1

DRUG METABOLISM: SIGNIFICANCE AND CHALLENGES

CHANDRA PRAKASH AND ALFIN D. N. VAZ

Department of Pharmacokinetics, Dynamics and Metabolism, Pfizer Global Research and Development, Pfizer Inc., Groton, CT, USA

1.1 INTRODUCTION

Searching for new drugs is a very time-consuming and expensive endeavor, taking approximately 10–12 years and on the order of $900 million to bring a new drug to market [1, 2]. It has been estimated that for every 5000 new chemical entities (NCEs) evaluated in a discovery program, only 1 is approved for market [3]. Even after a drug is marketed, there is the possibility of some undesired side effects, which were not seen in earlier clinical trials. In these cases, the drug is either withdrawn from the market or acquires a warning label (black box). Therefore, efforts are being made to reduce attrition of drug candidates during the various stages of their development to bring safer compounds to market. The major reasons for the failure of the NCEs are lack of in vivo efficacy, serious undesired side effects, and unfavorable drug metabolism and pharmacokinetics (DMPK). Therefore, in addition to potency and selectivity, drug candidates are selected on the basis of DMPK properties, such as desired clearance, oral bioavailability, low potential of drug–drug interactions, and acceptable metabolism/toxicology profiles in preclinical species [4, 5]. In support of this need, and as a consequence of increased knowledge within the drug metabolism discipline, new approaches have been developed that include extensive in vitro methods using human and animal hepatic cellular and subcellular systems, recombinant human drug-metabolizing enzymes, transgenic animals and cell lines stably expressing human transporters, increased automation for higher throughput screens, sensitive analytical
DRUG METABOLISM: SIGNIFICANCE AND CHALLENGES

Efficacy 27%
Clinical safety 13%
PK bioavailability 40%
Preclinical safety 20%
Commercial 20%
Others 10%

Efficacy 30%
Clinical safety 10%
PK 10%
Preclinical safety 20%
Commercial 20%
Others 6%

(a)
(b)

FIGURE 1.1 Reasons for attrition of NCEs in drug development: (a) 1990 and (b) 2000. (Modified from Ref. 6)

technologies, and in silico computational models to assess drug metabolism aspects of the NCEs. Recent data suggest that these approaches have reduced the attrition due to DMPK issues from 40% of total attrition in 1990 to 10% in 2000 (Figure 1.1) [6].

The predictive power of in vitro studies using animal and human hepatocellular and subcellular fractions and/or recombinant enzymes [7–9], in silico models [10–12], and in vivo studies using a range of experimental animal models [13, 14] has advanced considerably due to an ever increasing understanding of the relationships between in vitro and in vivo drug metabolism and disposition. These in vitro studies include

1. absorption/transport studies in Caco-2 cells or cell lines over expressing various transporters;
2. metabolic stability and metabolite formation in liver microsomes, S-9, hepatocytes or recombinant cytochrome P450 enzymes;
3. cytochrome P450 inhibition and induction;
4. plasma protein binding;
5. reactive metabolite (glutathione adduct formation).

The in vivo studies include

1. pharmacokinetic studies in laboratory animals via various routes of administration (oral, intravenous, subcutaneous, etc.);
2. tissue distribution (e.g., brain penetration);
3. metabolite identification and clearance pathways in animals and humans using radiotracers;
4. PK/PD relationship;
5. specific studies using genetically engineered mouse models.
Among ADME (absorption, distribution, metabolism, and excretion) properties, metabolism of an NCE by the host system can be one of the most important determinants of its pharmacokinetic disposition. It is the biochemical process by which compounds are converted to more hydrophilic (water-soluble) entities, which not only enhance their elimination from the body but also lead to compounds that are generally pharmacologically inactive and relatively nontoxic. However, metabolic transformation of an NCE at times can lead to the formation of metabolites with pharmacological activity [15] and/or toxicity [16–18]. Additionally, metabolism can be the main cause of poor bioavailability and drug–drug interactions via inhibition or induction of drug-metabolizing enzymes [19, 20]. Therefore, determination of metabolic rate and biotransformation pathways of an NCE, in animals and humans, and evaluation of pharmacological and toxicological consequences of its metabolites are very critical to pharmaceutical development [21]. At the lead optimization stage, information on the metabolic fate of the NCEs can direct medicinal chemists to synthesize metabolically more stable analogs by blocking sites of metabolism, and potentially creating NCEs with superior pharmacology and safety profiles. Knowledge of the major human metabolites of an NCE early in its development is useful to enable the judicious selection of animal species used for safety evaluation, to ensure that the selected animal species are exposed to all major metabolites formed in humans [22, 23]. Subsequently, major circulatory metabolites in humans can be synthesized for the evaluation of their pharmacological activity.

The metabolism of drugs has traditionally been classified into two reaction classes, phase I and phase II. Phase I reactions include hydroxylation, dealkylation, deamination, N- or S-oxidation, reduction, and hydrolysis. These reactions introduce or unmask a functional group (e.g., –OH, –COOH, –NH₂, or –SH) within a molecule to enhance its hydrophilicity. Phase II or conjugation biotransformations include glucuronidation, sulfation, methylation, acetylation, and amino acid (glycine, glutamic acid, and taurine) and glutathione (GSH) conjugation. The cofactors of these reactions react with functional groups that are either present on the NCE or are introduced during phase I biotransformation. Most phase II biotransformation reactions result in a large increase in drug hydrophilicity, thus greatly promoting the excretion of foreign chemicals via urine and/or bile, and, with few exceptions, are generally pharmacologically inactive. While phase I and phase II reactions are thought of as acting sequentially in the biotransformation of drugs, NCEs, and xenobiotics, these reactions occur independently, and often phase II enzymes become the primary metabolic route.

1.2 PHASE I DRUG-METABOLIZING ENZYMES

The phase I reactions are mediated primarily by liver enzymes such as cytochrome P450 (CYP450), FAD-containing mono-oxygenase (FMO), monoamine oxidase (MAO), molybdenum hydroxylase (aldehyde oxidase/xanthine oxidase; AO/XO), aldo-ketoreductase (AKR), epoxide hydrolase (EH), and esterase.
1.2.1 Cytochrome P450

CYP450 is a superfamily of hemoproteins, responsible for the oxidative metabolism as well as metabolic activation of the vast majority of xenobiotics (drugs, dietary components, and pollutants) and endogenous substrates (e.g., steroids, cholesterol, and bile acids). The CYP450 system possesses three known types of activities. CYP450 enzymes, acting as mono-oxygenases, activate molecular oxygen with electrons from NADPH via NADPH-CYP450 reductase, and insert one atom of molecular oxygen into the substrate while reducing the other atom of oxygen to water (equation 1.1). As a result, the xenobiotics can undergo hydroxylations, epoxidations, N-, S-, or O-dealkylations, deaminations, N- or S-oxidations, and oxidative dehalogenations:

\[
\text{RH} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NAD(P)}^+ \quad (1.1)
\]

The second activity commonly referred to as the oxidase activity of CYP450 involves electron transfer from reduced CYP450 to molecular oxygen with the formation of superoxide anion radical and \text{H}_2\text{O}_2 (equations 1.2a and 1.2b):

\[
\text{NADPH} + \text{O}_2 \rightarrow \text{O}_2^- + \text{NAD(P)}^+ \quad (1.2a)
\]
\[
2\text{NADPH} + 2\text{H}^+ + \text{O}_2 \rightarrow \text{H}_2\text{O}_2 + \text{NAD(P)}^+ \quad (1.2b)
\]

The third activity of P450 system, known as reductase activity, involves direct electron transfer to reducible substrates such as quinones and proceeds readily under anaerobic conditions.

CYP450 superfamily members are grouped into subfamilies on the basis of amino acid sequence homology. The drug-metabolizing CYP450 enzymes are confined to subfamilies 1, 2, 3, and 4. These subfamilies are further divided into isoforms. There are approximately 57 human CYP450 isoforms exhibiting major differences with respect to their catalytic specificity and patterns of tissue expression [24, 25]. Only a small group of these isoforms is involved in xenobiotic transformations. In the human liver, there are at least 18 distinct CYP450 isozymes while only 10 isoforms from families 1, 2, and 3 (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9,

![Diagram](https://example.com/diagram.png)

**FIGURE 1.2** Human cytochrome P450 genes in the drug-metabolizing CYP1, CYP2, and CYP3 families (Modified from Ref. 24).
CYP2C19, CYP2D6, CYP2E1, CYP2F1, and CYP3A4) are responsible for the hepatic metabolism of most of the marketed drugs (Figure 1.2) [24].

**CYP1A subfamily.** CYP1A subfamily consists of two members CYP1A1 and 1A2. CYP1A1 is present predominantly in extrahepatic tissue such as lung, small intestine, placenta and kidney, and at a very low level in the liver. On the other hand, CYP1A2 is mainly confined to the liver and expressed at a very low level in extrahepatic tissue. Both CYP1A1 and 1A2 play an important role in the metabolic activation of polycyclic aromatic hydrocarbons (PAHs), aromatic amines, and heterocyclic amines [26]. Despite the high similarity in their primary structures, CYP1A1 and 1A2 have different substrate specificities. For example, dibenzo[a]pyrene, a potent carcinogen, is oxidized almost exclusively by CYP1A1 in humans to highly mutagenic diol-epoxides [27] while acetanilide is metabolized primarily by CYP1A2. In human liver, CYP1A2 accounts for ~15% of the total CYP content and is involved in the metabolism of ~4% of marketed drug including acetaminophen, phenacetin, tacrine, ropinirole, riluzole, theophylline, and caffeine [25, 28].

**CYP1B subfamily.** CYP1B1 is expressed at a low level in heart, brain, placenta, lung, liver, and kidney [29], but it is present at much higher levels in tumor cells compared with the surrounding normal tissue [30]. CYP1B1 catalyzes 2- and 4-hydroxylation of 17β-estradiol [31]. It also participates in the metabolic activation of a number of procarcinogens, including PAHs, PAH-dihydrodiols, and aromatic amines [32, 33]. CYP1B1 is also involved in the metabolism of some clinically relevant anticancer agents used in the treatment of hormone-mediated cancer.

**CYP2A subfamily.** CYP2A subfamily includes CYP2A6, 2A7, and 2A13 in humans (Figure 1.2). CYP2A6 is expressed in the liver and accounts for ~4% of total hepatic CYP450, whereas 2A7 and 2A13 appear to be expressed at even lower levels. CYP2A6 is the principal and perhaps the sole catalyst for human liver microsomal coumarin 7-hydroxylation. CYP2A6 is involved in the oxidation of only 3% of drugs such as nicotine, cyclophosphamide, ifosfamide, and fadrozole [25, 34, 35]. In addition, CYP2A6 also plays an important role in the activation of several procarcinogens and promutagens especially the nitrosamines [36]. CYP2A6 might also be involved in the metabolic activation of 3-methylindole in human pulmonary microsomes [37]. CYP2A13 is predominantly expressed in the human respiratory tract and significantly involved in the activation of aflatoxin B1 [38].

**CYP2B subfamily.** CYP2B subfamily includes CYP2B6 and 2B7 isoforms. CYP2B6 is expressed in the liver and in some extrahepatic tissues, whereas CYP2B7 mRNA expression was detected in lung tissue. CYP2B6 accounts for ~1% of total hepatic CYP content. CYP2B6 is involved in the metabolism of only 4% of the marketed drugs, such as the anticancer drugs cyclophosphamide and tamoxifen [39], the anesthetics ketamine and propofol [40], and procarcinogens, such as the environmental contaminants aflatoxin B1 and
dibenzanthracene [41]. Significant interindividual differences from 25- to 250-fold have been reported in hepatic CYP2B6 expression [42]. Recent studies have reported that females express significantly higher amounts of CYP2B6 than males [43].

**CYP2C subfamily.** To date, four members of the human CYP2C subfamily (CYP2C8, 2C9, 2C18, and 2C19) have been identified and all together account for ~25% of the total CYP in human liver [25]. CYP2C8 and 2C9 are the major isoforms, accounting for 35% and 60%, respectively, of total human CYP2C, whereas CYP2C18 (4%) and CYP2C19 (1%) are the minor expressed CYP2C isoforms [44]. In addition to liver, CYP2C9 mRNA is also detected in the kidney, testes, adrenal gland, prostate, ovary, and duodenum, and 2C19 is detected in duodenum. CYP2C subfamily is involved in the metabolism of ~25% of drugs on the market. CYP2C8 is involved in the metabolism of retinol and retinoic acid, arachidonic acid, benzo[a]pyrene, and anticancer drug paclitaxel. CYP2C9 plays a critical role in the metabolism of a number of clinically significant drugs, including tolbutamide, phenytoin, S-warfarin, ibuprofen, diclofenac, piroxicam, tenoxicam, mafenamic acid, losartan, glipizide, and torasemide. CYP2C19 metabolizes (S)-mephenytoin, omeprazole, imipramine diazepam, some barbiturates, and proguanil.

**CYP2D subfamily.** In humans, only one isoform, CYP2D6, is expressed in various tissues including the liver, kidney, placenta, brain, breast, lungs, and small intestine. It is expressed at a low level in human liver, accounting for only ~3% of total CYP protein. In the small intestine, CYP2D6 is expressed in the duodenum and jejunum and not in the ileum and colon. CYP2D6 is responsible for the metabolism of numerous therapeutically used drugs [25, 45]. Approximately 7–10% of the Caucasian population shows an inherited deficiency in this enzyme due to the presence of one or several mutant alleles at the CYP2D6 gene locus [46]. These subjects are characterized by the poor metabolizer (PM) phenotype. Compared with normal or extensive metabolizers (EMs), PM subjects demonstrate markedly greater AUC values for parent drugs that are metabolized by CYP2D6, and therefore require lower doses to achieve therapeutic effects [47].

**CYP2E subfamily.** CYP2E1 is the only gene of this subfamily. It is expressed in many tissues, such as the nose, lung, and the liver. CYP2E1 accounts for ~7% of total CYP in the human liver and is involved in the metabolism of only 3% of the drugs, such as acetaminophen, caffeine, and chlorzoxazone, the latter being considered a marker of CYP2E1 activity [48]. CYP2E1 has a ubiquitous role in the metabolism and activation of an array of solvent carcinogens (which also induce its expression), such as N-nitrosamines, benzene, styrene, carbon tetrachloride, acrylonitrile, and ethylene glycol. CYP2E1 is the most active CYP enzyme in forming reactive oxygen intermediates, such as superoxide radical, causing tissue injury [49].

**CYP2F subfamily.** Only one functional gene has been found in the human CYP2F subfamily. The expression of CYP2F1 is highly tissue selective with highest
expression observed in the lung and little or no hepatic expression [50]. Substrates for CYP2F1 include ethoxycoumarin, propoxycoumarin, and pentoxyresorufin, but not ethoxyresorufin. Recombinant CYP2F1 is capable of activating two prototypical pneumotoxicant, 3-methylindole and naphthalene. CYP2F1 metabolizes naphthalene to its highly toxic intermediate, naphthalene-1,2-epoxide, and 3-methyl indole to its dehydrogenated pneumotoxic metabolite 3-methyleneindolenine [51, 52].

**CYP2J subfamily.** CYP2J2 is the only gene of this subfamily. It is known to be expressed in many extrahepatic tissues and may play a role in the oxidative bioactivation of arachidonic acid to form epoxyeicosatrienoic acids, which modulate bronchial smooth muscle tone and airway transepithelial ion transport [53]. CYP2J2 is also active toward other compounds such as linoleic acid and testosterone. Recently, it has been reported that CYP2J2 is involved in the intestinal first-pass metabolism of an antihistamine drug, astemizole [54].

**CYP2S subfamily.** In humans, CYP2S1 appears to be the sole member of a new subfamily, CYP2S. The CYP2S1 gene is located at the proximal end on chromosome 19q13.2 CYP2 gene cluster [55]. Of interest, CYP2S1 is closely related to CYP2F1 (47–49% identity) and is induced by dioxin in a human lung epithelial cell line, suggesting the possibility that CYP2S1 may participate in the metabolism of toxic and carcinogenic compounds [39]. Recent studies using heterologously expressed CYP2S1 in yeast have shown that CYP2S1 is able to metabolize naphthalene [56]. Therefore, it is speculated that CYP2S1 might also play a role in naphthalene-induced lung toxicity [57].

**CYP3A subfamily.** The CYP3A subfamily of CYP450 in humans is composed of several enzymes and accounts for ~28% of total hepatic P450 content. The human CYP3A family is clinically very important because it has been shown to catalyze the metabolism of an amazingly large number of structurally diverse xenobiotics and endobiotics. It is estimated that CYP3A forms participate in the metabolism of 35–50% of all marketed drugs [25, 58]. The human CYP3A subfamily includes CYP3A4, CYP3A5, CYP3A7 [59], and CYP3A43 [60]. CYP3A4 is the major human liver CYP3A enzyme, whereas CYP3A5 is present in only ~20% of human liver. CYP3A4 and CYP3A5 are also expressed in the stomach, lungs, small intestine, and renal tissue. Most of the CYP3A4 substrates are also metabolized by CYP3A5 [61]. CYP3A7 and CYP3A43 isozymes seem to play only a minor role in the metabolism of drugs. In fact, CYP3A7 is only present in fetal liver, whereas CYP3A43, which is expressed in liver, appears to be very restricted, both in terms of its activity and expression [62]. The highest concentration of transcript expression of CYP3A43 is in the prostate, whereas hepatic mRNA concentration is only 0.2–5% that of CYP3A4 [62]. Some examples of drugs metabolized by CYP3A are terfenadine, the benzodiazepines midazolam and triazolam, quinidine, lidocaine, carbamazepine, nifedipine, tacrolimus, dapsone, erythromycin, and dextromethorphan [63]. In addition to drugs, CYP3A is involved in the oxidation of a variety of endogenous substrates, such as steroids, bile acids, and retinoic acid [64].
1.2.2 Flavin Mono-Oxygenase (FMO)

FMOs are NADPH-dependent and oxygen-dependent microsomal flavoenzymes, which oxygenate a number of drugs and xenobiotics that contain a “soft-nucleophile” heteroatom such as nitrogen, sulfur, and phosphorus. Unlike CYPs, which generally use sequential one-electron transfer, the FMOs are two-electron oxygenating enzymes for N-oxidation. The microsomal FMO enzyme family is comprised of five isozymes (designated FMO1 to FMO5), whose expression is tissue specific. FMO1 is predominantly expressed in human kidney and FMO2 in lung and kidney [65]. FMO3 is the prominent isozyme in adult human liver, FMO4 is more broadly distributed in liver, kidney, small intestine and lung, and FMO5 is expressed in human liver, lung, small intestine, and kidney [66]. Of all the FMOs isozymes, FMO3 has a wide substrate specificity, including the physiologically and plant-derived tertiary amine, trimethylamine, tyramine, and nicotine; commonly used drugs including cimeti- dine, ranitidine, clozapine [65, 66], methimazole, itopride, ketoconazole, tamoxifen, and sulindac sulfide; and agrichemicals, such as organophosphates and carbamates [65, 66].

1.2.3 Monoamine Oxidase

MAOs are mitochondrial flavoproteins containing one covalently bound FAD cofactor. Two isozymes, termed as MAO-A and MAO-B, are known for the MAO enzyme family. They catalyze the oxidative deamination of structurally diverse amines including neurotransmitters dopamine, norepinephrine, serotonin, tyramine, and 2-phenylethylamine, and some drugs and xenobiotics that contain cyclic and acyclic alkylamine functional groups [67, 68]. The MAO reaction cycle involves two half reactions, as shown in equations 1.3a and 1.3c:

\[
\begin{align*}
RCH_2NH_2 + FAD & \rightarrow RCH = NH_2 + FADH_2 \quad (1.3a) \\
RCH = NH_2 + H_2O & \rightarrow RCH = O + NH_3 \quad (1.3b) \\
FADH_2 + O_2 + 2H^+ & \rightarrow FAD + H_2O_2 \quad (1.3c)
\end{align*}
\]

A two-electron oxidation results in the imine and reduced protein-bound FAD (equation 1.3a). The imine is then nonenzymatically hydrolyzed to the carbonyl compound (equation 1.3b). In the second half reaction, the reduced FAD (FADH$_2$) is reoxidized by molecular oxygen producing hydrogen peroxide (equation 1.3c).

MAOs are expressed in most mammalian tissues that complicate pharmacokinetic predictions when MAOs are involved in metabolism. Inhibitors of MAOs are used in psychiatry for the treatment of depressive disorders and in neurology for the treatment of Parkinson’s disease. MAO-A and MAO-B play a critical role in the bioactivation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to a toxic metabolite that induces Parkinson-like effects [68].
1.2.4 Molybdenum Hydroxylases

Molybdenum hydroxylases (i.e., AO and XO) are flavoproteins that contain in addition to a FAD, a pterine cofactor coordinated to a molybdenum atom, and an iron sulfur center for their catalytic activity. They catalyze the two-electron oxidation of substrates with transfer to molecular oxygen to produce H$_2$O$_2$, and insert an atom of oxygen from water into a wide range of $N$-heterocycles and aldehydes via two-electron redox reaction as shown in equation 1.4:

$$\text{RH} + \text{H}_2\text{O} \rightarrow \text{ROH} + 2\text{e}^- + 2\text{H}^+$$

AO and XO are cytosolic enzymes and are closely related. However, they differ in their substrate/inhibitor specificities. AO is involved in the metabolism of several clinically significant drugs such as famciclovir, zaleplon, zonisamide, and ziprasidone [69–72]. XO has a narrower substrate specificity than AO and is mainly active toward purines and pyrimidines. XO plays a role in the oxidation of several chemotherapeutic agents and has been implicated in the bioactivation of mitomycin B [73].

1.2.5 Epoxide Hydrolase

EH converts potentially reactive epoxides to $trans$-dihydrodiols. Originally, it was thought to be localized solely in the endoplasmic reticulum; subsequent studies demonstrated there are distinct microsomal (EH1) and cytosolic (EH2) forms of the enzyme. The EH1 gene is polymorphic and the expressed enzyme has two metabolic functions, detoxification and bioactivation, depending on the particular substrate. EH1 hydrolyzes epoxides, derived from aromatics and alkenes by CYP450 enzymes, to the corresponding dihydrodiols through the $trans$ addition of water. Dihydrodiols from PAHs can be substrates for further transformation by CYP450 enzymes to dihydrodiol epoxides such as $(+)$-anti–benzo[a]pyrene (B[a]P)-7,8-diol-9,10 epoxide, the most mutagenic and carcinogenic metabolite of B[a]P [74].

1.2.6 Esterase/Amidase

These enzymes are widely distributed in various tissue types and belong to the neutral, acidic, or metalloproteinase classes. While their roles have been recognized when drug structures contain ester or amide functions, very little characterization of the specific enzymes involved have been reported. Commonly, the ester function is introduced into drug structures as a means of masking carboxylate functions to increase absorption by increasing lipophilicity as with the antiviral drugs such as valganciclovir and oseltamivir that are converted $in$ $vivo$ to ganciclovir and oseltamivir carboxylate by intestinal and hepatic esterases [75, 76]. Amide functions are commonly used in linking drug substructures. These bonds are generally not susceptible to amidase activity since typically they are not natural amino acid derived. However, when such functions do present themselves in the NCEs, the potential for hydrolysis as a mechanism for clearance should be considered and stability in whole blood and hepatic subcellular matrices should be determined.
1.3 PHASE II CONJUGATIVE ENZYMES

Phase II reactions are catalyzed by conjugative enzymes, such as UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), glutathione S-transferase (GST), N-acetyltransferase (NAT) and methyltransferase (N-methyl-, thiomethyl-, and thiopurinemethyl-). Glutathione conjugates are further metabolized to cysteine and N-acetyl cysteine adducts. Most phase II reactions result in a compound’s concomitant increase in hydrophilicity and decrease in volume of distribution (VDss), which together greatly facilitate its excretion from the body.

1.3.1 Uridine Diphosphate Glucuronosyltransferase (UGT)

UGT family of enzymes catalyzes the transfer of glucuronic acid from UDP glucuronic acid to available substrates to form the water-soluble glucuronide conjugates, suitable for excretion. Thus, glucuronidation is a major detoxification pathway of endo- and xenobiotics in man and other animals. Beside human liver, the gut and kidneys are two important sites of glucuronidation. The UGT family of enzymes is subdivided into two subfamilies, UGT1 (1A1, 1A3, 1A4, 1A5 1A6, 1A7, 1A8, 1A9, and 1A10) and UGT2 (2A1, 2B4, 2B7, 2B10, 2B11, 2B15, 2B17, and 2B28), on the basis of sequence homology [77]. All classes of drugs containing a wide range of acceptor groups including phenols, alcohols, aliphatic and aromatic amines, thiols, acidic carbon atoms, and carboxylic acids are substrates for UGTs and this pathway has been estimated to account for ∼35% of all drugs metabolized by phase II drug-metabolizing enzymes [78]. Human UGTs can also form N-linked glucuronides of several tertiary amine drugs [79]. Some acyl glucuronides are reactive intermediates that bind covalently to macromolecules causing potential toxicity. Several drugs that contain a carboxylic acid such as diclofenac, ketoprofen, suprofen, and tolmetin are glucuronidated to form a reactive acyl glucuronide that could be associated with some of the observed toxicity of these drugs [80].

1.3.2 Sulfotransferase

SULT family of enzymes catalyzes the transfer of sulfite (SO$_3^-$) from 3’-phosphoadenosine 5’-phosphosulfate (PAPS) to a hydroxyl or an amino group on an acceptor molecule to form the water-soluble sulfonate or sulfamate conjugates and thereby aid in their excretion via the kidneys or bile. SULTs are capable of sulfonating hydroxyl group of a wide range of substrates including phenols, primary and secondary alcohols, N-hydroxy arylamines, and N-hydroxy heterocyclic amines. Amino groups of arylamines such as 2-naphthylamine are also sulfonated by SULTs. SULTs have a wide tissue distribution and play an important role in the detoxification, metabolism, and bioactivation of numerous xenobiotics, many dietary and environmental mutagens, drugs, neurotransmitters, and hormones [81]. In humans, three SULT families, SULT1, SULT2, and SULT4, have been identified that contain at least 13 distinct members. SULT1 and SULT2 families are the largest and are responsible for sulfonating the greatest number of endogenous and xenobiotic compounds.
Studies using recombinant enzymes demonstrated that many promutagens are activated with high selectivity by an individual SULT form.

1.3.3 Glutathione S-Transferase

The GST family of enzymes catalyzes the nucleophilic attack of the tripeptide (γ-glu-cys-gly) (GSH) on a wide variety of soft electrophiles such as epoxides and quinones, formed during phase I oxidation of xenobiotics, generally resulting in their elimination and detoxification. There are two GST superfamilies: (1) the membrane-bound GST isozymes and leukotriene C₄ synthetase and (2) the cytosolic-soluble enzymes, each of which displays different intracellular distribution and distinct catalytic as well as noncatalytic binding properties. Thirteen different human GST subunits, GSTA1 through GSTA4, GSTM1 through GSTM5, GSTP1, GSTT1 and GSTT2, and GSTZ1, have been identified belonging to seven distinct classes: alpha (α), mu (μ), omega (ω), pi (π), sigma (σ), theta (θ), and zeta (ξ) [82]. GSTs appear to be ubiquitously distributed in human tissues. Some examples of clinically significant drugs that form glutathione conjugates include acetaminophen, sulfonamides, irinotecan, carbamazepine, rotonavir, clozapine, procainamide, hydralazine, cyclosporine A, diclofenac, estrogens, and tamoxifen [83].

1.3.4 N-Acetyltransferase

NAT family of enzymes catalyzes the acetyl-CoA-dependent acetylation of aryl amines and arylhydrazines and N-hydroxyarylamine including many drugs and carcinogens. In most cases, this reaction is generally considered to result in the detoxification of potentially toxic exogenous compounds. However, NATs are also involved in bioactivation reactions via O-acetylation of N-hydroxyarylamines to unstable acetoxy esters that decompose to highly reactive mutagens that form adducts with cellular macromolecules. Humans express two distinct isozymes, designated NAT1 and NAT2 [84]. Clinically relevant substrates of NAT include isoniazid, procainamide, aminoglutethimide, sulphamethoxazole, 5-aminosalicylic acid, hydralazine, phenelzine, and dapsone. Also, NATs play an important role in the metabolism of industrial and environmental carcinogens, including 2-naphthylamine, benzidine, 2-aminofluorene, and 4-aminobiphenyl, as well as of potentially carcinogenic heterocyclic amines found in well-cooked red meat and cigarette smoke [85].

1.3.5 Methyl Transferase (MT)

MT family of enzymes catalyzes the O-, S-, or N-methylation of drugs, hormones, and neurotransmitters, and utilizes S-adenosyl-L-methionine (SAM) as a methyl donor. Catechol-O-methyltransferase (COMT) is the most extensively investigated drug-metabolizing MT. It plays an important role in the biotransformation of both endogenous and exogenous catechols. COMT has a rather broad substrate specificity for structures that contain catechol moieties and is often involved in the methylation of
drugs that have been metabolized by the CYP system to generate catechol functions [86, 87].

S-Methylation is also an important pathway in the biotransformation of many sulfur-containing drugs. At least two separate enzymes, thiol methyltransferase (TMT) and thiopurine methyltransferase (TPMT) are known to catalyze S-methylation in humans [87]. TMT, a membrane-bound enzyme, catalyzes the S-methylation of captopril, D-penicillamine, and other aliphatic sulfhydryl compounds such as 2-mercaptoethanol. On the other hand TPMT, a cytosolic enzyme, catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds including 6-mercaptopurine and other thiopurines. Recently, S-methyltransferase has been shown to play a critical role in the metabolism of the antipsychotic drug, ziprasidone, in humans [72, 88]. Both TPMT and TMT have been shown to be genetically polymorphic in humans.

Histamine-N-methyltransferase is a hepatic enzyme, although its inhibition by various drugs has been reported [87, 89], its ability to methylate drugs has not been established. Several protein arginine methyltransferases (PRMTs) are known and their physiological role in producing mono- and dimethylated arginine is known [90]. However, their role in N-methylating drug molecules has yet to be identified. Thus, except for COMT and S-methyltransferases the other MTs including DNA methylating enzymes are not known to play a role in drug metabolism.

1.4 DRUG EFFLUX TRANSPORTERS

Carrier-mediated transport of drugs and their metabolites has recently been recognized as an important issue in pharmaceutical research. There is a wealth of information that suggests that transporters are responsible both for the uptake and efflux of drugs and other xenobiotics, and may be key determinants of the disposition of a drug [91, 92]. Transporter proteins are divided into two categories: (1) the adenosine triphosphate (ATP) binding cassette (ABC) transporter superfamily and (2) the solute carrier (SLC) family of proteins.

1.4.1 ABCB1 (P-Glycoprotein, MDR1)

The first member of ABC efflux transporter family to be discovered was P-glycoprotein (P-gp; gene ABCB1 or MDR1). The importance of P-gp or MDR1 was first recognized in the multidrug resistance of tumor cells in response to chemotherapy treatment [93]. However, constitutive expression of P-gp in many normal tissues such as liver, kidney, intestine, blood–brain barrier, and lymphocytes demonstrates its role in modulation of the absorption, distribution, metabolism, excretion, and toxicology behaviors of some drugs and NCEs in development [94]. Experiments with mdr1a knock-out mice revealed that P-gp not only limits the CNS entry of drugs, but it also reduces the oral absorption of drugs by extruding them from enterocytes back into the intestinal lumen [95]. Therefore, the inhibition and induction of P-gp transporter can lead to significant drug–drug interaction and, most importantly, to drug treatment
resistance. Talinolol, fexofenadine, and digoxin are the most commonly used probe substrates for assessing the modulation of P-gp.

1.4.2 ABCC1 (Multidrug Resistance Related Protein1, MRP1)

ABCC1 or MRP1 is a 190-kDa transport protein that was originally cloned from a doxorubicin-selected lung cancer cell line. MRP1 has now been found to be expressed in a range of solid and hematological tumors, and has been demonstrated to transport a wide array of structurally diverse anticancer drugs such as oxorubicin, vincristine, and methotrexate [96]. In addition, MRP1 also transports many glutathione, glucuronide, and sulfate conjugated organic anions, such as leukotriene C₄, 17β-estradiol-glucuronide, and estrone 3-sulfate, respectively. MRP1 is also found in normal tissues throughout the human body and plays a significant role in tissue defense from toxic agents. Thus the expression levels and activity of MRP1 are important considerations in drug development and chemical toxicity.

1.4.3 ABCC2 (Multidrug Resistance Related Protein2, MRP2)

ABCC2 or MRP2 is located on the bile canalicular membrane and is involved in the biliary excretion of glucuronide, glutathione, and sulfate conjugates of lipophilic compounds (i.e., drugs) as well as endogenous compounds such as hormone and bilirubin conjugates. In addition, MRP2 can also transport uncharged compounds in cotransport with glutathione and thus can modulate the disposition of many drugs. MRP2 is predominantly expressed at the hepatocyte canalicular membrane [97]. It is also expressed in other tissues such as the kidney, as well as the intestinal enterocytes [97, 98] and apical membrane of brain capillary endothelial cells [99]. In humans, a genetic deficiency of MRP2 results in a disease known as Dubin–Johnson syndrome [100]. In addition, altered MRP2 function can change the ADME properties of many clinically important drugs including cancer chemotherapeutics (irinotecan, methotrexate, and vinblastine), antibiotics (ampicillin and rifampin), antihyperlipidemics, and angiotensin-converting enzyme inhibitors.

1.4.4 ABCG2 (Breast Cancer Resistance Protein, BCRP)

ABCG2 or BCRP, a 655 amino acid peptide with an ability to extrude a wide variety of chemical compounds from the cells, was first cloned from mitoxantrone and anthracycline-resistant breast and colon cancer cell lines [101, 102]. BCRP is a half-transporter efflux pump with an intracellular N and C terminus and an intracellular ATP binding site, followed by six putative transmembrane segments. It is expressed in liver, small intestine, placenta, kidney, mammary gland, and endothelial cells at the blood–brain barrier, and is believed to be involved in protecting tissues from xenobiotic accumulation and resulting toxicity [103]. It is capable of transporting sulfate and glucuronide conjugated organic anions, at least in vitro. It affects intestinal absorption/bioavailability, organ distribution, hepatic/renal elimination, and plasma clearance of substrate drugs such as topotecan [104]. Downregulation of BCRP was
suggested to contribute to cellular adaptation to folate deficiency and homeostasis [105].

1.5 DRUG UPTAKE TRANSPORTERS

The drug uptake (SLC) family of transporter is the largest superfamily of transporters. This family includes 31 transporters from organic anion transporter polypeptides (OATPs), organic anion transporters (OATs), organic cation transporters (OCTs), peptide cotransporters (PEPTs), and sodium–bile acid cotransporter classes. Only OATP, OAT, OCT, and PEPT are primarily involved with the transport of drugs/xenobiotics.

1.5.1 Organic Anion Transporter Polypeptides

The OATP transport proteins function as organic solute exchangers with broad substrate specificity that includes organic anions, cations, and neutral compounds [106]. The OATP transporters are expressed in organs such as the intestine, liver, and blood–brain barrier and considered to be the key determinant in the cellular uptake of many endogenous and exogenous chemicals, including drugs in clinical use. To date, eight different OATPs (OATP1A2, OATP1B1, OATP2B1, OATP3A1, OATP4A1, OATP1C1, OATP1B3, and OATP 2A1) have been cloned from human tissues [107].

1.5.2 Organic Cation Transporter

OCTs are responsible for reabsorption and excretion of a wide variety of organic cations, such as quinidine, cimetidine, procainamide, vecuronium, and cardiac glycosides as well as endogenous substances including, dopamine, epinephrine, norepinephrine, creatinine, and choline. The OCT transporters are expressed in human organs such as the intestine, liver, proximal tubules of kidney, brain, aorta, skeletal muscle, prostate, adrenal gland, salivary gland, and fetal lung. To date, three different OCTs (OCT1, OCT2, and OCT3) have been identified in humans [108, 109].

1.5.3 Organic Anion Transporter

OAT family plays a critical role in the renal excretion and detoxification of a wide variety of compounds including drugs, toxins, hormones, and neurotransmitter metabolites. OATs are primarily expressed in the kidney. In addition, OAT expression has been detected in liver, placenta, brain, and choroid plexus. At least five different OATs (OAT1, OAT2, OAT3, OAT4, and Urat1) have been detected in human tissues [109].

1.5.4 Peptide Transporter

The PEPTs are hydrogen ion-dependent transporters that transport small peptides and proteinlike compounds such as cephalosporins, penicillin, enalapril, and captopril. Humans express two distinct PEPTs designated PEPT1 and PEPT2. PEPT1
is involved with the oral absorption of drugs from small intestine while PEPT2 contributes to the proximal tubular reabsorption of drugs [108].

1.6 CHALLENGES IN DRUG METABOLISM

1.6.1 Prediction of Metabolism and Pharmacokinetics in Humans

1.6.1.1 In silico Computational Tools. In recent years, several in silico computational methods have become available for prediction of metabolism and pharmacokinetics of the NCEs and are increasingly becoming a part of the drug discovery process to select candidates with desirable ADME properties [110–112]. De Graaf et al. [110] have described how in silico computational approaches can be used to understand, rationalize, and predict the activity and substrate selectivity of CYPs. Mechanism-based quantum chemical calculations on substrates and the enzyme, pharmacophore modeling of ligands, and protein homology modeling in combination with automated docking and molecular dynamics simulations have been used to rationalize and predict ligand binding and formation of metabolites. Several of these models, especially for CYP2D6 [113] and CYP2C9 [114], have been shown to have a reasonably good predictive value concerning qualitative metabolism and substrate inhibitor selectivity. Programs such as Meteor and Metasite use database-based evaluations or de novo computational methods based on bond energies and active site docking to predict metabolic transformations and hot spots. While these methods assist in predicting metabolic outcomes, they are still limited and neither software is capable of predicting rates of metabolism at the sites that are predicted. These tools cannot be used to guide new chemical synthesis that incorporates metabolic concerns without experimental verification.

1.6.1.2 In Vitro–In Vivo Extrapolation. The ability to predict human pharmacokinetic from in vitro and in vivo models with a reasonable degree of accuracy (<2.0-fold variation) is an ongoing challenge with many different approaches yielding varying degrees of success. Interspecies allometric scaling of pharmacokinetic parameters is the earliest of the approaches and is still a tool used in predicting human pharmacokinetic behavior of drugs. The method is based on empirically observed relationships between physiological processes and body weights of mammals [115]. Several enhancements have been introduced to the original method such as maximum life-span potential, brain weight, body surface area, rule of exponents, and protein binding [115–117]. The allometric methods assume that clearance mechanisms are similar across species and do not consider interspecies specificity differences in drug-metabolizing enzymes and metabolic pathways. A recent analysis of 103 compounds for allometric scaling incorporating the enhanced methods where data were available for rat, dog, and monkey showed that the success rate for human projection from preclinical models was suboptimal (18–53%) with or without the enhanced methods [118]. A major limitation of the allometric methods is the lack of incorporation of metabolic differences in preclinical species and humans.
In vitro methods using liver slices, hepatocytes, and liver subcellular fractions, such as microsomes, have also been developed, and are used to varying extents in clearance prediction. Methods based on half-life of NCEs in metabolically active liver microsomes are used to project hepatic extraction from which clearance predictions are calculated [119]. The limitation of this approach is that prediction is limited to those NCEs for which hepatic metabolism by oxidative enzymes of the endoplasmic reticulum dominate the overall clearance mechanism. When drug-metabolizing enzymes other than CYPs or FMOs dominate the metabolic clearance, this approach falls short. Hepatocytes contain the entire cellular metabolic machinery and would be considered the better choice for hepatic clearance prediction. However, unlike human liver microsomes, which can be prepared from frozen liver tissue and effectively stored at \(-80^\circ\text{C}\), fresh human hepatocytes are not readily accessible, and the activities of some drug-metabolizing enzymes have been shown to decline with age of hepatocyte cultures, thus limiting their use. Cryopreserved human hepatocytes are used in a limited capacity to predict hepatic clearance and in evaluating overall metabolism of NCEs. When allometric scaling was combined with in vitro metabolism-based scaling for 10 highly metabolized drugs, a significant improvement was noted for the predicted human clearance compared to either method alone [120]. A recent retrospective analysis of pharmacokinetic data for a large set of diverse drugs suggests that human pharmacokinetic parameters can be predicted from rat pharmacokinetic data within a threefold variation by a fixed exponent approach [121]. This method allows for early binning of NCEs into low-, medium-, or high-clearance categories at a very early stage in drug discovery.

In the past two decades, success in predicting human pharmacokinetics from preclinical and in vitro techniques has dropped the attrition rate for NCEs due to failure to achieve the pharmacokinetic thresholds necessary for drug action in humans from \(\sim 40\%\) to less than \(10\%\) [6]. Thus, while success has been achieved in this area, clearly much progress is still necessary in preclinical models to achieve greater success in eliminating attrition due to pharmacokinetics. A more direct approach using drug microdosing in humans to predict pharmacokinetic behavior early in the drug development process is under investigation. In this approach, NCEs are administered at doses significantly below therapeutic levels (>100-fold lower than projected therapeutic dose or 100 µg) in order to get an early readout on their pharmacokinetic behavior [122, 123]. A microdosing study with five marketed drugs (warfarin ZK253 (schering), midazolam, erythromycin, and diazepam), known to be problematic in extrapolation to humans from either in vitro or animal models, has shown good concordance in the pharmacokinetic parameters obtained under micro- and therapeutic-dosing conditions for all except erythromycin [124]. Because of the extremely low dose, this method does not require the same extent of animal safety testing as is required by a traditional investigative new drug (IND) application to the Food and Drug Administration [124–126]. Microdosing has become possible because of newer analytical tools such as liquid chromatography coupled high sensitivity soft ionization mass spectrometry and more recently at significantly higher sensitivity with accelerator mass spectrometry [127].
1.6.1.3 Species Specificity and Limitation of Animal Models. The primary goals in the use of preclinical studies in animals, typically rodent (rat and mouse), dog, monkey, and rabbit, are to extrapolate pharmacokinetic parameters and establish clearance mechanisms applicable to human drug administration, determine toxicity limiting dosages, and evaluate long-term carcinogenic and teratogenic potential of the NCEs. Drug-metabolizing enzymes in mammals have common ancestral roots. However, the substrate and reaction rate specificities of NCEs are known to vary considerably among species [128, 129]. These variations in activity and specificity are particularly important for pharmacokinetic parameters when drug clearance is metabolically driven, and also when preclinical species are considered for the toxicological model. For toxicological studies, identification of major metabolites from *in vitro* and *in vivo* studies in rodent, dog, and monkey compared to those from human *in vitro* reactions with hepatocytes and microsomes are particularly useful to identify the preclinical species with optimal coverage of exposure to potential circulating metabolites in humans. Interspecies specificity of some drug-metabolizing enzymes can differ significantly. For example, when AO is involved in metabolic clearance, species selection for pharmacokinetics and toxicology testing is critical. AO activity is high in monkey and human, moderate in rodent, and not detected in dog liver. Complicating the situation is that significant species differences exist in both specificity and activity of this enzyme. Thus, in addition to identifying metabolites, the reaction phenotype for the major metabolites of an NCE at early stages in the drug development process can be critical in selection of the species for pharmacokinetic and toxicological testing [130].

1.6.2 Drug–Drug Interactions

Drug–drug interactions (DDIs) have received considerable attention in the pharmaceutical industry because in recent years several prominent drugs have been withdrawn from the market in the US and Europe due to serious adverse events as a result of significant DDIs [131]. DDIs are caused when the clearance of one drug is influenced by a coadministered drug [131]. Therefore, the consequences of these DDIs can range from loss of therapeutic efficacy to the introduction of potential lethal toxic effects. Although DDIs can occur during absorption, distribution, and elimination phases following initial drug administration, metabolism seems to be the predominant mechanism for such interactions. Since most marketed drugs are eliminated from the body at least in part by metabolism, inhibition and/or induction of DMEs by one drug could have a significant effect on the disposition of another drug. The NCEs can either be perpetrators or victims of such interactions, and early evaluation of NCEs for their potential to function in either capacity is critical to the development of new drug candidates and involves the identification of drug-metabolizing enzymes inhibited by NCEs (in particular CYPs) as well as the metabolic pathways by which they are cleared [130]. High specificity substrates for *in vitro*/*in vivo* use in drug interaction assays are known for six major isoforms of CYP enzymes involved in drug metabolism.
1.6.2.1 Biotransformation Reaction Phenotyping. As described earlier, a majority of drugs are cleared by metabolic transformation, generally first by oxidation to more polar metabolites, by hepatic enzymes. Knowledge of the specific enzyme(s) especially CYPs, involved in the metabolism of and inhibition by the NCE, particularly of the major pathways, is critical for evaluation of the potential liabilities that an NCE may have with respect to DDI [130]. Metabolic flux of an NCE through several CYP-dependent pathways where each pathway contributes less than 30% of the overall metabolic clearance is always preferable to flux through a unique CYP isoform since inhibition of any one of the pathways by a coadministered drug would not significantly increase the systemic exposure of the NCE, and consequently pose no significant risk. Terbinafine, an oral antifungal agent, is an example of such a drug where multiple pathways for its metabolism predict lack of significant clinical DDI for its clearance [132], which has been clinically confirmed [133]. However, terbinafine is an inhibitor of CYP2D6 and has been shown to increase the AUC of desipramine when coadministered to CYP2D6 EMs, suggesting that caution must be used with coadministration of terbinafine to patients on medications with narrow therapeutic index (TI), and its metabolism is mediated primarily by CYP2D6. Thus, terbinafine can be considered as an example of a drug that is only a perpetrator for a drug interaction that involves coadministered drugs that are metabolized primarily by CYP2D6.

If the metabolic flux of an NCE is greater than 60% through a unique CYP isoform, especially by a polymorphic enzyme (CYP2D6), the potential for DDI is extreme and careful consideration has to be given to the forward development strategy for such an NCE [134]. The case of the antihistaminic drug terfenadine stands out as an excellent example of this process. Terfenadine is primarily metabolized to its pharmacologically active form by CYP3A4. Inhibition of CYP3A4 by a variety of drugs, best exemplified by ketoconazole, results in dramatic increases in the AUC of terfenadine to toxic levels [135] that can prolong the QT interval and produce potentially fatal ventricular arrhythmias. This example shows how ketoconazole functions as the perpetrator of the DDI while terfenadine, because of its major flux through CYP3A4, becomes victim. When the TI for the NCE is narrow, knowledge of the CYP isoform(s) involved is critical and close attention is needed to the potential for DDI by coadministered drugs.

Cyclosporine, paclitaxel, tacrolimus, and warfarin are examples of drugs with narrow TIs where even dietary habits can play significant roles in toxicity. For example, in the late 1980s clinical observations noted a dramatic effect of grapefruit juice consumption on the levels of dihydropyridine calcium ion channel blockers. Flavanoids present in grapefruit juice were speculated on the basis of studies that showed Naringenin, the aglycone component of the flavanoid naringin, which is abundant in grapefruit juice, as inhibiting the in vitro metabolism of dihydropyridines by human liver microsomes [136]. Clinical studies on renal transplant patients showed that grapefruit juice significantly enhanced the systemic exposure of cyclosporine, while having no significant effects on prednisone or prednisolone [137]. Further investigations have established that the effect is on drugs that are primarily cleared by CYP3A4 and show low bioavailability due to presystemic metabolism by CYP3A4.
in the enteroocytes. The increase in systemic exposure arises from the inactivation of CYP3A4 in the enteroocytes.

Epoxyfuranocoumarins formed from grapefruit juice components like bergamottin and related natural products have been identified as responsible for self-inactivation of CYP3A4 [138]. The inhibitory effect of grapefruit juice on enteric metabolism by CYP3A4 is temporal and its duration is limited to the duration of consumption [139]. Dietary effects from the consumption of other foods and natural remedies on the metabolism of some drugs have also been reported [131–143]. Examples of such processes with other CYP450 enzymes abound in the literature with over 500 published articles since 1990 that examine *in vitro*, *in vitro–in vivo* correlations, and *in vivo* drug interactions via CYP450 enzymes demonstrating the importance of such studies in drug development. Thus, early phenotyping of metabolic pathways for an NCE and the inhibitory effect of the NCE on CYP isoforms are important aspects of the development process for NCEs as drug candidates that provide an understanding of potential clinical DDI issues that may arise for which early clinical evaluations can be conducted. Reaction phenotyping is done by several methods including the use of specific inhibitors for some CYP isoforms, recombinant CYP isoforms and relative activity factors, inhibitory antibodies, and interindividual correlations [130, 144–148].

As with CYP isoforms, UGTs are also more commonly encountered in the metabolism of drugs and xenobiotics. There are several drugs and xenobiotics where glucuronidation contributes to the overall metabolic clearance mechanism and in some instances is the predominant metabolic pathway. Examples include zidovudine (AZT) [149], mycophenolate [150], retigabine [151], mitiglinide [152], fenamates [153], and gemcabene [154]. In contrast to extensive DDIs caused by binding of drugs to CYP isoforms, DDIs with the UGT family enzymes are relatively few, and are confined to compounds where UGTs are either the primary clearance mechanism or contribute substantially to it. The cancer drug irinotecan is metabolized to its active form SN-38 (7-ethyl-10-hydroxy-camptothecin), which is cleared by glucuronidation by UGT1A1. Ketoconazole increases the circulating levels of SN-38 due to potent inhibition of its glucuronidation by UGT1A1 [155]. Since irinotecan has a small TI, patients should be genotyped for UGT1A1 prior to commencement of therapy. Another example of a UGT-dependent drug interaction is that of statins and the lipid-lowering drug, gemfibrozil [156]. A significant component of simvastatin’s clearance is via an unusual lactonization mechanism involving an intermediate acyl glucuronide catalyzed by UGTs [157]. Gemfibrozil, which is also metabolized to its glucuronide by UGTs, inhibits the glucuronidation of simvastatin with the resultant pharmacokinetic effect of an increase in simvastatin’s AUC and decrease in biliary levels of simvastatin lactone and glucuronide [158]. UGT1A1 has a critical physiological function in the elimination of bilirubin as its glucuronide. Surprisingly, no reports on drug interactions with UGT1A1 such as that of ketoconazole have been reported to show significant increases in the blood levels of bilirubin. A possible explanation for this may be that the *in vivo* concentrations of substrates or inhibitors cleared primarily by the particular UGT under consideration may often be below their corresponding $K_m$ and $K_i$ values, resulting in partial site occupancy and consequently
insignificant *in vivo* interactions. Hence, when considering the potential for drug interactions with the UGTs, dose and concentrations in the blood and in the liver are important parameters to consider. Reaction phenotyping of UGTs is less developed than that for CYPs. Few high specificity low $K_m$ or $K_i$ substrates or inhibitors have been identified, except for UGT1A1 where bilirubin is a highly specific substrate. However, it is only useful for *in vitro* inhibition assays. Phenotyping of UGT reactions is generally conducted *in vitro* using recombinant forms of the enzymes.

Drug interactions with MAOs are particularly relevant when patients are on therapy with MAO inhibitors and the MAOs are significant contributors to the clearance of a drug. As with UGTs, the $K_m$ or $K_i$ for MAO substrates tend to be generally high and drug interactions at therapeutic levels are less pronounced than with CYP enzymes. MAO-A and MOA-B can be selectively inactivated by Chlorgyline and Deprenyl, respectively [68]. Thus, distinguishing between these enzymes can be readily achieved using either liver microsomal suspension where MAO-A and B are present as contaminants from fractured mitochondrial membrane particles or recombinant enzymes [68].

AO has been shown to be responsible for the primary metabolism of several drugs that contain an aromatic nitrogen heterocycle [72, 88, 159, 160]. In general, when drug structures contain aromatic nitrogen heterocycles, a role for AO should be considered in preclinical and *in vitro* tests. The contribution of this enzyme to drug clearance can be defined by examining metabolism of an NCE by cytosolic preparations and inhibition by either menadione or raloxifene [161, 162]. DDIs with AO have not yet been reported in the literature.

Several *in vitro* models including cell-based, cell-free, and yeast systems as well as *in vivo* models such as genetic knock-out, gene-deficient, and chemical knock-out animals have recently been developed in order to understand the interaction between drugs and transports [163]. However, unlike the CYP-mediated drug interactions, the evidence for the transport-mediated drug interactions is often less conclusive due to a broad overlap of substrate specificities between transporters and drug-metabolizing enzymes. Some evidences for the interaction of digoxin, a good P-gp substrate, when coadministered with valsnoedar or verapamil (potent and selective P-gp inhibitors) have been reported [164].

### 1.6.2.2 Metabolism-Based Inactivation/Bioactivation

The metabolic activation of an NCE to a reactive intermediate is an important consideration in the development of NCEs as new drug candidates. Metabolic activation can be effected by phase 1 or phase 2 enzymes. Depending on the mechanism and the substrate, oxidative reactions can yield reactive intermediates such as epoxides, quinone methides, quinone imines, and quinones, which are alkylating agents, which react with cellular constituents such as DNA and proteins. A comprehensive listing of known bioactivation reactions and mechanisms has been presented [83, 165]. These modifications are thought to be responsible for DNA damage that can result in tumor initiation and promotion, and idiosyncratic reactions that are observed in patients post marketing of a new drug. The Ames test and micronucleus assay are routinely used to determine the carcinogenic potential of an NCE early in the development process. In addition, metabolism-based
inactivation (MBI) assays have been established to detect either an enzymatic activity loss at a single concentration or an IC50 shift when an NCE is preincubated with either pooled human liver microsomes or recombinant CYP enzymes, and human hepatocytes. However, the relationships between protein covalent binding by reactive intermediates and toxicity are not well understood.

An *in vitro* test commonly used to establish reactive intermediates is the trapping of a reactive intermediate by glutathione [166–170]. While this test shows the potential for a reactive intermediate to be generated from an NCE by the oxidative enzymes in hepatic microsomes, it does not imply that toxicity will necessarily result from such an intermediate. Acetaminophen (Tylenol) and ethynylestradiol are examples of drugs that, when used in the proper manner, have proven to be extremely safe, and yet are positive for reactive intermediates when tested with the glutathione reactive intermediate screen. Accordingly, a positive reaction for an NCE in this assay should by itself not constitute a kill shot, rather it should serve as a flag for enhanced vigilance in toxicological testing, and such information should be used cautiously when selecting between NCEs for progression of compounds for drug development [171].

As described earlier, conjugation of xenobiotics to glucuronide or sulfate is generally associated with detoxification. However, phase II enzymes can also catalyze the bioactivation of drugs. For example, acyl glucuronides formed by UGTs from carboxylic acid functions present in drugs, NCEs or their metabolites can be reactive intermediates that acylate macromolecules by transferring the aglycone moiety. The modified macromolecules may be recognized by the immune system and elicit an immunological response either to the aglycone or the modified macromolecule. Consequences of such events can be hypersensitization to the drug or induction of auto immunity [80, 172–175]. Acyl glucuronides undergo intramolecular acyl group migration, which is used as a measure of reactivity. A recent NMR study provides a rapid means of assessing the migration half-life and the competing hydrolytic process. A general observation is that increased substitution at the α-carbon of the carboxylic acid increases the migration half-life and decreases toxicity [176]. Clearly, a better understanding of the relationships between reactive intermediates, macromolecular adducts, and toxicity is necessary.

NATs are also involved in bioactivation reactions via O-acetylation of N-hydroxylamines formed from CYP-mediated N-hydroxylation of arylamines. These bioactivation reactions form unstable acetoxy esters that decompose to highly reactive species, which bind to cellular DNA [83]. The O-sulfonation of compounds catalyzed by SULTs can also result in the formation reactive intermediates. Recently, it has been shown that α-hydroxytamoxifen (derived from CYP-mediated hydroxylation of tamoxifen) is bioactivated by SULTs [177].

### 1.6.2.3 Induction and Repression of Drug-Metabolizing Enzymes.

Among the various classes of drug-metabolizing enzymes, members of the CYP family have been the most extensively studied for susceptibility to regulation by xenobiotic and endobiotic mediators. FMOs and MAOs have not been shown to be susceptible to induction or repression by xenobiotics, and UGTs have not been sufficiently investigated despite...
large interindividual variability. Induction of CYPs by xenobiotics and drugs has been known since early studies on this class of enzymes. Inductions of CYP1A, 2B, and 3A families by drugs such as phenobarbital, dexamethasone, rifampin, and xenobiotics like dioxins, benzo[a]pyrene and others have been well documented. An increase in functional expression of a drug-metabolizing enzyme can result in increased clearance of a drug, resulting in reduced efficacy. Repression of expression can result in toxic accumulation of a drug above its TI. Induction mechanisms are complex and involve the proximal promoter and xenobiotic-response enhancer regions of genes to which bind the various nuclear transcription factors. The various nuclear transcription factors can interact individually or in concert, creating an interaction network that affects the magnitude of the induction response [178, 179]. Critical to the development of new drugs is the dose-dependent magnitude of the induction response to an NCE [180]. It is recognized that major differences exist in the response to inducers in humans and preclinical models as a consequence of differences in xenobiotic receptors and nuclear transcription factors including differences in the binding affinities of receptors for xenobiotics. For example, the ligand binding domains of rabbit, rodent, and human pregnane X receptor (NR112) share only 75–80% sequence identity. Thus, induction in preclinical species may not appropriately reflect induction in humans or vice versa. The importance of nuclear receptors in regulation of drug-metabolizing enzymes has been established. However, much effort is needed to elucidate the molecular mechanisms involved in these processes. Primary cultures of human hepatocytes have been used to examine induction of drug-metabolizing enzymes by NCEs [181]. Recently, the use of an immortalized hepatocyte cell line (Fa2N-4) has been shown to be more reproducible for examining induction [182, 183].

1.6.3 Polymorphisms

Genetic polymorphisms in the form of single nucleotide polymorphisms (SNPs) have been observed in most genes that have been examined in mammals. SNPs are the most commonly encountered mutations that are observed either in the coding or promoter regions of genes encoding drug-metabolizing enzyme. Such changes can have effects that range from innocuous to extremely deleterious. The relevant forms of polymorphism from a drug metabolism perspective are those that affect the clearance and active transport of drugs. Genetic variability in drug-metabolizing enzymes is a significant contributor to the variability in human drug pharmacokinetics. An SNP that results in a stop codon within the coding region can result in a lack of expression, or expression of a nonfunctional or unstable protein that is phenotypically observed as a lack of metabolic activity. SNPs that result in amino acid changes can alter protein structure with variable effects ranging from a lack of activity to varying degrees of impaired activity. Other SNPs can be silent, resulting in no changes in the sequences or protein expression. SNPs in the promoter regions of the gene can affect levels of protein expression. Thus, for an NCE extensively metabolized by a particular drug-metabolizing enzyme, polymorphisms in the gene could cause plasma concentrations to rise to levels far above its TI with toxic consequences. This would be particularly critical for NCEs with low TIs. Hence, knowledge of the enzymes
involved in clearance of NCEs along with knowledge of polymorphisms that exist for these enzymes is critical to the development of safer drugs.

1.6.3.1 CYP450. Polymorphisms that have been discovered in the genes for CYPs, 2D6, and 2C19 are particularly good examples of the importance of knowing polymorphisms in drug-metabolizing enzymes and the challenges in the development of NCEs as new drugs. The “Debrisoquine Polymorphism” observed for the hypertensive drug debrisoquin was first recognized in the mid-1970s among hypertensive patients as a bimodal distribution in the ratio between debrisoquine and its 4-hydroxydebrisoquine metabolite, and via familial studies was suggested to be caused by a recessive allelic gene [184–186]. Subsequently this polymorphic effect was correlated with other drugs used in the 1970s and 1980s [187], and the enzyme responsible for the 4-hydroxylation of debrisoquine was identified [188, 189]. Extensive genotype/phenotype studies have been conducted in ethnically different populations around the globe and over 60 allelic variants have been identified for CYP2D6. The effect on enzymatic activity of these allelic forms is varied and extends from increased to complete loss in metabolic capacity of this enzyme. For drugs primarily cleared by CYP2D6 the consequence of increased metabolic capacity would be subtherapeutic levels of drug and loss of optimal efficacy. More critically, for patients with allelic variants moderately to extensively devoid of metabolic capacity, the effect would be increased drug levels with consequent toxicity. Hence, patient genotyping would appear to be critical prior to administration of drugs with low TIs and a significant contribution of CYP2D6 to their clearance. Consideration of CYP2D6 genotype can also be important when a drug such as Timolol is used topically. Timolol, a known CYP2D6 substrate, is known to have significant cardiopulmonary effects. The fraction absorbed systemically from a 0.5% aqueous ophthalmic application increases heart rate due to systemic absorption. With PMs the systemic exposure can reach levels sufficient to effect QT prolongation and consequent cardiac arrhythmia.

Similarly, for CYP2C19 over 20 allelic variants have been identified with several variants devoid of enzymatic activity. The activation of clopidogrel to its active metabolite is known to involve both CYP3A4 and CYP2C19. Clinical studies in healthy subjects have shown that in subjects carrying the CYP2C19*2 allelic variant, platelet responsiveness was markedly decreased [190]. Similarly, in the use of the anticancer drug Indisulam, which is metabolized primarily by CYPs 2C9 and 2C19, patients carrying allelic variants CYP2C9*6 or CYP2C19*2 can be susceptible to neutropenia due to increased systemic exposure to the drug [191]. SNPs have been identified among all the major CYP isoforms including the CYP3A family.

The majority of allelic variants identified for CYP3A4 and 3A5 either have no phenotypic effect or result in diminished catalytic activity, which can result in an increase in circulating drug levels to some extent. A rare allelic form of CYP3A4 recently discovered in a German subject with a midazolam clearance of 2.99 mL (min kg)^{-1} has been shown to be due to the lack of heme incorporation into the heterologously expressed protein [192]. The frequency of this variant in the general population has
not been established, and its effect on drug metabolism by such allelic carriers remains to be established. Tacrolimus, cyclosporine, and some other CYP3A cleared drugs that have low TIs are particularly sensitive to allelic variants with diminished metabolic capacity, and care in dose administration is critical. Clinical studies in renal transplant patients have shown that the CYP3A5 genotype correlated with dose, and the mean dose required to achieve efficacious concentrations was lowest for the CYP3A5*1/*1 allele. Tacrolimus is nephrotoxic and hence genotyping of subjects is critical for effective dose administration [193, 194].

Allelic variants for other CYP isoforms involved in the metabolism of drugs have been documented and in some cases effects of allelic variants have been examined. A compendium of allelic variants for human CYP isoforms in families 1, 2, 3, and 4 is available on the Web at the home page of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (http://www.cypalleles.ki.se/cypalleles.html).

1.6.3.2 UGTs and SULTs. Polymorphisms have been identified in all the UGT isoforms that have been examined. The mutations are observed in the coding and promoter regions of the genes. A compendium of allelic variants in families 1 and 2 is available on the Web at http://galien.pha.ulaval.ca/alleles/alleles.html. Sixty-two allelic variants have been reported for the UGT1A1 gene. Bilirubin is cleared as its glucuronide only by UGT1A1. Several syndromes are associated with mutations in the UGT1A1 gene. The type I Crigler–Najjar (CN) syndrome is lethal and is due to any of 36 allelic forms of UGT1A1 that result in complete loss of UGT1A1 activity. Systemic accumulation of bilirubin results in severe neurological toxicity. Currently, only liver transplant is the approach used to treat such patients [195]. Gene and cell therapies hold promise for future treatment of this disease [196, 197]. Sixteen allelic variants of UGT1A1 result in the less lethal, type II CN syndrome. Gilbert syndrome is a form of inherited mild hyperbilirubinemia that results from mutations in the TATA box upstream of the UGT1A1 gene resulting in altered gene expression [198]. The magnitude of severity in type II CN syndrome is determined by the intrinsic activity of allelic forms toward bilirubin and treatments vary from phenobarbital and phototherapy to dietary control [197].

The active metabolite of Irinotecan, SN-38, and the cancer drug Etoposide are drugs that are cleared primarily by UGT1A1 and have narrow TIs. To maintain drug concentrations within the therapeutic window, it would appear critical to genotype patients to be treated with such drugs. Therefore, from a drug development perspective, in vitro knowledge of the involvement of UGT1A1 in primary clearance of, or inhibition by, an NCE or its metabolite and their potential to limit bilirubin clearance would appear useful to conduct at an early drug discovery stage. Such knowledge is useful either to conduct early drug interaction studies to limit late stage attrition or as indicators for patient genotyping prior to commencement of therapy.

Polymorphisms have also been identified among several SULT members, and some secondary effects on metabolism have been documented. However, since this enzyme system has not yet been identified in any critical drug metabolism function, the effects of such mutations have been far less defined [199].
1.6.3.3 Other Drug-Metabolizing Enzymes. MOA-A and MOA-B are critical for maintenance of neurotransmitter homeostasis. Polymorphisms in these genes have been encountered and reports generally address polymorphisms in the promoter regions of these enzymes that result in alterations in enzyme levels [68]. No reports exist in the literature where polymorphisms encountered in the coding region of the genes have dramatically altered the catalytic activities for these enzymes.

There are five human flavin monooxygenase (FMO) gene families. Polymorphisms are known for these enzymes. However, due to a lack of significant primary roles in drug clearance or demonstrated DDIs, polymorphisms among these enzymes have not gained much attention [200]. FMO3 has received the highest attention because of its physiological role in clearance of trimethylamine. Polymorphic forms of FMO3 result in variable loss of trimethamine metabolic capacity from moderate to severe. This results in varying degrees of trimethylaminuria (fish odor syndrome) and consequent psychological effects [200].

AO/XO belongs to the family of molybdenum cofactor dependent enzymes as described earlier. XO has received the most attention because of its role in the metabolism of purines. Type I xanthinuria is caused by mutations in the XO gene that result in a lack of protein expression or inactive protein [201, 202]. Type II xanthinurias are caused by potentially inactive XO polymorphism and due to lack of the molybdenum cofactor caused by mutations in the molybdenum cofactor sulfurase gene [203, 204]. Literature on polymorphic forms of AO is limited. However, with increasing involvement of AO in drug clearance, attention needs to be focused on polymorphic forms of this enzyme. A recent report has identified Donryu rats with significant variability in clearance of the IND RS-8359. The variability was shown to be associated with mutations in the AO gene [205].

While no critical physiological function for the two forms of NAT1 and NAT2 have been identified, these enzymes have been associated with drug and xenobiotic metabolism, and with polycyclic aromatic amine-induced cancer. Humans have been classified as high, medium, and PMs with respect to these genes. Various reports have tried to link acetylator status with disease states. However, while trends have been noted, no definitive linkages have been established [206]. SNPs in the genes encoding for NAT1 and NAT2 are responsible for the high degree of variability observed in N-acetylator status. The antituberculosis drug isoniazid is cleared by NATs, and consequently the NAT genotype does influence the dose requirement for treatment of tuberculosis. Thus, from a drug metabolism perspective, it may be important to know if NATs play a role in clearance of NCEs. However, these enzymes have not become frontline screens in the drug discovery and development paradigm.

1.6.3.4 Transporters. If the NCEs are substrates for transporters, systemic or target tissue availability of the NCE may become limiting, and consequently influence the pharmacokinetics and pharmacodynamics of the NCE. Efflux transporters such as P-gp present in intestinal epithelia can have negative effects on the bioavailability of NCEs, particularly those that have poor membrane permeability. Whereas for NCEs that have good membrane permeability, efflux transporters do not play as critical role in intestinal absorption since a dose-dependent saturation of the efflux pump
is possible. While systemic exposure may be achieved by overcoming the intestinal efflux barrier, target tissues that express such transporters may function as efficient barriers to tissue penetration from systemic circulation. For example, P-gp present in the blood/brain barrier and in tumor cells is an effective hindrance to brain and tumor cell access of P-gp substrates [207, 208]. Uptake and efflux transporters in the liver and kidney can have similar influences on systemic exposure of NCEs [209]. Inhibition of transporter function can, depending on the endogenous role of a transporter, result in toxicity. For example, inhibition of bile salt transporters can result in cholestasis and hepatic failure [210]. Conversely, inhibition of P-gp efflux transporters can enhance efficacy of antineoplastic agents [211]. While extensive knowledge has been gained in transporters, techniques for rapid evaluation of their roles in pharmacokinetics is still lacking and major strides are needed to effectively incorporate transporters in the drug discovery paradigm.

SNPs in many classes of transporter genes are known, particularly among those involved in bile salt homeostasis [212]. Polymorphisms in P-gp have also been identified and are thought in part to contribute to variability in P-gp function. However, insufficient studies have thus far been conducted to establish distinct roles for SNPs [213].

1.7 SUMMARY

Since the early 1980s a majority of the human enzymes involved in drug metabolism such as the CYPs, UGTs, MAOs, FMOs, and some of the lesser encountered drug-metabolizing enzymes have been isolated or heterologously expressed and characterized, and more recently some crystal structures have been solved, providing a better understanding of the structural basis for their broad specificity. Knowledge of transporters such as P-gp, MRPs, OATPs, and OCTs has expanded with construction of cell systems incorporating specific transporters for use in characterizing their role in drug efflux or uptake. Progress in molecular genetics of drug-metabolizing and related enzyme systems has enabled some understanding of the molecular basis for inherited traits such as PMs and EMs. Several CYP isoform-specific inhibitors and substrates are known that allow for the elucidation of some primary metabolic pathways and an evaluation of the potential for DDIs. The enzyme kinetic parameters ($K_m$, $K_i$, $V_m$) for the isoforms involved in clearance or inhibition can be accurately determined and used to assess the potential for drug interactions and issues that may be associated with metabolism by, or inhibition of, polymorphic forms that can alter projected pharmacokinetic parameters. With this information preclinical and clinical studies can be designed to address and evaluate such concerns. Expansion of our knowledge of the reaction mechanisms of CYP enzymes, their substrate specificities, and crystal structures allows for in silico predictive models to be constructed. This can minimize exhaustive experimental testing by selectively examining compounds in a series. Advances in structure elucidation by soft ionization mass spectroscopy allow for rapid characterization of metabolites and identification of metabolic hot spots. When done in an iterative manner, this can aid in designing compounds with better pharmacokinetic properties.
Advances in our understanding of mechanisms of induction through nuclear receptors are expanding and allow for early screening to identify compounds with the potential to induce CYP isoforms using in vitro ligand binding assays for nuclear receptors rather than preclinical animal models where outcomes often do not translate to humans. This knowledge allows for appropriate nonclinical or clinical testing to be conducted at an early stage to mitigate late stage attrition due to enzyme induction.

Progress in efflux and uptake transporters allows for assessment of the role of the more commonly encountered transporters in achieving targeted drug concentrations at the site of action. This is particularly important in CNS and cancer programs where P-gp plays a critical role in the blood–brain barrier and tumor cell surface by preventing drug entry. Thus if CNS or cancer drugs are P-gp substrates, depending on the intrinsic efflux parameter, TI, and safety window, such drugs may not achieve their targeted concentrations for efficacy. For drugs not targeted to organs or sites with high P-gp content, the presence of P-gp in the intestinal wall, although limiting drug entry, is not as critical since saturation of the pathway can be achieved by increasing the oral dose to overcome the efflux. Biliary efflux pumps can also play a significant role in limiting systemic exposure. As tools to assess the roles of such transporters become available, the ability to more accurately predict behavior in human subjects becomes a reality.

Knowledge of non-CYP metabolic pathways has also expanded. Tools are available to assess if enzymes, such as MAOs, UGTs, aldehyde or xanthine oxidases, contribute to the clearance. Lacking are means of assessing the magnitude of the contribution made by these enzymes to the overall clearance. As medicinal chemists become more effective in limiting CYP-mediated metabolism of NCEs, non-CYP metabolic pathways will become increasingly involved in drug clearance and consequently better tools are necessary to evaluate the role such enzymes may play in metabolism of NCEs early in the drug development process.

Most drug testing is conducted in adult humans between the ages of 18 and 60 years. Pediatric use of drugs has generally been limited to allometric scaling from adult pharmacokinetic studies. Developmental changes in the hepatic expression of some drug-metabolizing enzymes have been noted; however, far greater understanding and statistical knowledge is needed for projection of pediatric pharmacokinetics and simulation thereof by predictive tools such as SimCYP can be effectively employed.

Despite major strides in understanding of drug metabolic processes, knowledge in several areas is still lacking, and predictive tools, while significantly improved, need further enhancement. In silico tools are improving with better ability to predict metabolic hot spots (Meteor and Metasite). As technologies evolve, strategies used in drug metabolism studies in early drug discovery will also evolve to fit changing paradigms.

REFERENCES


REFERENCES


REFERENCES


REFERENCES


