A4).

Reversible CYP inhibition is routinely evaluated in early drug discovery by means of rCYP enzymes and fluorescent probes, which are both amenable to high throughput screening. Assays such as this are useful in assessing CYP inhibition potential ($IC_{50}$ determination) or rank ordering of compounds or chemotypes and for the purposes of conducting SAR to eliminate a CYP inhibition liability. In order to determine a more accurate drug–drug interaction potential, most labs will perform inhibition studies with human liver microsomes ($IC_{50}$ or $K_i$ determinations) [106,109]. There is a considerable database of *in vitro* inhibition data related to human liver microsomes and clinical outcomes of drug–drug interactions. These correlations have resulted in a general categorization of potential drug interactions by means of the ratio of inhibitor concentration (efficacious plasma concentration, [I]) to the $K_i$.

$[I]/K_i > 1$ implies that a drug interaction is likely,

$1.0 > [I]/K_i > 0.1$ implies that a drug interaction is possible, and

$[I]/K_i < 0.1$ implies that a drug interaction is unlikely.

Going beyond the CYP enzymes, newer approaches evaluating other enzyme systems, such as glucuronosyltransferases or drug transporters and the use of human hepatocytes to predict drug–drug interactions are showing considerable promise to predict interactions with multiple enzyme systems and integrated pathways more accurately [27,109–111].

The second type of drug interaction known as *mechanism based-* or *metabolism-based inhibition* (MBI) is a result of inactivation of a drug-metabolizing enzyme by a metabolite or metabolic intermediate that forms an adduct with the enzyme (irreversible inhibition) or forms a metabolite-intermediate complex (MI complex) with the enzyme, also known as *quasi-irreversible inhibition* [18]. A classic example of irreversible inhibition was published by Trager *et al*. [112] with CYP1A2 and furafylline while a more recent example of MI-complex formation was published by Takakusa *et al*. [113] using lapatinib and CYP3A4 (both manuscripts provide detailed experimental protocols to assess these types of inhibition). Certain functional groups on drug molecules or natural products have a propensity to undergo reactive metabolite formation and cause irreversible inhibition. These functional groups include, but are not limited to, acetylenes, alkenes, organosulfo-compounds, arlyamines, tertiary amines, cyclopropyl amines, hydrazines, furans, thiophenes, dihaloalkanes, and methylene dioxyphenyl containing compounds [114,115]. Whenever such structures are present in a molecule, rigorous testing should be conducted to evaluate the potential for reactive metabolite formation that can lead to irreversible inhibition or covalent binding to other macromolecules such as proteins and nucleic acids.

Drugs that cause erreversible inhibition distinguish themselves from ordinary reversible inhibition by several characteristics: (i) they may display a delayed onset of inhibition, (ii) their inhibition properties are typically greater than what would be expected from ordinary reversible inhibition parameters ($IC_{50}$ or $K_i$), and (iii) the inhibition effects can persist after the inhibitor has been eliminated because of the fact that enzyme has been destroyed during the inactivation process and new enzyme must be synthesized to regain nominal enzyme levels. The mechanistic differences between reversible and irreversible inhibition require additional experimental methods to appropriately characterize the mechanism and potential drug–drug interaction from irreversible inhibition. For example, a simple test for reversible and irreversible inhibition is the $IC_{50}$ shift method. Here, liver microsomes are preincubated (30 min) with the compound of interest at several concentrations with and without NADPH. Each sample is then diluted ($\sim 10\times$) into an incubation containing a probe substrate and NADPH to measure enzyme activity. A left-ward shift in the $IC_{50}$ plot indicates an irreversible inhibitor. Irreversible inhibition is often referred to as *time-dependent inhibition* because the degree of inhibition increases with time, whereas with reversible inhibitors, the potency of inhibition does not change with time. The reference to time-dependent inhibition is for the most part correct, however, time-dependent inhibition can also be caused by formation of a metabolite that is itself a reversible inhibitor (and would show a similar left-ward shift). In this situation, the inhibition is referred to *reversible inhibition* but does characterize itself as time dependent because of the increasing concentration of the inhibitory metabolite with time. A more characteristic experiment, known as the *preincubation-dilution* method is commonly employed to determine the kinetic parameters of the inactivation process ($K_I$ and $K_{inact}$) [106]. The $K_{inact}$ is the maximum inactivation rate constant and the $K_I$ is the inhibitor concentration that causes half maximal inactivation (or the dissociation

constant of the initial enzyme-inhibitor complex). Using these kinetic parameters of the inactivation process, a reasonable prediction of the magnitude of the drug interaction can be determined. A review of industry practices and methods to evaluate and interpret irreversible inhibition was recently published [116].

***2.4.3.2 Enzyme Induction.*** The induction of drug-metabolizing enzymes or drug transporters can have significant consequences on the exposure and toxicity of drugs, that is, the efficacy of a drug is reduced if its clearance is increased by enzyme induction. For example, rifampin can decrease the AUC of coadministered drugs by as much as 70–90%, as in the case of (*R*)-verapamil coadministration with rifampin [117]. Another example of a clinically relevant induction drug–drug interaction is patients receiving cyclosporine (organ rejection), who are also treated with rifampin (tuberculosis) [118]. In contrast to inhibition where the drug immediately interacts directly with the enzyme and leads to toxic increases in drug exposure, induction is an indirect effect on the enzyme, generally taking days to result in a clinically relevant effect on drug elimination, which may persist for several days beyond cessation of the inducing agent. Induction of CYPs can also lead to toxicity by increasing reactive metabolite production or by increasing the CYP1A2-mediated activation of procarcinogens [119]. Recognizing the safety and health issues associated with exposure to inducing agents, the US Food and Drug Administration (FDA) and other regulatory agencies have placed more emphasis on determining whether a drug candidate has the potential to induce CYP or UGT enzymes, or drug transporters. In 2006, the FDA published a draft guidance on drug-interaction studies that presented details on the principles and conduct of *in vitro* induction studies. The Pharmaceutical Research and Manufacturer's Association (PhRMA) subsequently published a White Paper that addressed many of these issues and acknowledged the importance of appropriate study design [32].

The most common mechanism of CYP induction is transcriptional gene activation. For drug-metabolizing enzymes, transcriptional activation is mediated by transcription factors such as AhR (aromatic hydrocarbon receptor), CAR (constitutive androstane receptor), and PXR (pregnane X receptor or SXR-steroid X receptor) [120–125]. The general concept for nuclear receptor signaling, as exemplified by PXR, is that in the absence of a ligand (drug), the nuclear receptor is associated with corepressor complexes conferring a basal level of transcription. Drug binding to the ligand-binding domain (LBD) of the nuclear receptors induces conformational changes that lead to the release of corepressors and recruitment of coactivators. Subsequent dimerization with partner molecules (retinoid X receptor, RXR, for CAR/PXR and the AhR nuclear translocator, ARNT, for AhR) lead to chromatin remodeling and subsequent transcriptional activation. Regulation of target gene transcription is achieved through binding of the nuclear receptor DNA-binding domain (DBD) to respective DNA response elements present in the promoter region of target genes (drug-metabolizing enzymes) [125,126]. It should be noted that the LBDs of many nuclear receptors are different between various animal species and human [125]. Therefore, *in vitro* or *in vivo* animal models (e.g., rat and dog) of enzyme induction can be misleading and are generally not employed to assess the potential induction effect in humans.

The mechanisms of CAR- and AhR-mediated gene transcription differs somewhat from that of PXR, which is exclusively an agonist activated process. Both CAR and AhR can be activated by direct ligand binding as described above; however, they can also be activated by ligand binding independent mechanisms. Translocation of CAR

to the nucleus can be initiated by direct agonist binding to the receptor or through a partially elucidated ligand-independent mechanism involving kinases that dephosphorylate CAR. Phenobarbital is an example of a drug that does not bind to CAR yet causes nuclear translocation and transcriptional activation of the target gene, CYP2B6 [123]. Similarly, the inactive AhR resides in the cytoplasm and can be activated on ligand binding or via protein tyrosine kinases [127].

PXR-derived nuclear hormone receptor models (ligand binding and cell-based PXR transactivation assays) are the most common high throughput assays to evaluate enzyme induction due to the simplicity of ligand-based activation and the importance of PXR target genes, such as CYP3A4, CYP2B6, and CYP2Cs, in drug interactions. Although cell-based transcription activation assays exist for CAR (CYP2B6) and AhR (CYP1A); these are used less frequently and most often to address mechanistic questions.

Cultured primary human hepatocytes are the most widely accepted model for assessing the potential of drug candidates to induce human CYP expression [7]. The hepatocyte model is able to better predict the outcome of enzyme induction and drug interactions because of the ability to simultaneously capture multiple nuclear hormone receptor-mediated pathways rather than a single pathway as with the transcriptional activation models. Primary human hepatocyte culture systems have been shown to effectively model human *in vivo* induction responses and are recognized by regulatory agencies as an effective tool for assessing induction potential [128,129]. The enzyme induction data from *in vitro* methods are known to correlate well with clinical observations, provided the *in vitro* experiments are performed at pharmacologically relevant concentrations of drug [130]. In addition to CYP enzymes, numerous studies have been reported using primary hepatocyte culture systems to assess induction of a variety of gene targets from phase II enzymes and drug transporters [131–134]. The pharmaceutical industry and regulatory agencies recommend the analysis of RNA expression and catalytic activity of the major drug-metabolizing enzymes CYP1A2, CYP2B6, and CYP3A4 in freshly isolated or attachable cryopreserved hepatocyte cultures. Results should be obtained from at least three individual donors, treated with the test compound, vehicle, and positive controls for two to three days. A minimum of three test compound concentrations, based on the expected human plasma drug levels are suggested, one of which should be an order of magnitude greater than this concentration. In the absence of knowledge of human plasma levels, concentrations ranging over at least two orders of magnitude should be studied.

Immortalized hepatocytes have been shown to posses many of the characteristics of primary hepatocytes. The advantages of using immortalized cell lines primary hepatocytes for enzyme induction studies include (i) easy access and availability; (ii) the ability to propagate (i.e., continual supply); and (iii) more consistent induction response compared to the variability seen with multiple donors of human hepatocytes. Immortalized cell lines cited throughout the literature include HepG2, HepaRG, Fa2N-4, and BC2 [135,136]. HepG2 and HepaRG cells have been shown to respond, to varying degrees, to CYP1A1/2 and 3A4 inducers [137–139], Fa2N-4 cells respond to rifampicin and β-naphthoflavone in a similar manner as observed for primary human hepatocytes [135,136], and BC2 cells have been reported to respond to CYP1A inducers [140]. While these cell lines can be used for preliminary studies, they do not maintain all of the phenotypic characteristics of human hepatocytes, such as enzyme or receptor function or expression, and their use may result in erroneous conclusions.

Interpretation of *in vitro* induction results can be as simple as compared to known CYP3A4 inducers (e.g., rifampin) or rank ordering of potency ($EC_{50}$) both of which provide useful information. Accurate predictions of drug interactions incorporate therapeutic drug concentrations, such as therapeutic drug concentrations ($C_{max}$ or $C_{ss}$) along with induction data. Combining values, such as $EC_{50}$, $E_{max}$, and $C_{max}$ ($E_{max}$ mathematical model) have been used to assess induction effects for multiple induction models (transactivation, immortalized hepatocytes, and primary hepatocytes) [136,141,142]. More advanced mathematical models include such parameters as fraction unbound in hepatocyte culture, fraction metabolized, and a scaling factor ($d$), which allows better correlations between *in vitro* and *in vivo* outcomes of induction [32]. Another method of data interpretation is more empirical and involves correlations between the induction response found *in vitro* and the response *in vivo*. The most utilized form of correlation analysis utilizes the relative induction score (RIS), which has shown utility with both immortalized hepatocytes and primary hepatocytes [32,136].

## 2.5 FUTURE DIRECTIONS

The science of drug metabolism is continuing to move forward at an extremely fast pace, which is both a blessing and a curse. Without a doubt, the discipline is constantly growing with new information, methodologies, and instrumentation appearing as a continual stream in manuscript and presentation formats. At times, it can be challenging to keep abreast of the plethora of information reported in manuscripts across the field. Although this is a challenging time, it is also an exciting time to contribute to the wealth of information being generated today and to contribute to the future of drug metabolism.

One of the greatest challenges in drug metabolism is the predictive nature of our *in vitro* and animal models to human pharmacokinetics, metabolism, and toxicity. Most models appear to be qualitative or semiquantitative in their ability to accurately predict human outcomes. Moving down this "predictivity scale" to quantitative predictions with *in vitro* and animal models is the future direction of drug metabolism. This goal can be achieved through improvements of our existing models (e.g., hepatocytes), development of new models, and a better understanding of data analysis and data interpretation of both current and new models. Several of the new models that show great promise include the development of genetically modified animals [78,83] and stem cell derived hepatocytes [143,144]. Genetically modified animals provide an opportunity to evaluate human enzymes, receptors, transporters, or combinations of these, sometimes integrated events, in an *in vivo* setting. This should provide a significant improvement on evaluating events precipitated from these biological mediators through individual *in vitro* systems. Stem cell derived hepatocytes would significantly improve our ability to employ human hepatocytes across a number of existing *in vitro* methods, such as metabolite generation, predictions of hepatic clearance, and toxicity. Stem cell derived hepatocytes would provide a renewable and consistent supply of cells that are more stable in culture (expression and function of enzymes) and survive longer in culture so as to be useful in better predicting hepatic clearance or metabolite formation of slowly metabolized compounds, as well as toxic events that take longer to manifest in culture. Once these two advances become more established, the field will once again take a

large step forward, much like it did with the advent of molecular biology techniques in the 1980s and 1990s.

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