1 ADME Profiling in Drug Discovery and Development: An Overview

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1.1 INTRODUCTION

Discovery and development of a new drug is a risky, a time-consuming, and an extremely expensive business. Recent studies revealed that it takes an average time of 12–14 years and costs more than one billion dollars to discover, develop, and approve a new drug in the United States. The advances in chemistry, high throughput screening, molecular and cell biology, and human genomics have resulted in a multifold increase in the number of new chemical entities (NCEs) for preclinical and clinical evaluations. The applications of parallel synthesis and combinatory chemistry to expedite lead finding and lead optimization processes has shifted the chemical libraries toward poorer biopharmaceutical properties, that is, poor solubility and higher molecular weight (MW). It has been estimated that for every 5000 NCEs evaluated in a discovery program, only one is approved for market. At any point during the drug development process, a prospective drug may be terminated due to lack of *in vivo* efficacy, serious undesired side effects, and poor pharmacokinetic (PK) and absorption, distribution, metabolism, and excretion (ADME) properties. 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 early refinement of these ADME properties has been regarded as an essential part of the drug candidate selection process.

Encyclopedia of Drug Metabolism and Interactions, 6-Volume Set, First Edition.
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In support of this need, and as a consequence of increased knowledge within the drug metabolism discipline, new approaches have been developed, which include extensive *in vitro* methods using human and animal hepatic cellular and subcellular systems, recombinant human drug-metabolizing enzymes (DMEs), transgenic animals and cell lines stably expressing human transporters, increased automation for higher throughput screens, sensitive analytical technologies, and *in silico* computational models to assess drug metabolism aspects of the NCEs [1–3].

The ideal drug candidate should be able to provide sustained exposure, undergo balance clearance pathways with multiple enzymes, and not interfere with DMEs and transporters. Therefore, the design of drugs with optimal potency, PK properties, and less toxicity is still challenging, given the opposing requirements for absorption and metabolism. In the past decade, multiple *in silico, in vitro*, and *in vivo* ADME tools have been developed and implemented in various stages of the drug discovery and development process to alert chemists of potential ADME issues in the clinic [1–3]. Although significant improvements have been made to reduce the failure rate due to human PK-related issues, the overall attrition rate has not been improved [4]. The reason for attrition has been shifted to efficacy and safety. However, the ineffective efficacy, safety, and PKs are all interrelated and assignment of failure to a distinct factor might be misleading. For example, the poor efficacy of a drug candidate might be due to extensive metabolism, and clearance to inactive metabolites arising from induction of DMEs and its toxicity might be due to formation of toxic metabolites and/or its prolonged and unnecessary exposure arising from inhibition of the DMEs and transporters. Therefore, quantitative prediction of human PK parameters still presents the single most difficult challenge in the design and advancement of drug candidates to the development stage. Nonetheless, it could be argued that high throughput ADME screens contribute to the selection of the right molecule with the best PK properties for success in humans [4]. The objectives of these screens are to optimize absorption, plasma clearance, metabolic stability, plasma protein binding (PPB), and ratio of metabolic to renal/biliary clearance and to minimize intestinal/hepatic first-pass metabolism, inhibition/induction of DMEs and transporters, metabolism by polymorphic DMEs, and the formation of reactive metabolites of the NCEs. In this chapter, we will attempt to discuss the various ADME profiling approaches in drug discovery and development process and the latest technologies of the selected assays.

1.2 WHAT IS ADME?

When a compound is administered intravenously, the dose is delivered directly into the systemic circulation. All other routes of administration are collectively termed *extravascular routes* (e.g., oral, parenteral, topical, rectal, buccal, and sublingual). For drugs, oral administration is the preferred dosing route due to convenience and to enhance patient compliance. Delivering a drug to its desired target receptor site after an oral dose involves several complex steps of ADME.

The "A" in ADME represents the absorption, the processes that involve transferring the drug from the gastrointestinal (GI) fluid across primarily the jejunum and the ileum segments of the small intestine into the portal blood system. Thus, a drug must pass through to one or more layers of membranes either by passive diffusion, facilitated passive diffusion, or active transport to reach the systemic circulation. Drug

has to cross epithelial or mucosal cell walls, endothelial or capillary cell walls (blood stream), and cellular plasma membranes to exert effect. The extent and rate of absorption are dependent on physicochemical properties, drug formulation, and anatomy and physiology of the site of absorption.

Solubility and permeability are the key determinants of a compound's absorption after its oral administration, and in ultimately determining "how much compound is available to the body," a term also called *oral bioavailability* (OBA). During development of a chemical series, it is very important to ensure that the series has optimal solubility and permeability to avoid inaccurate results not only in biochemical and cell-based *in vitro* biology assays, which are designed to assess compounds' potency to target biological receptors, but also in basic *in vitro* ADME assays, designed to optimize "drug-like" properties of a chemical series. Poor solubility and poor permeability are two major reasons for misinterpretation (or complete lack) of structure–activity relation (SAR) in chemical series as well as for a disconnect between biochemical and cellular assays. Lead optimization of poorly soluble and permeable compound, based on *in vitro* assay results, is extremely risky and can cause compound failure in later stages of the drug development, when a significant amount of resources have already been invested. A common guide that is routinely used by discovery teams to get an estimate of the desirable solubility and permeability for a chemical series is given by the maximum absorbable dose (MAD) [5]. MAD is a function of solubility and permeability and is shown in the following equation:

$$\text{Target solubility(mg/mL)} = \frac{0.015 \times \text{Target dose(mg)(MAD)}}{\text{Permability}(\text{min}^{-1})} \tag{1.1}$$

(assuming all the administered drug is completely absorbed, i.e., target dose = MAD).

In general, for a compound of average permeability and a projected clinical dose of 1 mg/kg, the aqueous solubility should be greater than 50−100 μg/mL [6].

The "D" in ADME represents the distribution, that is, "the reversible transfer of a drug from one location to another within the body." The compound is distributed to different tissues of the body after entering into the systemic circulation (i.e., blood). In most cases, the volume of distribution at steady state (V_{dss}) is used to describe the distribution of a compound in the body. Distribution is generally uneven because of differences in blood perfusion, tissue binding, regional pH, and permeability of cell membranes. The distribution (which tissue and what rate?) of a drug is an important parameter in determining its pharmacological action. The acidic drugs such as warfarin and aspirin are highly protein bound and thus have a small apparent volume of distribution. The basic drugs (e.g., amphetamine and meperidine) are extensively taken up by tissues and thus have an apparent volume of distribution larger than the volume of the entire body.

The "M" in ADME represents the metabolism of a compound by enzymatic reactions. It is a biochemical process by which xenobiotics (e.g., drugs) are converted to more hydrophilic (water soluble) entities, which enhance their elimination from the body [7,8]. Metabolism occurs primarily in the liver and also can occur in other organs (e.g., lung, intestinal epithelial, and kidney). Drugs are metabolized in two different stages: phases I and II. Phase I, or functionalization, reactions include hydroxylation (aliphatic, aromatic, or nitrogen), epoxidation (aliphatic or aromatic), dealkylation (O–, N–, or S–), deamination, oxidation (N–or S–), reduction (nitro, disulfide, keto,

aldehyde, or olefin), and hydrolysis (amide, ester, carbamate, or epoxide). These reactions introduce or unmask a functional group (e.g., –OH, $-CO_2H$, $-NH_2$, 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. Most phase II reactions result in a compound's concomitant increase in hydrophilicity and decrease in volume of distribution (V_{dss}), which together greatly facilitate its excretion from the body. The rate and extent of metabolism of a drug determines the dose of drug and the duration of drug effect.

The "E" in ADME represents the elimination (i.e., irreversible removal) of a compound by liver, kidney, and other organs from the body. After oral absorption, drugs are eliminated from the body via excretion as unchanged forms in urine and bile and/or metabolism followed by excretion. For a drug whose clearance is dependent on metabolism, its intrinsic metabolic stability dictates the unbound drug concentrations and the duration of pharmacological effects.

1.3 FACTOR AFFECTING ADME

1.3.1 Physicochemical Properties

Several physicochemical properties of chemical compounds are determinants of intestinal drug absorption, and Lipinski's rule-of-five is a good starter set of rules to predict good absorption [9]. The compounds with MW <500, lipophilicity <5 (calculated log P), total hydrogen bond acceptors <10 (acceptors being O and N), and total hydrogen bond donors <5 (donors being O–H and N–H groups) should have good oral absorption. It was proposed that when a compound violates two or more of these five rules, it will likely have poor intestinal absorption.

1.3.1.1 MW and Lipophilicity. One of the basic and simple parameters that affect a compound's absorption is its molecular size, which can be estimated fairly reasonably by its MW. Lower MW compounds generally have better absorption and lower biliary excretion. Another metric, the polar surface area (PSA), which reflects a compound's capacity to form H-bonds, is a critical determinant of permeability. It is proposed that compounds with PSA $>140\text{Å}^2$ will be absorbed $<10\%$ across the intestinal wall and hence possess poor bioavailability [10]. This relationship holds when compounds are primarily absorbed via a passive transcellular diffusion route and there is no involvement of active transporters.

Lipophilicity, commonly denoted as log P, is defined as the ratio of the compound concentration between an organic phase and the aqueous phase at equilibrium. In general, a log P value of 0–3 is considered optimal for passive diffusion [11]. A log P value of <1 suggests that a compound will have good solubility by virtue of being hydrophilic but will have a poor permeability. Similarly, a log P value of >3 suggests that a compound is highly lipophilic and may possess low solubility or be prone to metabolism and/or biliary excretion.

1.3.1.2 Ionization Constant (pK_a). Ionization constant is a useful thermodynamic parameter to modulate the charge state and eventually solubility, permeability, and key

properties of an NCE. Therefore, accurate measurements of pK_a of an NCE allows for properly predicting and interpreting ADME properties. The ratio of the total drug, ionized plus unionized, in the aqueous medium and the organic phase is defined as the distribution coefficient, denoted as D and commonly reported as log D [11]. P (as in log P) is a pH-independent parameter, while D is a pH-dependent parameter. In general, a log $D_{7.4}$ value between -0.5 and 2 is considered optimal for oral absorption. Compounds with log $D_{7.4}$ values <-0.5 have poor permeability, while those with log $D_{7.4}$ values >2 are limited by poor solubility.

1.3.1.3 Solubility and Dissolution. Low solubility of NCEs remains a major drug development challenge since it not only causes aberrant results (artificially low potency) in *in vitro* cell assays [12] in the hit-to-lead stages but also can cause lack of efficacy in preclinical/animal models due to poor exposure, nonlinear PK, significant animal-to-animal PK variability, uncertainty in exposure prediction in humans, and formulation difficulties during clinical development. Significant time and revenues of research and development (R&D) are invested in developing a poorly soluble compound, via salt screens and formulation screens. Hence, it is highly desirable to avoid development of a poorly soluble compound from the early stages of drug development. Solubility is guided by two important parameters: lipophilicity and tightness of crystal structure, both of which are inversely related to solubility. Investigation of intrinsic solubility of poorly soluble marketed drugs revealed that several of these showed solvation-limited solubility (high lipophilicity), rather than solid-state-limited solubility (high energy of crystallization).

Several examples in the literature highlight medicinal chemistry efforts to improve solubility, via introduction of ionizable, N-containing basic groups [7,13,14], or disruption of planar crystal structure [15,16], as is shown in Fig. 1.1 for the selected literature examples.

Prodrug approaches have also been used to improve solubility of drugs such as that shown in Fig. 1.2. Fosamprenavir, a phosphate prodrug for the HIV protease inhibitor, amprenavir, showed a 10- to 1400-fold (in the pH range 3.3–7.4) improvement in solubility [17].

1.3.1.4 Permeability. Membrane permeability is not just critical for absorption across the biomembrane of the GI tract but also plays a very important role in its distribution to all other tissues of the body. Compounds can traverse the epithelial cell membranes via either (i) passive transcellular, (ii) passive paracellular, or (iii) active influx/efflux transporter-assisted routes. Majority of the neutral lipophilic drug candidates enter cells through the passive transcellular route, while hydrophilic or charged drugs do so via the passive paracellular route. Since the surface area of the tight junctions is about 0.01% of the small intestine surface area, the paracellular route of absorption is not a very efficient route as compared to the passive transcellular route, which takes place across the much larger surface area of the apical membrane of enterocytes' microvilli environment. Contribution of numerous transporters to drug uptake and efflux has also received a lot of attention in recent years.

Physicochemical properties of compounds alone sometimes fail to give insight into its permeability since there are various permeability pathways that involve specific transporters, which are essentially proteins that recognize the drugs in specific ways that cannot be predicted, based on physicochemical properties itself. In general, an increase

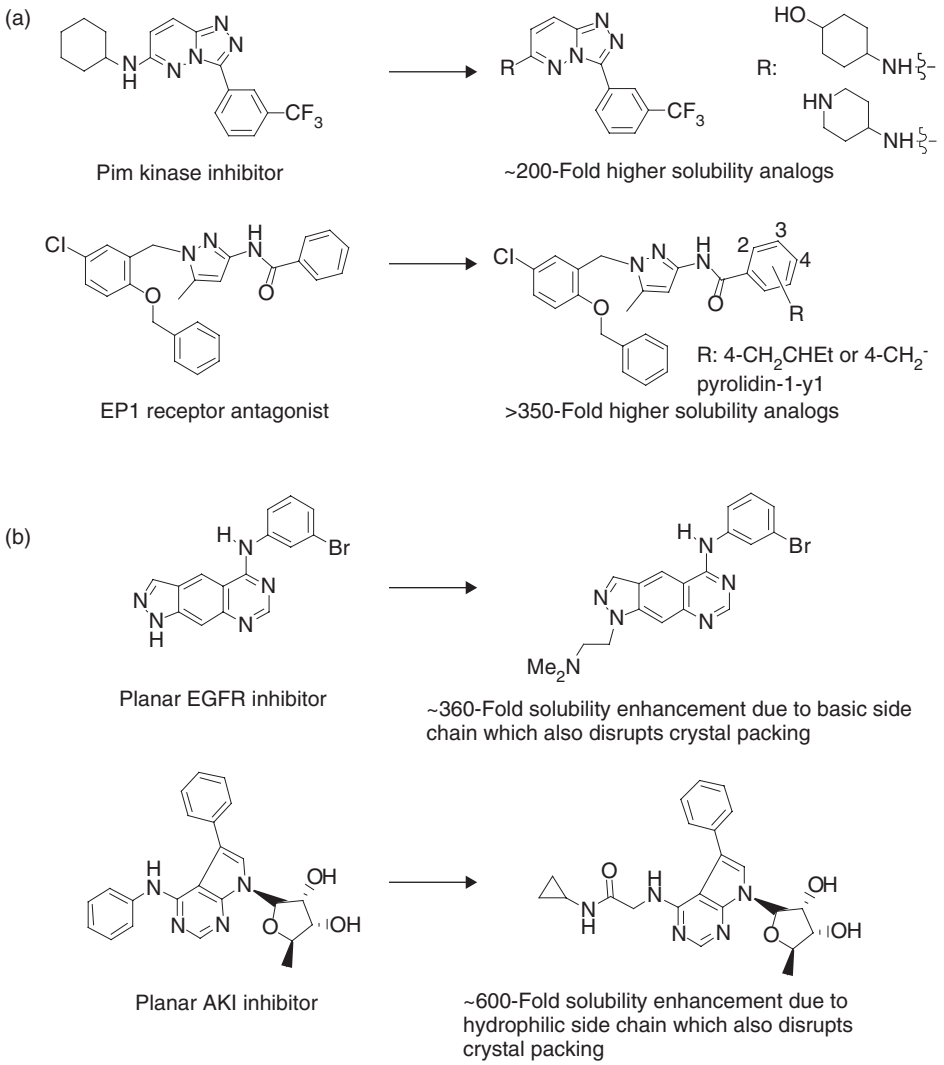

Figure 1.1 Literature examples of solubility optimization due to (a) introduction of ionizable groups and (b) disruption of crystal packing.

in lipophilicity, decrease in H-bonding, and reduction in polarity of a chemical series has been found to improve permeability of compounds [18–20] (Fig. 1.3). Sometimes, these changes do not always lead to an expected increase in the improvement of Caco-2 permeability as seen during structure optimization of a series of phenothiazine carboxylic compounds as novel histamine H_1 antagonist [21].

Attaining permeability of drug candidates across the blood–brain barrier (BBB) is a challenge in the development of central nervous system (CNS) drugs with only 2% of the CNS discovery compounds being able to cross the BBB to impart their therapeutic efficacy. Like Lipinski's rule-of-five, there are some predictive rules such as Clark's rule and Pardrigde's rule, which are able to predict permeability across

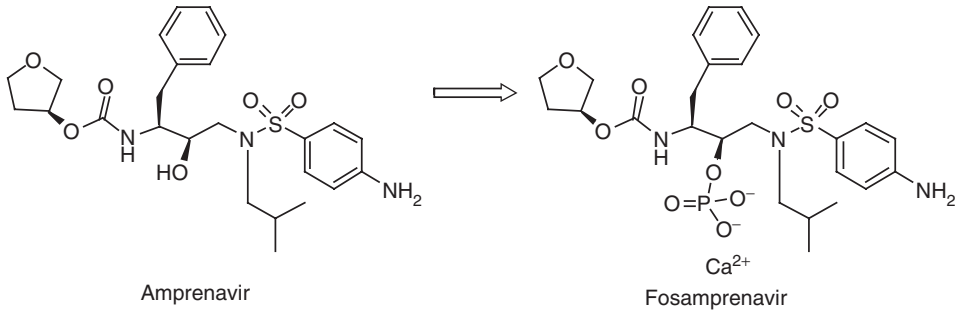

Figure 1.2 Overcoming poor solubility due to prodrug approach.

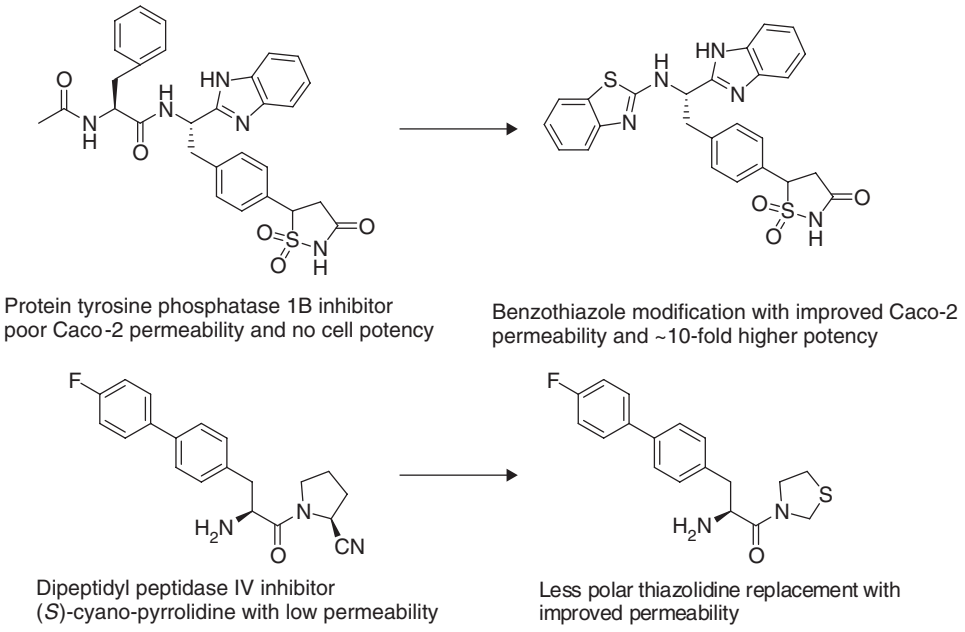

Figure 1.3 Literature examples of permeability optimization.

BBB with reasonable accuracy. The BBB membrane is negatively charged and this favors permeability of basic amine-containing compounds that are positively charged at physiological pH. Implication of charge can be seen in comparing indomethacin and trifluoroperazine. Indomethacin, which has a pK_a of 4.18 is negatively charged, while trifluoroperazine, which has a pK_a of 7.8, is positively charged at physiological pH, which results in inability of indomethacin to cross the negatively charged BBB [22]. Strategies that improve intestinal permeability, such as increased lipophilicity, decreased H-bonding, decreased polarity, also improve BBB penetrability. Uptake of morphine across BBB was found to be 32-fold higher than its M6-glucuronide, which has ~150-fold lower lipophilicity [23]. In another example, the substitution of one –OH group in morphine by a $-OCH_3$ in codeine reduced the intermolecular H-bonding and lipophilicity and increased BBB penetration increased by 10-fold. Replacement of

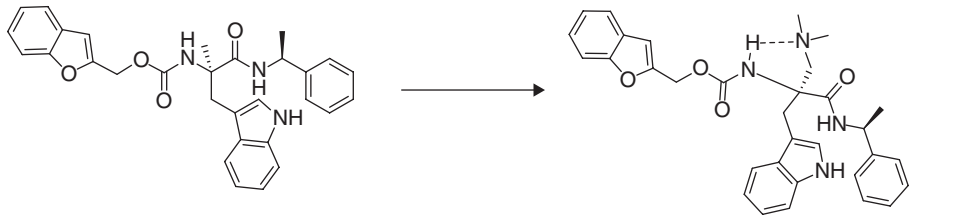

Figure 1.4 Introduction of intramolecular H-bonding to enhance BBB permeability.

both –OH in morphine by acetates to form heroine resulted in a 100-fold increase in brain permeability [23]. Introduction of intramolecular H-bonding has been shown to be effective in making a compound more lipophilic, hence increasing CNS penetration (Fig. 1.4) [24].

In general, ways to improve solubility include (i) reduction of log *P* via introduction of ionizable groups, increase in H-bonding, increase in polarity, decrease in lipophilicity and (ii) decrease in crystal packing via introducing out-of-plane functional groups. However, some of these changes result in undesirable modulation of intestinal permeability which is favored by increased lipophilicity, reduced H-bonding (reduced PSA), and reduced polarity. It is often easier to fix solubility of compounds than permeability since solubility of a chemical series can be modulated over a million-fold range, while the difference between a poorly and highly permeable compound is only by ~50-fold [5].

1.3.2 Metabolic Stability

The great majority (>75%) of the marketed drugs are eliminated by metabolism. High metabolic liability usually leads to poor PK properties (high clearance, low exposure, short half-life, and low OBA), and therefore, it is desirable to have a metabolically stable compound, (except in cases of prodrugs) to ensure minimal variations in interindividual responses for its exposure and duration of action. Determination of metabolic stability, also commonly referred to as *in vitro intrinsic clearance* (Cl_{int}), is one of the key *in vitro* ADME parameters that is evaluated and optimized at a very early stage of discovery to transform a less desirable, metabolically unstable chemotype into one that has favorable Cl_{int}. Metabolic stability *in vitro* is also believed to predict hepatic clearance *in vivo*, which in turn is a major contributor to total body clearance of the majority of xenobiotics. High metabolic stability and hence low Cl_{int} are often desirable. In most cases, low Cl_{int} also leads to favorable PK properties such as good OBA and long half-life, which result in good exposure and desired duration of action, respectively. Compounds that possess high Cl_{int} are also more susceptible to drug–drug interactions (DDIs) and possess less-than-desirable exposure and duration of action required for therapeutic efficacy of a drug.

1.3.3 Plasma Protein Binding

PPB is believed to have a significant influence on the rate of drug diffusion between plasma and tissues (influx and efflux) [25] and therefore influence Cl and V_{dss} of

drugs. In general, it is desirable to avoid highly plasma protein bound drug since small changes in PPB of a highly bound drug can lead to significant fluctuation in its free fraction.

1.4 ISSUES IN DRUG DEVELOPMENT

1.4.1 Failure of *In Vitro–In Vivo* Correlation for Prediction of DDIs

In early stages of drug discovery, as actual human data are not available, results obtained from human subcellular fractions are extremely valuable in predicting the human PK parameters and assessing the potential of an NCE as a successful human drug candidate. But before a significant amount of effort is dedicated to optimize and modulate drug metabolism and pharmacokinetics (DMPK) properties of a new chemotype, it is very important to evaluate the ability of the *in vitro* ADME assays to predict the *in vivo* situation, that is, establish *in vitro–in vivo* correlations (IVIVCs) at very early stages of drug discovery. The following are some underlying reasons that are being made in predicting Cl_{hep} from Cl_{int} for the failure of IVIVCs [7]:

1. Assume that metabolism is the major route of Cl of the NCE.
2. Assume that liver is the primary organ responsible for the Cl (additional relevant *in vivo* clearance pathways, such as renal or biliary, that are not captured in *in vitro*).
3. Assume that cytochrome P450 (CYP)-mediated oxidative metabolism is the key metabolism pathway and contributions by other phase I and phase II enzymes, and hence DDI mediated by these, are nonexistent.
4. Failure to account for shift in major metabolic pathway *in vivo* as compared to *in vitro* (not predicted by *in vitro*).
5. Failure to account for formation of inhibitory (or inducing) metabolites *in vivo* (or vice versa).
6. Assume that Cl_{int} is similar in microsomes and hepatocytes *in vivo*.
7. Assume that enzyme expression and activities in isolated subcellular fractions is representative of those in the physiological milleu.
8. Assume that no uptake (not modeled by microsomes) or efflux transporters (not modeled by hepatocytes) are involved in drug uptake and Cl.
9. Failure to account for intestinal efflux.
10. Failure to account for effect of f_u in the intestine.
11. Uncertainty in quantitative effect of inhibitors toward intestinal clearance.
12. Failure to incorporate high PPB or concentration-dependent PPB of an NCE.
13. Failure to account for nonspecific binding of an NCE to microsomes, hepatocytes, or blood cells.
14. Failure to identify correct enzyme inhibition mechanism [reversible vs mechanism-based inactivation (MBI)] or inaccurate determination of k_{deg} in case of MBI.
15. Assume that the key kinetic parameters, such as K_i, k_{cat}, K_I, k_{inact}, k_{deg}, EC_{50}, and E_{max}, that are crucial in DDI assessment are similar *in vivo* to those determined *in vitro*.

16. Inaccurate determination of $f_m \times f_{m,CYP}$ (in the case of CYP-mediated DDI).
17. Effect of genetic polymorphism leading to interindividual variability in exposure, species, and gender difference in DME and transporter expression and activity.
18. Failure to quantitatively account for concurrent inhibition and induction by drugs (e.g., ritanoavir and CYP3A4).
19. Lack of physiological scaling factors for transporters and DMEs other than CYPs [DMEs such as flavin monooxygenase (FMO), uridine glucuronosyl transferase (UGT), *N*-acetyl transferase (NAT), and sulfotransferase (SULT) and transporters such as Pgp, Breast cancer resistance protein (BCRP), Multidrug resistance-associated protein (MRP), Organic anion transporter (OAT), and Organic anion transporter polypeptide (OATP)].
20. Inability of *in vitro* systems to evaluate complex interplay of DMEs and transporters.
21. Assume that no atypical kinetics between the NCE and the enzyme responsible for its Cl.

It can be simply summarized that due to species differences in the expression levels, tissue localization, substrate specificity and gene regulation of transporters and DMEs, and extrapolation of data from *in vitro* studies and *in vivo* preclinical species to predict drug disposition *in vivo* in humans is very challenging.

1.4.2 Projection of Human PK

Accurate projection of human PK parameters (Cl, V_{dss}, and F) and efficacious dose is extremely valuable in drug discovery and ensures that the NCEs with poor PK properties or safety concerns (due to high dose requirement) are not selected as potential drug candidates for development. Over the past two decades, numerous methodologies using the drug metabolism and disposition data from *in vitro* human tissues such as microsomes or hepatocytes and *in vivo* preclinical studies have been developed, each with its own advantages and disadvantages [26,27]. Using these techniques, an overall success rate of 60–80% of compounds in prediction of human PK parameters within twofold of actual human PK parameters [26–28] has been achieved. Recently, Hosea *et al*. [29] have shown that the either single-species *in vivo* data or *in vitro* human liver microsomes can quantitatively predict human *in vivo* PK within twofold of actual values by conducting a retrospective analysis of 50 proprietary compounds for which *in vitro*, preclinical PK data and oral single-dose human PK data were available. The success rate of 60–80% is good to rank order the NCEs in drug discovery but is insufficient to discriminate closely related analogs within a series [28]. In drug discovery, the ability to predict the human PK of an NCE from *in vitro* and *in vivo* models with a reasonable degree of accuracy (<twofold variation) is one of the most challenging task.

Human PK projection can be performed via some common methods (Fig. 1.5) [7]: (i) allometric scaling of preclinical *in vivo* PK parameters; (ii) *in vivo* Cl projection from *in vitro* Cl_{int}; (iii) species invariant time methods such as equivalent time, kallynochrons (elementary Dedrick plot), apolysichrons, dienetichrons, or syndesichrons, each of which are some modifications of the elementary Dedrick plot method; and (iv) C_{ss}-mean residence time method. For human OBA estimation, the fraction of dose appeared into the portal vein ($F_g \times F_a$) of each preclinical animal species can be calculated, the average of which can be used to estimate human $F_g \times F_a$. This estimate

Simple allometry:

$Cl = a\,BW^{b}$

$V_{dss} = c\,BW^{d}$

BW = body weight

MLP = maximum life span

a = coefficient (log a is the y-intercept) of Cl

b = exponent (b is slope of log–log plot of Cl and BW) of Cl

c = coefficient (log a is the y-intercept) of V_{dss}

d = exponent (b is slope of log–log plot of V_{dss} and BW) of V_{dss}

ROE used for Cl prediction:

if $0.55 < b < 0.7$ then simple allometry best (above equation)
if $0.71 < b < 0.99$ then $Cl \times MLP = a\,BW^{b}$
if $b \geq 1$ then $Cl \times$ brain weight $= a\,BW^{b}$

Elementary Dedrick plot

$$Y = \frac{\text{Concentration}}{(\text{Dose/BW})} \qquad X = \frac{\text{Time}}{BW^{1-b}}$$

Complex Dedrick plot

$$Y = \frac{\text{Concentration}}{(\text{Dose/BW}^{d})} \qquad X = \frac{\text{Time}}{BW^{d-b}}$$

Dienetichrons

$$Y = \frac{\text{Concentration}}{(\text{Dose/BW}^{d})} \qquad X = \frac{\text{Time}}{MLP \times BW^{1-b}}$$

Calculation of OBA in human

$$f_a \times f_g = \frac{F_{PO,\,animal}}{\left(1 - \frac{Cl_{hep,\,animal}}{Q_{h,\,animal}}\right)}$$

$$F_{PO,\,human} = f_a \times f_g \times \left(1 - \frac{Cl_{hep,\,human}}{Q_{h,\,human}}\right)$$

Cl_{hep} = hepatic clearance

Q_h = hepatic blood flow

f_a = fraction absorbed

f_g = fraction escaping gut first pass

F_{po} = bioavailability

Figure 1.5 Various methods used to predict human pharmacokinetics.

can be combined with the predicted human Cl, obtained by methods outlined above, to yield the predicted human OBA. Human OBA values can further be used to estimate the predicted human exposure at a desired dose. It is highly advisable to use multiple scaling methods to obtain a range of values for each of the predicted PK parameters.

1.4.3 Prediction of Human Metabolites

Metabolism facilitates the removal of chemicals from the body. In general, metabolites are pharmacologically less active and less toxic than their corresponding parent compound. However, the biotransformation reactions can sometimes lead to undesirable consequences, such as too rapid drug clearance, formation of pharmacologically active metabolites, DDIs via inhibition or induction of DMEs, and/or formation of toxic metabolites. In addition, drug-metabolizing ability is age dependent, influenced by the genetics of the individual (polymorphisms), reduced by certain disease states and can be altered when coadministered with other drugs.

Knowledge of the metabolic site(s) of an NCE in early drug discovery is essential for selecting compounds with favorable PK credentials and aiding medicinal chemists in modifying metabolic "soft spots" and to design better, lower metabolic-clearance analogs. In development, elucidation of biotransformation pathways of a drug candidate by identifying its circulatory and excretory metabolites is vitally important to understand its physiological effects. In discovery, metabolite characterization is performed *in vitro* using hepatic subcellular fractions from human and multiple animals to guide species selection for safety assessment studies in preclinical development. The species that most closely resembles human with respect to metabolite profile are selected as the preferred species for toxicity evaluations. Metabolite identification is, therefore, very important in drug discovery for the NCE progression and should be incorporated at very early stages to eliminate structural liabilities in the later stages of drug development. Efforts directed to improve metabolic stability are represented by some selected examples from literature, as shown in Fig. 1.6 [7].

Structure elucidation of metabolites may also indicate the formation of reactive intermediates, which have the potential to bind to biomolecules and cause immune-mediated toxicity. Knowledge of formation of reactive metabolites in the early drug discovery stages is very helpful in minimizing the risk associated with reactive intermediates/metabolites. It is postulated that reactive metabolites bind to crucial proteins and result in either direct organ damage or the modified protein elicits a hapten-mediated immune response. A complex cascade of signaling results in unpredictable toxic responses, also called *idiosyncratic toxicity* or *idiosyncratic drug*

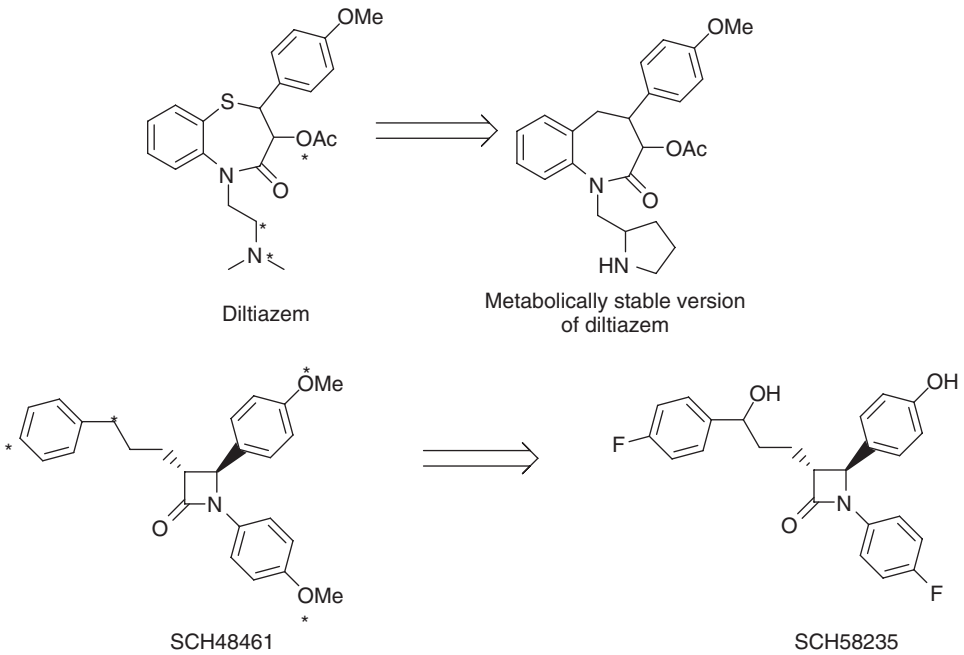

Figure 1.6 Examples to highlight structure optimization to improve metabolic stability: compounds shown on right side of the arrows are the metabolically stable version of the compounds on the left of the arrows. *Represents sites of metabolism that were blocked.

reaction (IDR). It is called idiosyncratic because the toxicity is not only unpredicted but also occurs in 1 of 1000 patients receiving the drug/NCE, thus making it virtually impossible to detect earlier at the preregistration phase. IDR risk is a significant liability and increases cost and risk of development of an NCE. It is therefore crucial to detect reactive metabolites very early on during the drug discovery phase and strategically remove the toxicophore moiety to avoid costly drug failures in development or worse, postmarketing. Structural characteristics of compounds forming reactive intermediates/metabolites have been extensively reviewed [7,30]. Compounds containing functional groups such as anilines, nitrobenzenes, benzyl/cyclopropyl amines, thiophenes, furans, thiazoles, sulfonylureas, hydrazines, carboxylic acids (those with α-protons), methylenedioxy, alkynes, terminal alkenes, halogenated hydrocarbons, and Michael acceptors [7,30] are all recognized to produce reactive metabolites.

In some cases, metabolites, due to structural similarity with their parent, could also be pharmacologically active, which results in disconnect between pharmacokinetics/pharmacodynamics (PK/PD) data. Several active metabolites have been developed and marketed as drugs. The examples of active metabolites of marketed drugs that have been developed as drugs include acetaminophen, fexofenadine, desloratadine, cetrizine, oxazepam, oxyphenbutazone, morphine-6-*O*-glucuronide, and minoxidil sulfate. Each of these drugs provides a specific benefit and has a better PK and/or safety profiles over the parent molecule.

1.4.4 Drug–Drug Interactions

Another major challenge in drug discovery is DDI. In recent years, several drugs have been withdrawn from the market in the United States and Europe due to serious adverse reactions as a result of significant DDIs [31]. DDIs are caused when the PK of one drug is influenced by a coadministered drug. The consequences of these DDIs can range from loss of therapeutic efficacy to the introduction of toxic effects. Most marketed drugs are eliminated from the body at least in part by metabolism, and many of the DMEs are inducible and can be inhibited; therefore, 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. The DDI risk is more profound if the victim drug has high intrinsic clearance or is selectively cleared via one major pathway. So, development of an NCE that is either itself a modulator of DME or affected by modulation of the DMEs, especially if the NCE also possesses high Cl_{int}, is not favorable. Therefore, inhibition and induction of DMEs by an NCE and the DME responsible for its metabolism are routinely assessed at very early stages of the drug discovery.

DDIs should not be viewed as solely undesirable as there have been several cases where PK of one drug has been modulated by another via a well-planned design to improve the exposure (and hence the efficacy) of the affected drug [7]. Kaletra is a coformulation of lopinavir and ritonavir, whereby ritonavir-mediated CYP3A4 inhibition results in higher plasma levels of lopinavir and boosts its anti-HIV protease activity. Ketoconazole (KTZ), a potent CYP3A4 inhibitor, is commonly used in combination with cyclosporine A (CsA) to enhance the immunosuppressive properties of

the latter as a result of increase in CsA exposure to due KTZ-mediated inhibition of CsA metabolism.

1.4.4.1 Drug-Metabolizing Enzyme Inhibition. Inhibition of enzymes can be reversible or irreversible and always results in reduction of intrinsic clearance of the pathway that is inhibited [32]. Reversible inhibition, also known as *direct inhibition*, can be typically classified as competitive, uncompetitive, noncompetitive, or mixed, with competitive inhibition being the most commonly observed pathway of inhibition. *In vitro* inhibition studies are valuable in assessing potency of an NCE as an inhibitor as given by $[I]/K_i$ ratio (details in Section 1.5.2). In addition to the $[I]/K_i$ ratio of the inhibitor, contribution of the inhibited pathway to the overall metabolism of an NCE, $f_m \times f_{m,CYP}$ (f_m = fraction of the NCE cleared via metabolism, $f_{m,CYP}$ = fraction of metabolism via CYP), enzyme interaction at the intestinal site and parallel routes of metabolism, all play a vital role in risk assessment due to potential DDI. In situations where an NCE can inhibit multiple CYPs with $[I]/K_i$ ratio >0.1 for each of the CYP-mediated pathways, a rank order approach is recommended for conduct of clinical DDI [7,33].

For MBI, *catalytic bioactivation of an NCE by an enzyme* is a *prerequisite*. This results in irreversible inhibition of the enzyme, and therefore, potency of both these types of inhibition increase over time (with preincubation). MBI of CYPs have been extensively investigated and the presence of functional groups [7,30] such as aniline, nitrobenzene, hydrazine, benzyl/propargyl/cyclopropyl amine, hydantoin, thioureas, thiazole, furan, thiophene, epoxides, methylene dioxy, methyl indoles, alkyne, isothiocyanate, and terminal alkenes, on an NCE warrants immediate and early assessment of inactivation potential of the NCE to avoid severe DDI liability in late stage development. It must be borne in mind that if an NCE does possess a structural alert, it does not automatically imply that it will be a potent inhibitor. Distinguishing MBI from simple reversible inhibitor is critical in predicting a clinical DDI, since applying a reversible inhibition model to an MBI may result in significant underprediction of a DDI risk.

The magnitude of a DDI is dependent on the degree of contribution of the clearance pathway that is modulated (inhibited or induced); higher the fraction of the dose metabolized by the enzyme that is modulated, greater is the magnitude of DDI. It is very favorable for an NCE to have multiple pathways of clearance, so that when one of these is affected, it is compensated for by the other pathways. Drugs that are metabolized by polymorphic enzymes (e.g., 2D6, 2C9, and 2C19) are prone to risk of either lack of drug efficacy (due to increased clearance of the drug in the rapid metabolizers) or increased toxicity (due to accumulation of drug in the poor metabolizers) and may warrant a dose adjustment based on the population genotype. Hence, identifying major pathways that are responsible for the clearance of an NCE is crucial in eliminating DDI risk later on in drug development [7]. A significant amount of work has been done in the area of the CYP enzyme phenotyping due to the availability of cDNA-expressed enzymes, normalization methods, and isoform-specific substrates and inhibitors (chemical and antibody) [34]. CYP phenotyping involves rigorous kinetic analysis of metabolite formation, to give an estimate of $f_{m,CYP}$, which represents *in vivo* contribution of a particular isoform(s) toward a biotransformation pathway of an NCE. The magnitude of the product of $f_{m,CYP}$ and f_m reflects the propensity of an NCE to be subjected to DDI. Higher the value of $f_{m,CYP} \times f_m$, higher will be the risk

Exposure change due to inhibition or induction of CYP-metabolism

$$\frac{AUC_{PO,\ inhibitor}}{AUC_{PO,\ control}} = \frac{f_{g,\ inhibitor}}{f_{g,\ control}} \times \frac{1}{\left(\frac{f_m\, f_{m,CYP}}{CI_{int,\ control}/CI_{int,\ inhibitor}}\right) + [1-(f_m\, f_{m,CYP})]}$$

$$CI_{int,\ control} / CI_{int,\ inhibitor} = 1 + [I]/K_i \text{ for reversible competitive or noncompetitive inhibition}$$

$$= 1 + \frac{k_{inact}}{K_I} \bullet \frac{[I]}{k_{deg\ for\ mechanism\text{-}based\ inhibition}}$$

AUC = exposure
f_g = fraction surviving gut first pass
f_m = fraction of total clearance due to all CYP-metabolism
$f_{m,\ CYP}$ = fraction of total CYP-metabolism catalyzed by the inhibited CYP isoform
$[I]$ = inhibitor concentration
k_{inact} = rate constant of CYP inactivation
K_I = half maximal inactivation rate
K_i = dissociation constant of enzyme–inhibitor complex

Exposure change due to inhibition of intestinal metabolism (for drugs that this pathway is significant)

$$\frac{AUC_{PO,\ inhibitor}}{AUC_{PO,\ control}} = \frac{Q_{gm} + f_u\, CI_{int,\ gm}}{\left(Q_{gm} + \frac{f_u \bullet CI_{int,\ gm}}{[1 + (I_{gm}/K_i)]}\right)} \bullet \left(1 + \frac{I_u}{K_i}\right)$$

I_{gm} = unbound inhibitor concentration in intestine
I_u = unbound inhibitor concentration in liver
Q_{gm} = blood flow to GI mucosa
f_u = fraction unbound of inhibitor in plama
K_i = inhibition constant

Figure 1.7 Exposure change in the presence of inhibitor.

of DDI. Changes in exposure of an NCE in the presence and absence of a modulator (inhibitor or inducer) can be simulated by the equation shown in Fig. 1.7 [7].

Estimation of f_m can be most accurately made from a human radiolabel study. A good estimate can also be obtained from a single-dose phase I human study. In many projects, due to the absence of human data in early stages of development, estimates of f_m are not available and $f_{m,\,CYP}$ data are used to guide clinical DDI studies via a "rank order" approach [33].

Unfortunately, the assessment of the contribution by other enzymes such as FMO, SULT, and UGT are limited to qualitative evaluation only due to limitation of availability of cDNA-expressed enzymes, normalization methods, and isoform-specific substrates and inhibitors.

1.4.4.2 Drug-Metabolizing Enzyme Induction. DDI can also occur through induction of nuclear receptors and modulation of the transcription and the expression of DMEs and transporters. This results in elevation of enzyme levels or increase in activity, which leads to faster clearance of the NCEs. Faster clearance of an NCE leads to lower than expected exposure, as demonstrated by significant decrease in exposures of several drugs such as midazolam, amitriptyline, cyclosporine, digoxin, indinavir, irinotecan, warfarin, phenprocoumon, alprazolam, dextrometorphane, simvastatin, and ethinylestradiol in the presence of St. John's wort, which has been shown to be a potent inducer of both CYP3A4 and Pgp [7]. Reduced exposure sometimes severely compromises therapeutic efficacy as seen with coadministration of cyclosporine (CYP3A4 substrate) and rifampin (potent CYP3A4 inducer) to organ transplant patients. Induction

of DMEs and transporters is regulated by a large family of nuclear receptors, such as aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), glucocorticoid receptor (GR), and peroxisome proliferator-activated receptor-α/β/γ (PPAR-α/β/γ) [35]. Owing to regulation of several DMEs and transporters via common receptors, it is advised to monitor a few sentinel CYP isoforms that are indicative of activation of the principle nuclear receptors. CYP1A2, 2B6, and 3A4 induction are suggested to be indicative of activation of AhR, CAR, and PXR, respectively [36].

1.4.4.3 Interplay between Drug-Metabolizing Enzymes and Transporters. There are numerous examples in the literature where a DDI may be the result of modulation of a DME, as exemplified by the DDI between terfenadine-KTZ, fluvoxamine-astemizole, cisapride-erythromycin, statins-protease inhibitors, felodipine-nifedipine, codein-quinidine, β-blockers-ritonavir, and rifampin-cyclosporine/simvastatin/verapamil/midazolam. Sometimes it may be due to modulation of transporters such as those exemplified by digoxin-quinidine, probenecid-acyclovir/allopurinol/cephalosporins/ciprofloxacin, and talinolol/verapmil. More often the DDIs result from an intricate interplay of modulation of both DMEs and transporters. Gemfibrozil causes six- to eight fold increase in exposures of coadministered drugs such as cerivastatin and repaglinide (antidiabetic) not only due to inhibition of uptake inhibitor OATP1B1 but also due to inhibition of CYP2C8, CYP3A4, and Pgp. Increase in plasma and Ceribrospinal fluid (CSF) concentration of the HIV protease inhibitors ritonavir and squinavir, due to coadministration with KTZ, has been attributed to potent inhibition of both CYP3A4 and Pgp by KTZ.

Transporters can also modulate the extent of metabolism of an NCE by affecting its exposure of an NCE to the DMEs [37,38]. Ability of transporters to modulate metabolism is also dependent on the Biopharmaceutics Classification System (BCS) class of the NCE under investigation [37,39]. Uptake or efflux transporters are unlikely to affect metabolism/exposure of class 1 (high solubility and high permeability) compounds, while uptake and efflux transporters are expected to modulate metabolism/exposure of NCEs, belonging to class 2 (low solubility and high permeability), class 3 (high solubility and low permeability), and class 4 (low solubility and low permeability).

1.4.4.4 Plasma Protein Binding. It is a common concern when the free drug concentration of a highly bound drug A increases due to PPB displacement by a strong binding drug B. The increased free drug A's concentration could potentially cause toxicity (due to more free fraction available to interact with target receptors) and may need dose adjustment. In addition to target-mediated efficacy/toxicity, PPB information is also helpful to estimate unbound/effective concentration of drugs that mediate inhibition of DMEs and transporters. Disease models are extensively validated in animals to progress a compound into development. *Unbound drug concentration* and therefore PPB across species is very informative in establishing safety margins and guiding selection of First in human (FIH) dose and human efficacious dose.

CNS drugs have been highlighted to be extremely sensitive to PPB and PPB-displacement situation. It must be recognized that BBB transporters, permeability, and protein binding to blood versus brain *all* play an equally important role in the rate and extent of drug uptake into CNS [40]. Increase in nonspecific binding in brain

tissue is considered to be a driving force to overcome effect of efflux transporters and therefore, Pgp substrates can still penetrate the brain because partition into brain tissue can provide a significant drug concentration gradient at the BBB to overcome effects of efflux transporters. Usually, increase in lipophilicity of a drug, not only increases its CNS penetration/permeability, but it also increases its nonspecific binding to brain tissue, resulting in lesser unbound/pharmacologically active drug available in brain for efficacy. Several examples of compounds that demonstrate high target affinity, high plasma concentration, and high brain/plasma (B/P) ratio, and yet fail to show efficacy, warrant extreme caution in optimization of the total B/P ratio only, for CNS drugs. CSF has been widely believed to be a surrogate for estimation of the unbound, pharmacologically active drug concentration in CNS [41]. Owing to a very low protein content of CSF, drug–protein binding in CSF is assumed to be negligible and CSF concentration is considered to represent the unbound drug in CNS, which is equal to the unbound drug concentration in plasma at steady-state equilibrium.

1.4.5 Polymorphism of Drug-Metabolizing enzymes

Many of the DMEs show genetic polymorphism, which can influence the levels of expression, enzyme stability, and catalytic properties [42–45]. Polymorphisms that have been discovered in the genes of CYP2C19, CYP2D6, UGT1A1, NAT, and thiopurine methyl transferase (TMT) are particularly good examples of the importance of knowing polymorphisms in DMEs and the challenges in the development of NCEs as new drugs. Genetic variability in DMEs is a significant contributor to the variability in human drug PK. 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 drug.

1.4.6 Drug-Induced Toxicity

Drug-induced toxicity remains one of the major reasons for failures of new pharmaceuticals and for the withdrawal of approved drugs from the market. The combined *in vitro*, *in vivo*, and *in silico* computational models have been used to assess the toxicity of the NCEs. While using these approaches, many compounds that might have serious adverse reactions associated with them are effectively eliminated before reaching the clinical trials; some toxicities such as those caused by idiosyncratic responses, however, are not detected until the drug is in late stages of clinical trials or have become available to the market. One of the proposed mechanisms for the development of idiosyncratic drug toxicity is the bioactivation of drugs to form the reactive metabolites by DMEs (Section 1.4.3). Efforts are being made to detect these reactive metabolites in the early stages of drug development [46].

1.4.7 Metabolites in Safety Testing

In February 2008, Food and Drug Administration (FDA) issued the final Guidance for Industry: Safety Testing of Drug Metabolites. The guidance recommends that the exposure of human metabolites that exceed 10% of the parent drug exposure [ICH (International Conference on Harmonisation) revised to total drug-related material] at steady state should be equal or higher in at least one of the preclinical animal species be used for long-term safety assessment [47,48]. Additional toxicological testing on

metabolites that display higher exposure in humans than preclinical animal species may be required by direct administration of metabolites to the test animals. Therefore, metabolite safety qualification strategy needs to be established as soon as practical in drug development. There are a number of examples when human metabolism was quantitatively different from preclinical species.

1.5 ADME ASSAYS IN DRUG DISCOVERY

Multiple *in silico*, *in vitro*, and *in vivo* ADME studies have been developed and implemented in various stages of the drug discovery to weed out weak candidates and to select a compound with favorable PK properties for further development. Table 1.1 summarizes some of the commonly used ADME assays that DMPK scientists rely on to not only help in the iterative screening of compounds in discovery but also for risk mitigation as well as to gain insight into mechanism of certain ADME pathway or process. A list of useful guidances and white papers for the design and conduct of metabolism and DDI studies are described in Table 1.2 [47–55].

1.5.1 *In Silico*

Availability of experimental data, molecular descriptors (molecular size and shape, hydrophilic and hydrophobic regions, hydrophilic and lipophilic balance, and H-bond donors and acceptors), and statistical analysis methods (multiple linear, partial least squares, artificial neural nets, Bayesian neural networks, and clustered regression) are the three essential prerequisites for *in silico* modeling. *In silico* models for solubility take into account log P, melting point, crystal packing, intrinsic solubility, and ionization effects [56–60]. From the simple general solubility equation, to more complex models based on Monte Carlo simulations using 2D or 3D properties, and fragmental or atom-based or quantum-chemical methods have all been used to predict aqueous as well as Dimethyl sulfoxide (DMSO) solubility of compounds with fair accuracy. There are several programs (ACD/logs, SLIPPER, CSlogWS, KLOGS, CERIUS, and WSKOWWIN) available for these predictions and are highly recommended for solubility prediction in early discovery [61].

Molecular descriptors such as H-bond donors and acceptors, hydrophobic groups, aromatic rings, and basic nitrogen atoms are all important for the development of permeability and Pgp substrate models [61]. Computational Volsurf-based model [62], conformation-dependent, GRid-Independent descriptors (GRIND) descriptor method, and recent QSAR models [63] have all been valuable in aiding medicinal chemists to optimize permeability in drug discovery stages. Recent development in predictive models for BBB penetration has also gained significant popularity, which is also directed to gain better insight into transporters at the BBB junction [64,65]. Methods include 2D-QSAR techniques, built on pharmacophore modeling using Catalyst, DISCOtech, and 3D-QSAR techniques, using SYBYL, comparative molecular field analysis (CoMFA), and comparative molecular similarity index analysis (CoMSIA).

Composite QSAR models that incorporate solubility and permeability and programs (iDEA and GastroPlus) have been very useful to predict fraction of dose absorbed and bioavailability [66]. Bioavailability predictions are not very simple since it necessitates integration of absorption prediction (combination of solubility and permeability) and first-pass metabolism (gut and liver). QSAR models and adaptive fuzzy portioning

TABLE 1.1 List of Commonly Used ADME Assays Used is Various Stages of Drug Discovery and Development

Parameter	Type	Method	Throughput
Solubility	Kinetic	Nephelometry	High
		Direct UV	High
		Turbidimetry	High
	Thermodynamic	Equilibrium shake flask	Low
		Potentiometric	Low
Permeability	Passive diffusion	Artificial membrane-based (IAM and PAMPA)	High
		Cell-based (Caco-2, MDCK, LLC-PK1, and CHO)	Moderate
	Transporter assisted	Cell-based Calcein-AM	High
		Inverted membrane vesicle-based ATPase	High
		Cell-based (Caco-2, MDCK, LLC-PK1, CHO, HEK293, KB-V-1, and MCF-7)	Moderate
		Transient transfection (oocytes, CHO, and COS)	Moderate
		BMECs for BBB	Moderate
		Gene KO animal studies (*in vivo*)	Low
	Rate of absorption	*In situ* tissue (liver, intestine, and brain) perfusion	Low
		Intraduodenal and portal vein cannulated rats (*in vivo*)	Low
Metabolism	Clearance	cDNA-expressed enzymes	High
		Subcellular fractions (microsomes and S9)	High
		Cell-based (fresh or cryopreserved hepatocytes)	Moderate/high
	Enzyme Inhibition	cDNA-expressed enzymes	High
		Subcellular fractions (microsomes and S9)	High
		Cell-based (cryopreserved hepatocytes)	Moderate/high
		Liver slices	Low
	Enzyme induction	Reporter gene assay (PXR binding and transactivation)	High
		Cell-based (sandwich-cultured) hepatocytes	Low
	Reaction phenotyping	cDNA-expressed enzymes	High
		Subcellular fractions (microsomes and S9)	High
		Cell-based (cryopreserved hepatocytes)	High
	Metabolite ID	Subcellular fractions (microsomes and S9)	High
		Cell-based (cryopreserved hepatocytes)	High

(*continued overleaf*)

TABLE 1.1 (*Continued*)

Parameter	Type	Method	Throughput
		Biological matrices (plasma, bile, and urine)	Moderate
PPB	N/A	Biacore technology (fluorescence spectroscopy)	High
		Chromatography and capillary electrophoresis	Moderate
		Ultrafiltration	High
		Ultracentrifugation	High
		Equilibrium dialysis (traditional/rapid-RED)	Low/high
		Microdialysis (*in vivo*/*in situ*)	Low

TABLE 1.2 List of Some Useful Guidances and White Papers for Design and Conduct of Metabolism and DDI Studies

Guidance	Purpose	References
The conduct of *in vitro* and *in vivo* drug–drug interaction studies: a PhRMA perspective	DDI study design and conduct	49
The conduct of *in vitro* studies to address time-dependent inhibition of drug-metabolizing enzymes: a perspective of the PhRMA	TDI study design and conduct	53
PhRMA white paper on ADME pharmacogenomics	Polymorphic DME and transporters	55
Drug interaction studies—study design, data analysis, and implications for dosing and labeling	Design and conduct DDI	52
Drug metabolism/drug interactions in the drug development process: studies *in vitro*	Design and conduct *in vitro* metabolism and DDI	50
in vivo drug metabolism/drug interaction studies—study design, data analysis, and recommendations for dosing and labeling	Design and conduct *in vivo* metabolism and DDI	51
The International Transporter Consortium: A Collaborative Group of Scientists from Academia, Industry, and the FDA	Decision tree for transporter DDI assessment	54
Safety testing of drug metabolites	Safety assessment of metabolites	47
ICH guidance of nonclinical safety studies for the conduct of human clinical trials and market authorization for pharmaceuticals	Safety assessment of metabolites	48

(AFP) models have been observed to yield a 60–65% prediction at best [67,68]. *In silico* intestinal device (ISID) model has also been developed to model absorption for compounds that are dual substrates of CYP and Pgp [69].

QSAR models to predict drug metabolism have been extensively developed using various statistical techniques [MLR (Multiple Linear Regression), PLS (Partial Least Square), NBC (Naïve Bayes Classifier), k-NN, SOM (Self Organizing Map), RP (Recursive Partitioning), ANN (Artificial Neural Networks), and SVM (Support Vector Machines)] [70–72]. A recent MLR approach for human hepatic clearance prediction using *in vitro* experimental data and six molecular descriptors (MW, number of total atoms, number of aromatic rings, number of single bonds, TPSA, and SKlogP) yielded ~85% success [70]. Randon Forest and Bayesian methods [based on MOE (Mixture of Experts) and E-state descriptors] to predict human microsomal stability have also yielded about 75–80% prediction success. Availability of X-ray crystallographic structures of mammalian CYP enzymes has prompted development of ligand- or CYP enzyme-based 3D-QSAR, quantum mechanics (QM), and pharmacophore models [73], which have predicted compounds' ability to be CYP substrates with reasonable accuracy. Softwares such as MetabolExpert, META, METEOR, and MetaSite are readily available to discovery teams for prediction of metabolic sites. Significant effort has been directed toward developing *in silico* models for CYP inhibition especially for CYP3A4 and 2D6, since these are the two isoforms that metabolize a vast majority of the marketed drugs. Several approaches such as RP ensemble method, PLS and ANN models, MLR analysis, NN, and Bayesian methods have been developed, some with as high as 99% prediction accuracy of CYP inhibitors. Pharmacophoric models and 3D-QSAR models (CoMFA and GOLPE/GRID) have also been used to predict CYP inhibitors with ~70% accuracy. It is interesting to note that for different CYP isoforms, different molecular descriptors are important, based on physicochemical nature of substrate binding at the CYP-active site. For example, molecular volume is important for CYP1A2 inhibition, while H-bond acceptor and hydrophobicity are more important for CYP2C9 models and MW, H-bonding capacity and number of aromatic atoms are more important for CYP3A4 models [74].

In silico modeling of CYP induction is very challenging due to the requirement of availability of crystal structure of nuclear receptors along with well-understood structural biology [75]. In addition, due to large binding pocket of some of the receptors, especially PXR, combined with the ability of the ligands to bind to several regions of the nuclear receptors further complicates modeling. Crystal structure of PXR, along with experimental data, has been used to build *in silico* PXR-binding model. Introduction of polar groups in the regions that show high affinity to the hydrophobic binding pocket of PXR, as well as making compounds more rigid to prevent binding, have been successful strategies, to synthesize compounds that do not bind to PXR. Pharmacophore modeling of hCAR also was able to model hCAR ligands with reasonable success.

In general, for CYPs and nuclear receptors, flexibility of their active sites, promiscuous nature due to which they accommodate very diverse ligands, the presence of multiple binding sites, binding modes, binding orientation, ability of ligands to alter protein structure, unavailability of unambiguous structural biology mechanism for some pathways, and the presence of multiple metabolic pathways have all contributed to make *in silico* modeling of CYP inhibition and CYP induction very challenging.

In silico metabolism prediction softwares such as Meteor and Metasite, coupled with several metabolite identification data processing softwares (offered by Agilent,

Applied Biosystems, and Thermo Scientific), have made biotransformation prediction for an NCE much easier. However, a strong organic chemistry background coupled with good expertise in liquid chromatography (LC)/tandem mass spectrometry (MS/MS) methodologies and data interpretation are the key prerequisites for successful metabolite identification.

1.5.2 *In Vitro* Methodology

1.5.2.1 Solubility. Solubility measurements can be of two different types—kinetic and thermodynamic [67]. For kinetic solubility, it is common to start with a stock solution of the compounds of interest in organic solvent, mostly DMSO, which is then diluted in buffer over a wide concentration range and solubility of compounds assessed via UV or optical light scattering methods. Kinetic solubility measurements are quite common in early discovery for rank ordering compounds and are most valuable for assessing solubility of compounds in a certain *in vitro* assay (biochemical or cellular) medium. Thermodynamic solubility measurements of NCEs are usually performed, in their solid state and hence this type of solubility measurements takes into account disruption of crystal lattices and dissolution process of the compounds. Although the gold standard for solubility measurements, thermodynamic solubility measurements are quite labor intensive and variations of a classical thermodynamic solubility assay are gaining popularity, one of these involves starting with DMSO stocks as in kinetic solubility measurements, but the DMSO is completely removed to dry the compound down to a powder form before adding the buffer [76].

1.5.2.2 Permeability/Transporters.

1.5.2.2.1 Cell-Based. Several cell-free and cell-based systems have been employed to investigate permeability and role of transporters in permeability of an NCE [61]. The cell-free parallel artificial membrane permeability assay (PAMPA) has been widely used as a high throughput, inexpensive, and simple tool to screen discovery compounds for their potential for passive permeability [77]. Caco-2 cell permeability is a widely used, "gold standard," *in vitro* screening and investigative assay to rank order compounds in the discovery stages, for their intrinsic permeability across intestinal epithelium and to investigate basic mechanistic studies involving absorptive transport (paracellular vs transcellular transport and the presence of saturable active transporter) [78]. Madine–Darby canine kidney (MDCK) cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) 293 cells, porcine proximal tubular epithelial (LLC-PK1) cells, human metastatic breast cancer cells (MCF-7), and Multidrug resistance protein (MDR) human KB carcinoma (KB-V-1, human cervical carcinoma/epidermoid cell line) cells are some of the other commonly used cell lines, transfected with transporter(s) of interest, to perform diagnostic/mechanistic transporter studies [79]. Cultured brain microvessel endothelial cells (BMECs), which form a confluent monolayer and retain morphology and functional capacity of BBB, are very valuable in investigating transport mechanisms involving passive diffusion and polarized uptake/efflux across the BBB [80]. Double/triple transfected cell lines (most of the time with one uptake and another efflux transporter) such as MDCKII-BCRP/OATP-B, MDCKII-NTCP/BSEP, and MDCK II-BCRP/OATP2 are very useful in investigating synergistic effect of uptake and efflux transporters across intestinal epithelium and hepatobiliary membrane [79].

1.5.2.2.2 Membrane-Based. Membrane-based transporter assays are important tools to study transporter-mediated interaction/disposition of drugs [61]. ATPase assay is a plasma membrane-based simple, rapid, and high throughput assay that can assess whether a compound is a substrate or inhibitor of ATP-binding cassette ABC transporters [81,82]. The ATPase assay endpoint is release of inorganic phosphate due to adenosine triphosphate (ATP) hydrolysis. The release of inorganic phosphate is monitored via a simple colorimetric assay. However, this assay cannot be used for Solute carrier transporters (SLC) transporters since SLC transporters lack ATPase activity. Isolated membrane vesicles, such as the rat liver canalicular membrane vesicle (CMV) [83] and rat intestinal and renal brush-border membrane vesicles (BBMVs) [84], are very useful for studying ABC transport. Inside-out vesicles present ATP-binding site to the NCEs being tested and substrates of ABC transporters are taken up in an ATP-dependent process across the vesicles.

1.5.2.2.3 Oocytes. Xenopus laevis oocytes, transfected with SLC transporters, have also been useful for studying SLC-transporter-mediated uptake studies. Recently, MDR1-mediated drug transport has also been investigated in this model [85]. Hepatic disposition of irinotecan and its glucuronide metabolite and the effect of OATP1B1 polymorphism on its uptake were investigated using this model [86].

1.5.2.2.4 B-CLEAR. B-CLEAR hepatocytes model has been very useful in investigating biliary excretion of compounds—primary sandwich-cultured human hepatocytes (SCHHs), used in this model, permit the canalicular network to be formed and maintained [87]. Since the uptake and efflux transporters are present in the SCHHs, this model is very valuable not only for biliary excretion prediction of compounds but also for interspecies differences in hepatobiliary disposition (rat vs human), hepatobiliary—transporter-mediated DDI prediction and hepatobiliary toxicity.

1.5.2.3 Metabolism. Since liver is considered to be the primary site of metabolism for the majority of the xenobiotics and endogenous compounds, subcellular fractions, intact cells, and tissue slices, derived from liver, are powerful *in vitro* tools and are routinely used in the pharmaceutical industry to assess the metabolism of NCEs [7]. Subcellular fractions (liver microsomes, microsomes with cDNA-expressed metabolic enzymes, and S9-homogenates) from a variety of rodent and nonrodent animal species, along with human, are commercially available, easy to handle, and amenable to long-term storage. Liver microsomes are the most commonly used *in vitro* system and although they contain enzymes located only in the endoplasmic reticulum, such as CYP, FMO, UGT, and esterases, microsomes are viewed as a very good system for assessing oxidative potential of NCEs. cDNA-expressing microsomes have the advantage of expressing high concentration of one particular enzyme/isoform and are often used when assessing the DDI potential of NCEs. S9 homogenates, although have the advantage over microsomes of containing both oxidative and conjugative enzymes, have not attained as much popularity as the microsomes due to relatively lower enzyme activity levels. Predictability of *in vivo* clearance from the subcellular-fraction-derived *in vitro* Cl_{int} is often questionable because of the need to fortify the subcellular fraction with supraphysiological proportions of cofactors [e.g., NADPH (Nicotinamide adenine dinucleotide phosphate), NADPH reductase, $MgCl_2$, and cytochrome b5]. In spite of the mixed success of *in vivo* Cl_{int} predictions from subcellular-metabolism-derived data (more discussed in the IVIVC section later), it is well accepted that in the early

stages of drug discovery, the subcellular fractions play an extremely important role in rank ordering compounds. Intact cells (cryopreserved and fresh hepatocytes) and tissue slices have certain distinct advantages over subcellular fractions in that they possess intact cellular morphology and are therefore believed to mimic the *in vivo* situation more closely than subcellular fractions. It must be remembered that cryopreserved hepatocytes, although mimics compound uptake through the cell membrane as in an *in vivo* situation, however, due to internalization of efflux transporters, compound efflux is not captured. They also do not need any added cofactors to facilitate metabolism, and metabolism studies can be carried out over much more extended time periods than with the subcellular fractions. However, handling and storage of intact cells and especially tissue slices are also lot more complicated than subcellular fractions, which are by far the most commonly used *in vitro* tools to evaluate metabolism of NCEs in drug discovery stages. Several excellent reviews have been published recently to compare the advantages and disadvantages of these various *in vitro* models and overview of experimental conditions required.

1.5.2.3.1 Prediction of Human PK. Metabolic stability at the early discovery stages is commonly expressed as either percent parent compound remaining after incubation for a certain period of time (up to 30 min for microsomes and up to 2/4 h for hepatocytes), but it is highly advisable to translate the percent parent remaining to Cl_{int} (Eq. 1.1, Fig. 1.8). Commonly used methods to calculate Cl_{int} involves determination of either the *in vitro* half-life ($t_{1/2}$) or by calculating the enzyme kinetic parameters; maximum velocity of the enzymatic reaction (V_{max}), and substrate concentration yielding the half of V_{max} (K_m), as shown in Equation (2) in Fig. 1.8 [7].

To gain a better physiological perspective of Cl_{int}, it is further converted to Cl_{hep} via several different methods, such as the well-stirred (Eq. 3, Fig. 1.8) or venous equilibrium model, sinusoidal perfusion or parallel tube model, and physiological-based dispersion mode. In most cases, when the NCE in question is a low clearance compound ($E < 0.25$), Cl_{hep} predicted from either well-stirred or parallel tube models will not be much different. However, for a high clearance compound ($E > 0.75$), Cl_{hep} predicted from parallel tube model yields a higher value than that from a well-stirred model. Hence, it is prudent to take into consideration the nature of the model with high clearance compounds. Once the predicted Cl_{hep} is calculated, the values can be expressed as percentage hepatic blood flow to classify compounds as low (<25%, Q_h), moderate (25–75%, Q_h), and high (>75%, Q_h) Cl. With the recent surge of knowledge in transporters, the traditional Cl_{hep} equation has been modified to incorporate the transporter contribution to total hepatic clearance (Eq. 5, Fig. 1.8).

In early stages of drug discovery, metabolite identification is routinely performed in liver subcellular fractions, cDNA-expressed enzymes, or hepatocytes. Indeed, the results of a recent study suggested that all these systems reliably predicted human excretory and circulating metabolite profiles [88]. Although it is highly preferable to maintain low NCE concentration (1 μM) in incubation mixture to mimic physiologically relevant conditions, analytical difficulties in detection of metabolites that are formed in low quantities force most metabolite identification reactions to be performed around 10 μM or higher (depending on the extent of metabolism of the NCEs). At supraphysiological incubation conditions of most met Identification (ID) studies, the biotransformation pathways can sometimes be different from *in vivo* situation. In majority cases, this difference is mostly quantitative rather than qualitative—in other words,

Calculation of Clint

$$Cl_{int} = \frac{0.693}{\textit{in vitro } T_{1/2}} * \frac{\text{mL incubation}}{\text{mg microsomes}} * \frac{\text{45 mg microsomes}}{\text{gm liver}} * \frac{X \text{ gm liver}}{\text{kg BW}} \quad (1)$$

X = 20(human); 25-(dog, Pig); 30(Cyno); 45(Guinea pig, rat); 87.5(Mouse)

$$Cl_{int} = \frac{V_{max}[S]}{K_m + [S]} \text{ at } S << K_m, \text{ simplifies to } Cl_{int} = \frac{V_{max}}{K_m}$$

(Involvement of one enzymes)

$$Cl_{int} = \frac{V_{max1}[S]}{K_{m1} + [S]} + \frac{V_{max1}[S]}{K_{m1} + [S]} \text{ at } S << K_m, \text{ simplifies to } Cl_{int} = \frac{V_{max1}}{K_{m1}} + \frac{V_{max2}}{K_{m2}} \quad (2)$$

involvement of two enzymes

Calculation of Cl_{hep} via Well-stirred model

$$Cl_{hep} = \frac{Q_h Cl_{int}}{Q_h + Cl_{int}}$$

(No protein binding)

$$Cl_{hep} = \frac{Q_h \left(\frac{1}{B/P}\right)(f_{u,serum}/f_{u,microsome})\, Cl_{int}}{Q_h + \left(\frac{1}{B/P}\right)(f_{u,serum}/f_{u,microsome})\, Cl_{int}} \quad (3)$$

(Plasma and microsome protein binding and blood : plasma partitioning)

$$Cl_{hep} = \frac{Q_h f_{u,blood}(Cl_{int}/f_{u,microsome})}{Q_h + f_{u,blood}(Cl_{int}/f_{u,microsome})}$$

(Blood and microsome protein binding)

Equation for extraction ratio

$$E = \frac{f_{u,plasma} Cl_{int}}{Q_h + f_{u,plasma} Cl_{int}} \qquad E = \frac{f_{u,plasma} Cl_{influx} Cl_{int}}{Q_h Cl_{efflux} + Cl_{int}(Q_h + f_{u,plasma} Cl_{influx})} \quad (4)$$

Incorporating transporters

Calculation of Cl_{hep} incorporating transporters

$$Cl_{hep} = \frac{Q_h f_{u,plasma} Cl_{influx}(Cl_{int,sec} + Cl_{int,met})}{Q_h (Cl_{efflux} + Cl_{int,sec} + Cl_{int,met}) + f_{u,plasma} Cl_{influx} (Cl_{int,sec} + Cl_{int,met})} \quad (5)$$

Cl_{int} = intrinsic clearance
Cl_{hep} = hepatic clearance
$[S]$ = substrate concentration
V_{max} = maximum reaction velocity
K_m = substrate concentration that results in half of maximum velocity
Q_h = hepatic blood flow
$f_{u,blood/serum/plasma}$ = fraction unbound in blood/serum/plasma
B/P= blood : plasma partition ratio
Cl_{influx} = intrinsic clearance of influx
$Cl_{int,sec}$ = intrinsic clearance of biliary secretion
$Cl_{int,met}$ = intrinsic clearance of metabolism
Cl_{efflux} = intrinsic clearance of efflux

Figure 1.8 Methods to calculate Cl_{int} and Cl_{hep}.

even at artificially higher NCE concentrations, commonly used for met ID studies, the profile of metabolites would not alter significantly (in most cases), although their relative amounts and the enzymes involved in their formation may vary significantly. Very sensitive analytical LC/MS/MS- or LC/NMR-based methods have revolutionized for metabolite identification, quantitation, and characterization [89–97].

1.5.2.4 Reactive Intermediate Trapping. Reactive metabolites such as electrophilic intermediates or radicals are unstable, usually formed in small amounts, and rapidly react with nucleophiles and can, therefore, easily be missed in routine metabolism studies. Hard electrophiles react with basic groups in DNA and lysine residues in proteins ("hard nucleophiles"), while soft electrophiles react with the thiol groups of GSH and cysteine residue in proteins ("soft nucleophiles"). Radicals do not covalently bind to proteins but abstract a hydrogen atom from macromolecules. Direct detection of these reactive intermediates has proven almost impossible both *in vitro* and *in vivo* due to their inherent reactive nature. The basic methods to trap the reactive intermediates have been well established and have been used for screening of new chemical entities in a high throughput manner in early drug discovery. Majority of reactive metabolites generated are "soft" and are trapped by the endogenous thiol GSH and *N*-acetyl cysteine, while the "hard" electrophiles are trapped by the hard nucleophiles such as cyanide, methoxylamine, and semicarbazide. The trapped adducts are detected and identified by LC/MS/MS and Nuclear Magnetic Resonance (NMR).

In addition to commonly used GSH and Cyanide (CN) trapping of reactive intermediates, some of the recently emerging methods to measure "biochemical markers" of toxicity, resulting from reactive intermediate formation is also gaining popularity [98]. For example, increase in alanine transaminase level as an indicator of heptotoxicity, induction of GSH transferase and quinone reductase as indicators of cells stress, and measure of mitochondrial function for cell toxicity have been proposed as methods that could be used for risk assessment toward reactive metabolite-mediated IDRs. Use of gene chips to explore gene expression patterns to identify individuals susceptible to IDRs has also been proposed. However, as with other existing methods, quantitative risk assessment and its clinical relevance remains a challenging hurdle for all these methods. Covalent binding assessment has also been extensively used as the gold standard for reactive intermediate screening [99]. However, expert opinion in this area emphasizes that the *in vitro* covalently binding studies are best used to *retrospectively* investigate mechanism of toxicity when encountered in clinical setting and is valuable in identifying imminent bioactivation pathways of NCEs and rank ordering NCEs in discovery stages. However, efforts to quantitatively link the degree of covalent binding to clinical toxicity should not be attempted *prospectively* [7,100–102].

1.5.2.5 CYP Identification/Mapping. In early discovery phases, a qualitative idea of the major route of elimination contributing to the total clearance of an NCE is sufficient. To tease out contribution of FMO- and CYP-mediated metabolism, it is common to incubate microsomes at 50°C for 2–3 min, as FMOs, but not CYPs, are thermally labile enzymes which can be denatured by incubating the reaction mixture in the absence of NADPH at 50°C.

During the late discovery stage, a little more rigorous method called *enzyme mapping* is implemented. Enzyme mapping helps identify the DMEs that are responsible for the overall clearance of an NCE. In early discovery stages, mapping is most commonly

done via the use of commercially available recombinant human enzymes (CYP, UGT, FMO, and NAT). Major caveat of this method is that since recombinant enzymes do not accurately represent physiological expression and specific activities of the enzymes, the results observed in recombinant enzymes are unable to give relative contribution of an enzyme to the total metabolism of the NCE *in vivo*.

When NCE is moved further into late stage discovery or early development, a more rigorous CYP phenotyping is performed where a more quantitative estimation of contribution by a CYP isoform to total NCE clearance is evaluated via a stepwise integrated approach [7,34,103].

Step 1. Assessment of metabolite formation of an NCE in a pooled Human liver microsomes (HLM) (20–50 donors to reduce intersubject variability) and ruling out non-CYP-mediated pathways.

Step 2. Enzyme kinetic parameters V_{max} and K_m for each major biotransformation pathway are determined under linear conditions (time and protein) and careful analysis of resulting Eadie–Hoftsee plots (rule out multiple CYPs).

Step 3. Effect of CYP-isoform-specific chemical inhibitors on the particular biotransformation pathway (in HLM) is assessed (with $[S] \leq K_m$ of the NCE and $[I]/K_i > 10$ of the CYP-specific inhibitor).

Step 4. CYP-isoform-specific inhibitory antibodies are also used to evaluate relative contribution of different CYP isoforms toward NCE metabolism to complement data from chemical inhibitors (step 3).

Step 5. Relative activity factor method or intersystem extrapolation factor method are also utilized to gain an insight into relative contribution of the CYPs.

Sometimes other methods, such as, total normalized rate method, correlation analysis, or use of genotyped liver (in case where polymorphic enzymes are involved) have all been reported [34,103].

1.5.2.6 CYP Inhibition. Inhibitory potential of an NCE is routinely determined in early compound optimization stages by a simple, model-independent, IC_{50} determination assay in liver microsomes or cDNA-expressed protein [104,105]. Use of liver microsomes is more common in the case of CYPs which have specific/highly selective probe substrates and inhibitors available, while use of cDNA-expressed enzyme is more common in the case of UGT, NAT, SULT, and GST, for which isoform-specific/selective substrates and inhibitors are not well established and/or validated. Use of human hepatocytes-plasma system to predict inhibition-based DDI has also been demonstrated [106]. IC_{50} determination, in *in vitro* assays, is usually performed at one substrate concentration ($S \leq K_m$), and a wide range of inhibitor concentrations to monitor the decrease in reaction velocity with increasing inhibitor concentration. An NCE is classified as potentially high, medium, or low DDI risk based on whether its IC_{50} value for a particular enzyme is <0.1, $0.1-1$, or $>1.0\,\mu M$, respectively. IC_{50} values should be compared to maximum predicted NCE plasma concentration (C_{max}) at steady state and only if IC_{50} values are greater than C_{max}, a potential DDI risk *in vivo* can be anticipated. Drawback from risk prediction based on IC_{50} values is that IC_{50} values are heavily dependent on assay-dependent parameters such as the nature of subcellular fraction (pooled microsomes, cDNA-expressing microsomes, and heptocytes); concentrations of the substrate, inhibitor, and enzyme; incubation time; solvent effect;

choice of marker substrate used especially for CYP3A4 and 2C9; solubility limit of inhibitor and substrate; and extent of protein binding of inhibitor.

A more rigorous assessment of inhibitor potency, usually performed for late stage discovery compounds, is via determination of K_i, which requires thorough kinetic analysis of a specific biotransformation pathway of interest. Eadie–Hoftsee (or Lineweaver–Burke) plots are generated using multiple substrate and inhibitor concentrations and the mechanism of inhibition (competitive, noncompetitive, uncompetitive, or mixed) is assigned based on model-fit of the data, defined by statistical criteria. K_i value, which unlike the IC_{50} value, is a more intrinsic number, is substrate independent, and yields a superior ranking method than IC_{50} determination. Evaluating an NCE's inhibitory potential involves assessment of its $[I]/K_i$ ratio, where $[I]$ represents the mean C_{max} (peak plasma) of inhibitor at steady state, after highest proposed clinical dose and K_i is the dissociation constant of the enzyme–inhibitor complex. An inhibition is considered high, medium, or low risk based on whether its $[I]/K_i$ ratio is >1, $0.1-1$, or <0.1, respectively.

1.5.2.7 CYP Induction. Reporter gene assays, such as the PXR–nuclear receptor binding assay and PXR-transactivation assay are rapid and high throughput assays are used to evaluate an NCE's potential for CYP induction. Induction has also been studied in other human hepatocytes cell lines such as HepG2, Fa2N-4, BC2, HepaRG, human intestinal cell line LS174T, and breast cancer cell line MCF-7 [79]. Each of these cell lines have their limitations, which need to be thoroughly considered before using these cell lines for predicting enzyme induction. Primary SCHHs model is deemed as the "gold standard" by DMPK scientists. Under optimal experimental/culture conditions, primary human hepatocytes maintain physiologically relevant receptors, DMEs, and transporters, all under their regulatory control and hence is a very good model to concurrently evaluate metabolism, inhibition, and induction. It has been observed that induction results obtained with primary hepatocytes cultures correlate well with clinical results. Readers are advised to refer to these excellent reviews that discuss in detail *in vitro* methodologies and optimization of experimental conditions such as culture format, number of donors, culture medium, confluence number, culture period, and so on, for studying enzyme induction with primary cultured hepatocytes. Endpoint readout in induction studies usually involves a combination of measuring the mRNA levels or protein levels or activity of the target enzyme(s) [36,107,108].

Frequently, in early discovery stages, a simple rank order approach based on C_{max}/EC_{50} ratio (C_{max}, maximum plasma concentration of inducer after administration of the highest anticipated clinical dose; EC_{50}, concentration of inducer where 50% of maximal induction is observed) is used. A high, medium, or low risk is associated to whether the C_{max}/EC_{50} ratios are >1, $0.1-1$, or <0.1, respectively. A more reliable method, called the *relative induction score* (RIS) method is also used to better predict clinical DDI based on *in vitro* induction data (Fig. 1.9) [108]. RIS takes into account the maximum induction response in addition to C_{max} and EC_{50}. The RIS method has also been modified to incorporate an additional No adverse effect level (NOAEL) term (Fig. 1.9), which is the highest concentration at which no induction is observed for an NCE. Generally, the DDI guidance by FDA recommends that NCEs that increase a particular isoform's enzyme activity by 40% of the positive control should be regarded as an inducer for that particular enzyme (assay done in freshly isolated or attachable cryopreserved hepatocytes in *in vitro* systems from at least three human donors).

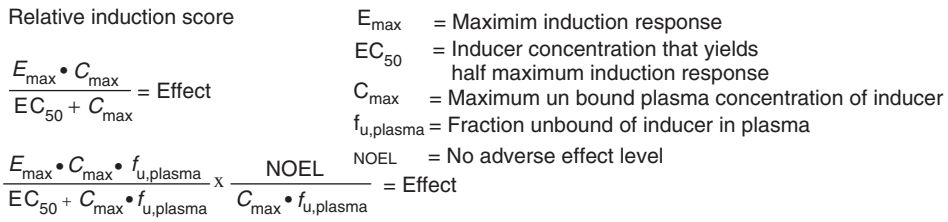

Figure 1.9 Calculation of RIS.

1.5.2.8 DDI Prediction in Transgenic Mice. There has been a significant recent interest in the use of genetically modified mouse models to assess DDI potential and toxicity prediction [109]. Double transgenic mouse (expressing human CYP3A4/PXR, CYPs 3A4/2D6, and OATP1B1), knockout (KO) mouse (specific drug disposition enzyme- and transporter-null mouse and SOD2 null mouse), humanized mouse (mouse expressing human CYPs PXR and PPAR), chimeric mouse with humanized liver have truly revolutionized ways to investigate the mechanism of human-specific biotransformation and toxicity [7]. However, *quantitative* prediction of clinical DDI and toxicity in human from these models is yet to be thoroughly established.

1.5.2.9 Plasma Protein Binding. The "gold standard" methods for PPB are equilibrium dialysis, ultrafiltration, and untracentrifugation [110–112].

1.5.2.9.1 Equilibrium Dialysis. Its simple setup involves two chambers, usually plasma and buffer, separated by a dialysis membrane (MW cutoff) and an NCE (known volume and concentration) is introduced in the plasma chamber. Equilibrium between the two chambers is allowed to establish, whereby concentration of *free* NCE should be same across both sides of the membrane via simple diffusion. Quantitation of an NCE in the buffer side and the plasma side gives an estimate of percentage free NCE. This generates rapid, accurate, and quantitative data, with simple experimental setup. Caution should be exercised to minimize high membrane binding, volume shift due to osmosis, and Gibbs–Donnan effects.

1.5.2.9.2 Ultrafiltration. The assay principle is to separate a small volume of protein-free phase from plasma, containing known NCE concentration, by filtration through a semi-permeable membrane, and measure the concentration of unbound NCE in the filtrate. Also it provides the rapid, accurate, and quantitative data with simple experimental setup. Attention should be paid to membrane binding, sieve effects, Gibbs–Donnan effects, along with temperature and pH control.

1.5.2.9.3 Ultracentrifugation. This method involves application of a high centrifuge force ($100,000 \times g$ or more) to a drug solution (no membrane is required) for a prolonged period (e.g., 15 h) to sediment the protein to the bottom of the tube. The unbound drug concentration in the protein-free supernatant is measured. Although this technique is devoid of membrane-related issues or change in total drug concentration, sedimentation of drug molecules, floating lipid layer, extensive experimental setup and time, along with the need to control temperature and pH may pose as hurdles.

1.5.2.9.4 Ex Vivo *PPB.* *Ex vivo* PPB refers to PPB determination of a compound, via conventional PPB methods, in blood samples obtained after dosing the animals or individuals with the compound (most of the time, done with [^{14}C]-labeled compound). *Ex vivo* PPB values have been shown to yield slightly higher values than *in vitro* PPB determination [113] as reported for celecoxib. It is desirable to evaluate *ex vivo* PPB, especially in cases where a compound yields significant amount of metabolite *in vivo*. Metabolites, due to their more polar nature than the parent drug, are usually less plasma protein bound than the parent drug but an *ex vivo* evaluation in these cases captures any interaction between drug and metabolites at physiologically relevant concentration/therapeutic doses.

1.5.3 *In Vivo*

1.5.3.1 Absorption. OBA is defined as the fraction of drug that reaches systemic circulation after oral administration of a drug. It is therefore heavily dependent on how efficiently the drug is absorbed after an oral dose. For orally administered drugs, fraction of dose absorbed systemically F (also defined as *OBA*) is dependent on the fraction absorbed F_a, fraction escaping gut metabolism, F_g, and fraction escaping hepatic metabolism, F_h, given by the following equation:

$$F = F_a \times F_g \times F_h$$

Knowledge of fraction of dose absorbed is very informative for orally administered drugs. Although very accurate information about fraction of dose absorbed is obtained with radiolabeled compounds, yet availability of radiolabeled compound is very limited in early discovery stages and simple mechanistic studies with unlabeled compounds gives a reasonable estimate of this parameter [11,114]. For example, comparison of dose-normalized AUC between oral and intraportal vein dosing gives a reasonable estimate of $F_a \times F_g$, fraction of dose absorbed into portal vein, after an oral dosing (assuming hepatic clearance remains same between the two routes of dosing). $F_a \times F_g$ can also be estimated after oral dosing of a compound and comparison of compound concentration (dose-normalized AUCs) in portal vein versus systemic circulation. Fraction of dose escaping hepatic metabolism F_h can be estimated from either comparing dose-normalized AUCs after intraportal and intravenous (IV) dosing or after an oral administration comparing compound exposure in portal vein versus in systemic circulation (portal vein vs mesenteric artery).

KO and mutation of transporter expression in rodents is a widely used strategy to understand involvement of the silenced or mutated transporters in drug disposition in humans [115] and to explain transporter-mediated toxicity to certain xenobiotics. For example, studies in Npc1L1-null mice revealed the essential role of this transporter in cholesterol uptake across intestines, while Dmt 1-mutated rats showed that this is an important transporter for intestinal absorption of iron. Other KO models have also been valuable in investigating role of OAT, OCT, MRP2, MRP3, MRP6, ABCA1, BCRP, MDR1, MDR2, and MDR3 in hepatobiliary, renal, and brain disposition and toxicity of xenobiotics. Concurrent double and triple-transporter gene KO mice, such as Mdr1a/b/Mrp2 KO [116], Bsep/Mdr1a/Mdr1b KO [115], and Mdr1a/Mdr1b KO [117,118], have also been very useful in understanding the role of multiple transporters in drug disposition.

Metabolite identification of *in vivo* samples is usually done in biological matrices such as plasma, urine, bile, and feces. Ion suppression from a variety of endogenous compounds in the biological matrices is a commonly encountered hurdle in *in vivo* metabolite identification and leads to drastic decrease in MS signal intensity of parent and its metabolites. A strong expertise in sample preparation and novel sample introduction into the LC/MS/MS [e.g., Ultra Performance Liquid Chromatography (UPLC) and nano-flow techniques] tremendously improves success in detection and identification of *in vivo* metabolites. There are a multitude of very good articles discussing several of these modern analytical strategies involved in metabolite identification and are highly recommended for further reading [89–97].

1.6 ADME STUDIES IN DRUG DEVELOPMENT

As described in Section 1.5, most of the ADME studies in drug discovery are conducted using cold material. However, ADME studies in drug development are generally conducted using radiolabeled (^{3}H or ^{14}C) material [119]. The use of radiolabeled material offers a unique mode of quantification of total drug-related molecules.

1.6.1 Absorption

Determination of the amount of drug-related material absorbed proves to be quite cumbersome using unlabeled compound. The use of a radiotracer allows an accurate quantitation of drug and drug-related material that is absorbed and this technique has been employed in studying absorption for several years. The radiolabeled version of the drug is administered orally and intravenously to intact or bile-duct cannulated animals and humans. Urine, bile, plasma, and feces are collected for as long as is necessary to obtain a full recovery of radioactivity [120]. Several methods have been then used to determine the fraction absorbed (F_a).

1. Calculating the percentage of cumulative excretion of radioactive drug-related material in urine and bile from bile-duct animals following oral administration as

$$\text{Absorption}(F_a) = \frac{\%\text{total dose excreted in urine and bile}}{100}$$

2. Measuring the amount of unchanged drug in feces after oral administration from intact animals using as

$$\text{Absorption}(F_a) = 1 - \frac{\%\text{dose of parent drug excreted in feces}}{100}$$

3. Comparing the amounts of total radioactivity excreted in the urine after oral and IV dose as

$$\text{Absorption}(F_a) = \frac{\%\text{dose excreted in urine after PO dose}}{\%\text{dose excreted in urine after IV dose}}$$

4. Comparing the exposure (AUC) of total radioactivity after oral and IV dose as:

$$\text{Absorption}(F_a) = \frac{\text{AUC}_{\text{total radioactivity after PO dose}}}{\text{AUC}_{\text{total radioactivity after IV dose}}} \times \frac{\text{dose IV}}{\text{dose PO}}$$

The extent of absorption of lasofoxifene was investigated in the intact and bile-duct rats after oral administration of single dose of [^{14}C]lasofoxifene [121]. The cumulative excretion amounted to 1.72% in the urine and 95.0% in the feces of intact rats. While in a separate bile-duct cannulated study in rats, 83% of the administered radioactivity was excreted in the bile following oral administration of a single dose of [^{14}C]lasofoxifene. These data suggest that at least 85% of dose (percentage recovery in urine and bile) was absorbed and that the fecal excretion in intact animals likely reflects excretion in the bile rather than incomplete absorption.

Barbhaiya *et al*. [122] and Shukla *et al*. [123] have demonstrated the differences in the extent of absorption of drug-related material and the systemic bioavailability of nefazodone in humans and dogs after oral and IV administration of [^{14}C]nefazodone. Plasma was analyzed for nefazodone and its metabolites, and plasma, urine, and feces were analyzed for total radioactivity. The bioavailability of nefezodone was estimated to be 14% and 15% in dogs [123] and humans [122], respectively (Table 1.3). The recovery of total radioactivity in urine and feces was similar after Oral (route of dosing) PO and IV administration for both dogs and humans, indicating complete absorption of the drug after oral administration (Table 1.3). The low bioavailability of nefazodone was due to first-pass metabolism rather than poor absorption.

1.6.2 Distribution

Drug regulatory agencies require that tissue distribution studies should be conducted with laboratory animals (rats) as a prerequisite for projecting tissue exposure to radioactivity before administration of radioactive drugs to humans. The objectives of this study are to determine the radioactivity in each organ of interest after administering the radiolabeled drug to animals. The concentration of drug-related material (total radioactivity) is determined by whole body autoradiography/whole body autoradioluminography (WBAL) or liquid scintillation counting of tissues of interest.

TABLE 1.3 Mean Total Excretion of Radioactivity and Exposure (AUC) of Nefazodone in Dogs and Humans following Oral and IV Administration of [^{14}C]Nefazodone

	Analyte	Dose (mg/kg)	Route	AUC0-inf	% Dose Excreted	
					Urine	Feces
Dog	Radioactivity	10	IV	25,642	24.9	61.1
		10	Oral	23,997	27.7	59
	Nefazodone	10	IV	6023	—	—
		10	Oral	829	—	—
Human	Radioactivity	5	IV	—	51.6	20.1
		20	Oral	—	49.1	24.1
	Nefazodone	5	IV	160	—	—
		200	Oral	1327	—	—

Prakash *et al*. [121] examined tissue distribution in male and female pigmented rats (Long–Evans) using WBAL following administration of a single oral dose of [^{14}C]lasofoxifene. [^{14}C]Lasofoxifene-related radioactivity distributed rapidly in the rat with most tissues achieving maximal concentrations at 1 h. Tissue exposure was similar in male and female rats, with the greatest exposure in the uvea. The GI contents had the highest concentrations of [^{14}C]lasofoxifene radioequivalents following oral administration. Half-life of radioactivity was longest in the uvea (124 h) and shortest in the spleen (~3 h). Radioactivity became undetectable in some tissues by 8 h postdose. At 168-h postdose, radioactivity could only be measured in the intestinal contents and uvea. The projected human exposure to ^{14}C radioactivity is estimated by direct extrapolation from the exposure data obtained from the rat quantitative whole body autoradiography (QWBA) tissue distribution study [124–126].

1.6.3 Metabolic Profiling and Identification

In drug development, the metabolite profiles of new drug candidates are generally accomplished by mass balance and excretion studies in which radiolabeled drugs are administered to laboratory animals and humans. The biological fluids are collected, analyzed for total radioactivity, and evaluated for a quantitative profile of metabolites. Metabolite profiling of samples is generally conducted using high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) and radiometric detector. Metabolite quantification is performed by measuring radioactivity in the individual peaks that are separated on HPLC, using a radiometric detector or collecting fractions followed by counting in a scintillation counter.

As an example, the metabolic fate of torcetrapib a cholesteryl ester transfer protein (CETP) inhibitor was investigated in rats, monkeys, mice, and humans following oral administration of a single dose of [^{14}C]torcetrapib [127,128]. More than 28 metabolites were identified in all species, and they were products of oxidation and conjugation pathways [127]. The metabolites identified in humans were detected at least in one preclinical species [128].

In some cases, oxidative cleavage such as N-dealkylation or hydrolysis of the ester and amide could result in splitting of a molecule into two large fragments. In these cases, it is useful to label both sides of the molecule either with one isotope at two different positions or a mixture of two radioisotopes. This strategy has been successfully used in *in vitro* studies of a $GABA_A$ receptor partial agonist [129] and *in vivo* Absorption-metabolism-excretion (AME) studies of torcetrapib [128], ziprasidone [130,131], and gemopatrilat [132].

1.6.4 Excretion

Excretion studies in preclinical animal species used for long-term safety assessment and human volunteers with the aid of the radiolabeled drug provide the total fate of drug-related material. One of the primary objectives of these studies is to demonstrate that the administered dose is readily eliminated from the body, ideally after exerting the intended therapeutic effect. By quantitative collection of the excreta for sufficient time, it not only provides valuable information on mass balance, route, and extent of excretion of the administered radioactive dose but also provides critical information on the clearance mechanism of the drugs.

Several studies describing the procedure for determining the excretion of drugs using [^{14}C]- and [^{3}H]-labeled compounds have been published [128,130,131]. We have described a mass balance and excretion study of torcetrapib in rats, monkeys, and mice by oral administration of [^{14}C]torcetrapib, labeled either at the trifluoromethyl-3,4-dihydro-2*H*-quinoline moiety or at the benzylic carbon of the bis-trifluorophenyl ring [128]. The administered radioactive dose was quantitatively recovered in all species. Excretion of the radioactivity was rapid and nearly complete within 48 h after dosing. In the rats and monkeys, the majority of dose was excreted in feces, while in the mice; the dose was recovered equally in urine and feces. In the separate studies using bile-duct cannulated rats and monkeys, only $<10\%$ of the administered radioactivity was recovered in bile. This suggested that a major portion of dose excreted in the feces of rats and monkeys was mainly due to unabsorbed dose. There were no discernible gender differences in the excretion pattern of radioactivity in these species after oral administration of [^{14}C]torcetrapib [128].

1.7 SUMMARY AND FUTURE PERSPECTIVES

Drug discovery is an extremely time-consuming, expensive, and challenging process, where success is heavily dependent on very thorough understanding of chemistry and mechanism of metabolism of NCEs in humans. Unavailability of data in humans in early drug discovery stages makes it imperative to have well-validated, rapid *in vitro* ADME tools, in complement with relevant *in vivo* preclinical animal models, to accurately predict ADME/PK of an NCE in humans to minimize risk of failure in late stages of development. With advancement of technology, there is a plethora of *in silico* and *in vitro* assays available to model and evaluate each pathway of the complex ADME process such as involvement of transporters and DMEs, possible risk associated with their inhibition or induction, implications of enzyme polymorphism, insight into mechanistic biotransformation pathways, and reactive intermediate formation. It must be borne in mind that each of these *in silico* modeling techniques and *in vitro* assays and systems (e.g., recombinant enzymes, microsomes, hepatocytes, and membrane vesicles) have their limitations, depending on the experimental conditions tested (e.g., types of molecular descriptors in the case of *in silico* models and substrate concentration, buffer, incubation time, pH, and protein concentration, in the case of *in vitro* assays) and results must be carefully interpreted during ADME optimization of an NCE. Preclinical animal models should also be very carefully evaluated since there is significant species and gender difference in biotransformation pathways of NCEs, not only due to differential expression and activity of the transporters and DMEs but also in animal physiology and genetic polymorphism. Designing the correct *in vitro* and *in vivo* experiments and intuitively interpreting and integrating all ADME/PK data are challenging but key steps toward risk mitigation of an NCE in a clinical setting. More often than not, even with the most sophisticated modeling and the best designed experiments, extrapolation of animal data to human fails due to highly species-specific expression levels, tissue localization, substrate specificity and gene regulation of transporters and DMEs, and differences between human and animal physiology. To address these issues, IV microdose and pharmacologically active oral dose strategies for exploratory phase I studies should be employed.

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ABBREVIATIONS

ADME	Absorption, Distribution, Metabolism and Excretion
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
CYP	Cytochrome P450
DDI	Drug–Drug Interactions
DME	Drug-Metabolizing Enzymes
DMPK	Drug Metabolism and Pharmacokinetics
ER	Extraction Ratio
FMO	Flavin Monooxygenase
GI	Gastrointestinal
IDR	Idiosyncratic Drug Reaction
IVIVC	*In Vitro–In Vivo* Correlation
LC	Liquid Chromatography
MAD	Maximum Absorbable Dose
MBI	Mechanism-Based Inactivation
MRM	Multiple Reaction Monitoring
MRT	Mean Residence Time
MS	Mass Spectrometry
MW	Molecular Weight
NAT	*N*-Acetyl Transferase
NCE	New Chemical Entity
OBA	Oral Bioavailability
PAMPA	Parallel Artificial Membrane Permeability Assay
PK	Pharmacokinetics
PK/PD	Pharmacokinetics/Pharmacodynamics
PPB	Plasma Protein Binding
R&D	Research and Development
QWBA	Quantitative Whole Body Autoradiography
SAR	Structure–Activity Relation
SULT	Sulfotransferase
TMT	Thiopurine Methyl Transferase
UGT	Uridine Glucuronosyl Transferase

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