The Role of Absorption, Distribution, Metabolism, Excretion and Toxicity in Drug Discovery

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Abstract: Major reasons preventing many early candidates reaching market are the inappropriate ADME (absorption, distribution, metabolism and excretion) properties and drug-induced toxicity. From a commercial perspective, it is desirable that poorly behaved compounds are removed early in the discovery phase rather than during the more costly drug development phases. As a consequence, over the past decade, ADME and toxicity (ADMET) screening studies have been incorporated earlier in the drug discovery phase. The intent of this review is to introduce the desirable attributes of a new chemical entity (NCE) to the medicinal chemist from an ADMET perspective. Fundamental concepts, key tools, reagents and experimental approaches used by the drug metabolism scientist to aid a modern project team in predicting human pharmacokinetics and assessing the "drug-like" molecule are discussed.

INTRODUCTION

In recent decades, the process of drug discovery (from leading to the selection of a clinical candidate) and development (from a clinical candidate to final FDA approval) in the pharmaceutical industry has become an increasingly time-consuming and costly endeavor [1]. On average, it will take approximately 10-15 years and $600-$800 million to develop a successful drug product [2]. Major reasons preventing drugs reaching the market are because of pharmacokinetic (PK) difficulties and toxicities in man (50%) and a lack of efficiacy (29%) [3]. The failure rate due to PK and toxicity problems may be greater than that which was reported because inappropriate PK properties such as lack of oral absorption, unfavorable distribution, or rapid metabolism or excretion and a drug-induced toxicity may be clinically manifested as a lack of efficacy [2].

Hence, it is very important to design "drug-like" molecules. While the phrase "drug-like" is used in slightly different ways by different authors [4-6], it is generally means "molecules which contain functional groups and /or have physical properties consistent with the majority of known drugs" [7], which directly refer to those compounds that have sufficiently acceptable PK and toxicity profiles to survive through the completion of human Phase I clinical trials [1]. As a result, a paradigm shift has occurred in the initial phases of drug discovery. In addition to paying attention to the traditional concern of attaining potency and selectivity towards the biological target of interest, companies also take account of PK and toxicity considerations at an early stage [8].

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There are four major components of PK, which are commonly referred to as absorption, distribution, metabolism and excretion (ADME). Absorption is the process by which a drug proceeds from the site of administration to the systemic circulation. Distribution is the movement of drug molecules from the systemic circulation to extravascular sites. Metabolism describes the enzymatic breakdown of a drug to metabolites that are subsequently cleared from the body. Excretion describes passive or active transport of intact drug molecules into the urine and/or bile [9]. For the purpose of this review, toxicity it defined as the adverse effects of drug molecules on animals or humans.

An ideal drug candidate possesses both good pharmacological activity (potency and selectivity) as well as good ADME and toxicity (ADMET) properties. For example, compounds that show high potency *in vitro* may prove later to have no *in vivo* efficacy, or to be highly toxic in *in vivo* models. Lack of *in vivo* efficacy may be attributed to undesirable pharmacokinetic properties, such as poor absorption or short half-life. The toxicity may result from the molecule itself or formation of reactive metabolites.

In the past 15 years, combinatorial chemistry, a new approach to the identification and optimization of drug leads in medicinal chemistry, has been enormously successful in synthesizing large number of compounds for pharmacological screening [10]. In response, high throughput screening (HTS) methods are being developed to rapidly identify lead compounds in combinatorial libraries that interact with specific receptors or show desirable pharmacological effects. As a consequence, a demand for high throughput (HT) ADMET screening has also arisen. One challenge has been the need for quantitative analysis of a large number of diverse compounds. Fortunately, advances in mass spectrometry have aided this task by providing

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selectivity and sensitivity that is frequently unavailable with UV, fluorescence, or radioactivity detection [11]. The availability of robotic liquid-handling systems now has resulted in the automation of liquid-liquid or liquid-solid phase extraction in 96-well plates [12], thereby significantly reducing the sample preparation time and have a significant positive impact on throughput of *in vitro* ADMET and *in vivo* cassette dosing PK [13, 14].

Due to ethical constraints, relevant ADMET properties have to be extensively assessed in human *in vitro* systems and /or laboratory animals. Although the accuracy of prediction from human *in vitro* systems and /or laboratory animals to human is still evolving, it is generally believed that the current predicting tools can be reasonably well extrapolated to human with the application of appropriate pharmacokinetics principles [15, 16].

The intent of this review article is to introduce the medicinal chemists to desirable attributes of a new chemical entity (NCE) from a drug metabolism discipline perspective.

Fundamental concepts, key tools, reagents and experimental approaches used by the drug metabolism scientist to aid a modern project team in predicting human pharmacokinetics and assessing the potential "drug-like" human agent are discussed. The scope of this article is limited to small molecules with oral delivery (immediate release preparations) as the intended route of administration in man.

Figure **1** summarizes common experimental screening tools/reagents available in drug discovery that can be used to help avoid undesirable ADMET properties in the lead optimization process.

INTESTINAL ABSORPTION

In vivo drug absorption may be broadly described as the process by which unchanged drug proceeds from the site of administration to the systemic circulation [17]. The primary sites of absorption include the gastrointestinal tract, and skin. Drug absorption may occur from other sites depending on the route of administration. An ideal "drug-like" molecule undergoes rapid and complete absorption from a commercially viable dose formulation with limited inter-subject variability and minimal food effects. Extravascular dosing (or any route other than intravenous) requires an absorption step. Intestinal absorption is not the complete picture of absorption in the systemic circulation but a starting point for assay development in early drug discovery.

To maximize patient compliance, oral dosing is the most preferred and convenient route of administration. Following oral administration, a drug must pass through intestinal cell membranes via passive diffusion, carrier mediated uptake, or active transport processes prior to reaching the systemic circulation. Many potential drugs will inevitably fail due to poor oral absorption. Several screening paradigms that include absorption have been employed to enhance the

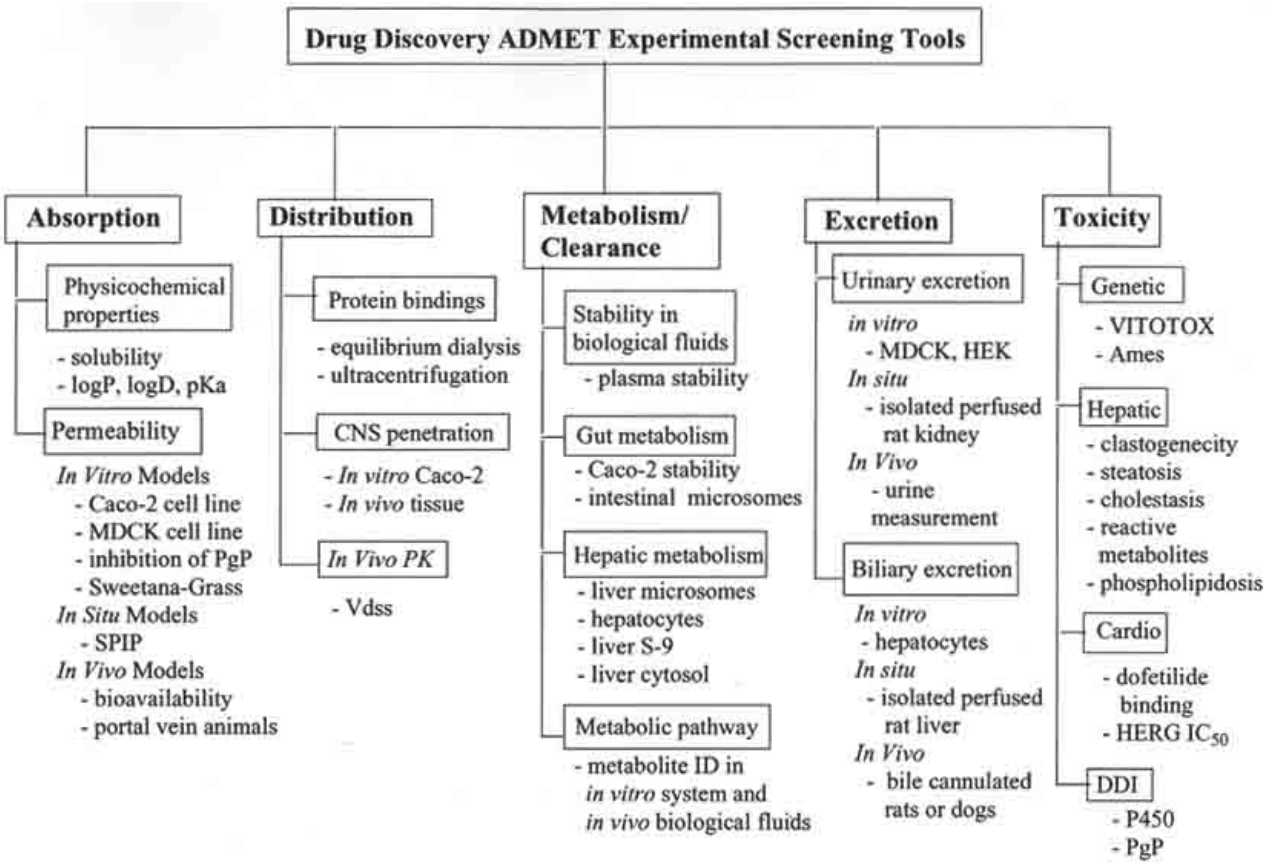


Fig. (1). Summary of ADMET experimental screening tools for small molecule drug discovery

probability of success through the drug development stage. Methods to assess absorption rely on *in situ* or *in vivo, in silico or in vitro* models used alone or in combination.

Passive diffusion, a dominant mechanism by which drugs cross the gastrointestinal tract, occurs primarily through the transcellular route (through cells), and a lesser extent, the paracellular route (between cells) [18, 19]. Simple estimates of passive diffusion can be obtained using physicochemical properties such as LogD, LogP, ionization state (pKa), hydrogen bonding, molecular size and solubility [20, 21]. Whole cell models such as Caco-2 and MDCK have been used to model intestinal permeability [19, 21, 22]. Artificial membrane (parallel artificial membrane permeation assay (PAMPA), and immobilized artificial membrane (IAM) columns) methods have evolved with efforts to increase throughput, reduce variability and/or assign rank order permeability values [23, 24]. Computational (*in silico*) models have evolved to predict permeability based on more easily obtained physicochemical, molecular properties, or molecular descriptors [25]. *In silico* approaches offer the highest throughput potential and are useful for virtual chemical libraries, but may lack enough detail to rank order drug absorption. Experimental approaches for predicting human absorption of drugs have been extensively reviewed elsewhere [18, 26-30], and a summary of the current experimental models is shown in Table **1** [20-24, 27, 31-39].

Absorption and Bioavailability *In Vivo*

While the gastrointestinal tract represents the first barrier to drug absorption after oral administration, it is the dynamic interplay between absorption, metabolism, distribution, and elimination that determines the amount of drug in the plasma. Several parameters define the complete pharmacokinetic profile of a drug and are described in detail elsewhere [17, 40]. Two important pharmacokinetic parameters for understanding absorption following an extravascular dosing are area under the curve (AUC) and bioavailability (F).

Rate limiting steps to oral bioavailability may include: dissolution, permeability, gut motility, and degree of ionization. Additional determinants of bioavailability include first-pass effects. First-pass effects refer to the loss of drug as it passes, for the first time, through sites of elimination following absorption through gastrointestinal tract and liver cell membranes. First-pass effects lower the amount of drug passing to the general circulation by

Table 1. Summary of Models for Absorption Prediction

System	Models	PROS	CONS	Reference
In Silico	Various computational approaches; Using SAR/Molecular Properties	HTS (~1000s or more/day); Easy to use; Currently work is ongoing to capture active transport and metabolism parameters.	Currently best models predict passive permeability; Model is only as good as the quality/quantity of data used in the training set; Models can be series/species specific	20, 25, 31
	PAMPA (Parallel Artificial Membrane Permeation Assay)	HTS (100s-1000s/day); Easy to Use; Easy analyze; Relatively low variability.	Transcellular only; No active transport; High drug concentration needed - solubility issues.	23
	IAM (Immobilized Artificial Membrane) Chromatography	Easy to use; Easy to analyze; Relatively low variability.	Transcellular only; No active transport; Not HTS.	24, 32, 33
In Vitro	Cell Based Systems (Caco-2 /MDCK)	Can be moderate to HTS (~10s to 100s/day); Active and passive transport included.	Can be analytically challenging; can underestimate paracellular; More variability if culturing/assay conditions not controlled.	21, 22, 34
	BBMV (Brush Border Membrane Vesicles)	Moderate throughput; Active and passive transcellular transport	More labor intensive than simple transwell systems; No paracellular transport information; Can be analytically challenging; Can be variable.	35, 36
	Sweetana-Grass	Versatile – can evaluate different species	Low throughput	37
In Situ	Single Pass Intestinal Perfusions (SPIP)	Closer to *in vivo* Situation; Relevant transporters; Relevant tight junctions.	Labor intensive; Species differences; Affect of anesthesia/surgery on transporter expression; Low throughput.	38, 39
In Vivo	Portal Vein Animals	Relevant transporters; Relevant tight junctions; Information on first pass metabolism.	Labor intensive; Species differences; Low throughput.	27

extracting it through metabolic and transport mechanisms. Oral bioavailability may, therefore, be represented as the product of the fraction absorbed by the gastrointestinal tract (F_a) and the fractions in the gastrointestinal tract (F_g), liver (F_h), and lung (F_l) which are not subject to first pass effects: $F = F_a \cdot F_g \cdot F_h \cdot F_l$ or $F=F_a(1-E_g)(1-E_h)(1-E_l)$ where E represents extraction ratios of gut (E_g), liver (E_h), and lung (E_l). For brevity, when the molecule is mainly metabolized by the liver (i.e, $E_g = 0$ and $E_l = 0$), the bioavailability F can be expressed as Equation 1.

$$F=F_a (1-E_h) \text{ -- } (1)$$

where E_h is the metabolic hepatic extraction ratio and Fa is the intestinal fraction absorption.

In vivo measures of drug absorption in laboratory animals are often used to predict human absorption. Rat is the primary species used in toxicology studies and often used to determine F. When comparing the percent of an oral dose absorbed in rat versus humans, a significant correlation was observed; however, it is unclear whether this correlation holds for all chemical series [41]. To determine the percent of bioavailability, animals are dosed both orally and intravenously, and drug concentrations in plasma are determined as a function of time. AUC is determined from these studies as a primary measure of the amount of drug present in the systemic circulation. AUC is derived directly from plasma concentration-time curves and calculated using a numerical method (linear trapezoidal). The AUC represents the amount of drug absorbed into the systemic circulation. F is defined as the amount (and rate) of drug reaching the systemic circulation [40]. Absolute F is defined as Equation 2 and commercially available software programs, such as *WinNonLin 3.2* (Pharsight Inc.) provide rapid PK parameter calculations.

$$F = (AUC_{oral}/Dose_{oral}) / (AUC_{iv}/Dose_{iv}) \text{ -- } (2)$$

In Vitro Models of Intestinal Absorption

In vitro models are generally focused on determining membrane permeability to predict intestinal absorption. While the highest throughput potential remains with *in silico* approaches, *in vitro* measures of permeability offer an added level of confidence for developing *in vitro-in vivo* correlations. Many *in vitro* approaches focus on the prediction of passive permeability while some offer potential for the evaluation of active transport processes.

(1). IAM/PAMPA.

In vitro chromatographic models, such as immobilized artificial membranes (IAM) and parallel artificial membrane permeability assays (PAMPA), have been designed to mimic phospholipid bilayers for the permeability predictions. IAM/PAMPA permeability has shown correlation both *in vitro*, with Caco-2 cells [27] and *in vivo* in whole animal rodent studies [23, 33].

(2). Caco-2 and MDCK Cells.

Caco-2 cells, derived from a human colorectal carcinoma, are one of the most widely used *in vitro* models for understanding intestinal absorption and deciphering mechanisms of transport. With respect to passively absorbed drugs, a correlation between Caco-2 cell permeability and human intestinal absorption has been established [19, 21].

In order to measure cell membrane permeability in an *in vitro* monolayer-based assay, test compounds are added onto the apical side (A) of a monolayer, and their appearance in the basolateral compartment (B) indicates membrane permeability. Permeability measurements for *in vitro* cellular models are generally described by an apparent permeability coefficient (Papp) and calculated according to equation 3.

$$Papp=dQ/dt * 1/A*Co \text{ -- } (3)$$

where dQ/dt is the permeability rate (nmol/sec); Co is the initial substrate concentration in the donor compartment,; and A is the surface area of the monolayer (cm^2).

Papp values (cm/sec) < $1x10^{-6}$, 1-$10x10^{-6}$, and > $10x10^{-6}$, may be associated with low, moderate and complete absorption, respectively. It is important to note that the Papp ranges associated with absorption predictions can vary greatly from laboratory to laboratory [42]. Inter-laboratory variability in Caco-2 cell populations and culturing methodology may be the primary reason for the lack of correlation when comparing results from different laboratories [28]. Compounds such as mannitol, a paracellularly absorbed compound, can show 50-fold variation in Papp values determined from different sources [43]. Similarly, inconsistent results from laboratory to laboratory are also seen with transcellularly-transported substrates [42].

Despite the inter-laboratory variability observed with Caco-2 cells, they are useful, not only for human absorption predictions but, also for the study of several transport systems. This is due to the fact that they express transporters for sugars, amino acids, bile acids, and; thus, are a relevant system to assess transport for many drugs [44]. Most transporter-based studies, however, focus on the use of Caco-2 for evaluating MDR1 (P-glycoprotein, PgP) interactions. The significance of PgP in limiting drug absorption and bioavailability is well established [45]. Several factors are of concern, when assessing PgP activity in Caco-2 cells. PgP expression levels may vary several-fold in both human intestine [46], and Caco-2 cells [47]. Caco-2 culture time and passage number appear to modulate PgP expression [47]. Cell trypsinization and passage prior to confluence have revealed that the higher passage numbers express more PgP than lower passage numbers [47]. This means that culture conditions must be held constant to ensure consistent levels of PgP when testing chemical series over several passages of cells. Since Caco-2 cells express many transporters in addition to PgP, they can also be used to assess drug-drug interactions with multiple transporters, the details of which will be discussed in the Toxicity section of this review. This however, can also be a limitation for Caco-2 cells as one can have difficulty in definitively assigning efflux activity to MDR1.

For HTS screening purposes, the use of MDCK, derived from dog kidney, has become increasingly popular. While

Caco-2 cells require a three-week culture time (21 days in culture) to fully differentiate (i.e. establish tight junctions, cell polarity, and membrane transporter expression) MDCK cells form complete monolayers after 3 days of culture [27]. In addition, human MDR1-transfected MDCK cells are available that significantly overexpress human MDR1 when compared to endogenous transporters. As such, these cells offer superior resolution of transporter-specific efflux as compared to Caco-2 cells and; thus, are more amenable to definitively identifying human PgP-specific substrates.

While Caco-2 and MDCK cells are useful screening models to predict human absorption, both systems lack the expression of clinically significant drug metabolizing enzymes such as CYP3A4, an enzyme that is highly expressed in human intestine. The role of both intestinal CYP3A4 and PgP in limiting oral bioavailability has been established [48]. Thus, cell monolayer permeability assays such as Caco-2 or MDCK can lead to over prediction of absorption potential if a compound is significantly metabolized by intestinal CYP3A4.

However, Caco-2 cells in combination with metabolic lability (i.e. hepatocytes or microsomal assays) may be used to improve bioavailability within a structural series of compounds. Using a graphical method, permeability data from Caco-2 studies and half-life data from microsomal studies may be used to predict bioavailability [49]. In that study, a plot of Caco-2 cell permeability versus parent disappearance after 30 minutes of microsomal incubation yielded an oral bioavailability estimation map. Compounds were binned into low, medium, and high bioavailability based on their location within the x, y plot.

(3). Sweetana-Grass

The Sweetana-Grass method represents a modified version of the Ussing chamber for the measurement of drug movement across intestinal tissue [37]. To measure permeability, tissue is mounted between two acrylic half-cells and drug concentrations are measured in both the donor and receiver chambers with time of incubation.

In Silico Models and Physicochemical Properties

In silico approaches to predict permeability or absorption potential are based on physiology and physicochemical properties including: solubility, charge, molecular weight, molecular shape, polar surface area, hydrogen bonding, lipophilicity, gastrointestinal anatomy and physiology. A retrospective study of the World Drug Index led to the definition of the Rule of Five, a widely used criteria for determining absorption potential as defined by permeability. Poor permeability is predicted when two of the following are true: (a) molecular weight>500, (b) LogP>5, and (c) H-bond donors>5 and/or (d) H-bond acceptors>10 [20].

Aqueous solubility is a primary focus for many computational approaches because it is critical for drug absorption across the gastrointestinal tract. This is because permeation across the intestinal membranes is proportional to the concentration gradient between intestinal lumen and blood [20]. Poor solubility limits absorption resulting in low bioavailability [20, 50]. The solubility(s) of organic nonelectrolytes may be determined using melting point and octanol-water partition coefficient (LogP): $\log S_{predicted}$ (mol/L) = {0.5 – 0.01 (m.p. – 25) – logP} [51]. Melting point and Log P may be obtained in silico (QMPRPlus™) or experimentally. Log P may be also calculated with commercially available software such as CLOGP (www.biobyte.com).

Lipophilicity defines the affinity of a substrate for the lipid bilayer. It can be determined experimentally by measuring compound distribution (partition coefficient) in a biphasic system (i.e. 1-octanol/water) or using artificial membranes [33, 50]. These experimental approaches are less labor intensive than living cell models. In general, cell membrane permeability increases with lipophilicity; however, highly lipophilic compounds (Log P > 5) are poorly absorbed most likely due to solubility limitations [20]. Negative Log P values may be indicative of hydrophilicity and may also be associated with poor absorption because of an inability to cross the lipid bilayer. H-bonding is often a contributor to the potential lipophilicity of a molecule [50]. H-bond donors in a molecule are roughly determined by summation of the NH and OH bonds [20]. H-bond acceptors may be estimated by the sum of Ns and Os attached to at least one H atom in their neutral state [20].

The ionization state (pKa) of a drug has been associated with absorption both *in vivo* and *in vitro*. *In vivo*, the stomach environment is acidic while the intestinal pH can vary from slightly acidic to basic. The stomach preferentially absorbs weak acids whereas weak bases exhibit greater absorption in the intestine. However, it should be noted that it is only the uncharged species that is absorbed via transcellular passive diffusion.

In addition to physicochemical properties, descriptors representing structural fragments are employed in predictive *in silico* models. Physicochemical properties and molecular fragments represent the majority of descriptors currently used in computational approaches. Software providers, including SimulationsPlus, Tripos, and Accelrys, provide user friendly modules to calculate physicochemical properties and structural elements. Software programs such as QMPRPlus™ (www.simulationsplus.com) and IDEA™ (www.lionbioscience.com) can predict bioavailability from structural data with *in vitro* and *in vivo* input (e.g. experimental permeability and metabolic lability data).

In summary, several physicochemical properties are determined experimentally or calculated from the molecular structure alone and have been incorporated into high-throughput discovery computer models. A caveat to the use of *in silico* models is that they are limited to permeability predictions of passively absorbed compounds (i.e. substrates for membrane transporters may not be applied to Rule of Five criteria) [20]. More theoretically derived predictions of absorption may be calculated with the use of physiologically based absorption models [23, 31], but these models presently require experimental input such as Caco-2 or MDCK permeability and rate of dissolution [52].

A refinement of descriptors may be necessary to better predict absorption, and incorporation of metabolic lability and permeability data can aid in the prediction of bioavailability. Physicochemical properties are not independent of each other when used to assess [53]. Increasing molecular weight tends to increase log P which in turn decreases solubility [54]. The most important or correct combination of descriptors for predicting passive transcellular diffusion remains a topic of debate, and is often a function of the particular series under investigation.

Recommendation For Assessing Absorption in Drug Discovery

A variety of *in vivo, in vitro* and *in silico* models are available to measure permeability or determine bioavailability. This has resulted in an ability to rank order drug-like molecules in a series-specific manner to generate structure-absorption relationships. Whole animal studies are the most widely used method of gathering oral bioavailability information, and while *in vivo* studies are the most relevant when considering the complex interplay of parameters that determine bioavailability, they are also the most time consuming and expensive. These caveats have resulted in an impetus to develop *in vitro* and *in silico* approaches to predict *in vivo* absorption and bioavailability. Thus, investigators have a wide variety of tools available to them to measure/predict absorption and bioavailability and should look to establish *in silico-in vivo* correlations or *in vitro-in vivo* correlations when evaluating the absorption potential and bioavaiability of their chemical series.

DISTRIBUTION

Drug distribution describes the movement of drug molecules from the systemic circulation to extravascular. Since most drug targets are not in the vasculature, access to target organs commonly relies on drug distribution. The desired attribute of an ideal "drug-like" molecule will require sufficient drug concentrations attained for the appropriate duration in the target organ for the desired pharmacological effect. The distribution process will be controlled by the passive diffusion across lipid membranes, the presence of carrier-mediated active transport processes involving the xenobiotic, and the protein binding in the blood and tissues [55].

The process by which drugs transverse capillary membrane includes passive diffusion and hydrostatic pressure. Passive diffusion is the main process by which most drugs across cell membranes, which indicates drug molecules move from a high concentration area to a low concentration area. Hydrostatic pressure represents a pressure gradient between the arterial end of the capillaries entering the tissue and the venous capillaries leaving the tissue [56].

Distribution of drugs is generally rapid, and most small lipophilic molecules permeate lipid membranes easily by passive process. Large or more polar molecules do not across lipid membranes by passive diffusion and will require specific transporters to enter the tissue [56]. If a drug does enter a tissue by an active transporter mechanism, its concentration in the tissue may be much higher than that in the plasma.

While the drug molecules can be carried into the target tissues, such as brain, skin, tumors and other organs for drug action (receptor), some drugs can also be distributed to elimination organs, such as liver and kidney.

The drug binding to plasma proteins, lipids and various tissue proteins is very important in drug distribution. Generally, the higher the lipophilicity (logD) of a drug, the stronger its binding to tissue protein and the greater its distribution [57]. High lipophilicity also increases oral absorption and volume of distribution. However, liphophilic molecules also become more vulnerable to metabolism, leading to a higher clearance [58], thus decreasing the drug half-life and lowing the bioavailability.

In this section, three aspecets of the distribution of a molecule will be discussed, i.e., drug protein binding, brain pentration and volume of distribution.

Drug Protein Binding

Many drugs interact with plasma or tissue proteins to form a drug-protein complex named drug-protein binding that may be a reversible or an irreversible process. In plasma, there are two major proteins are likely to bind drugs, i.e., α_1-acid glycoprotein (AAG) with molecular weight at 44,000 D and serum albumin with molecular weight at 65,000 D [59].

The drug-protein bound complex is a macromolecule, which cannot easily cross cell membranes and therefore has a restricted distribution to tissues. Moreover, the protein-bound drug is usually pharmacological inactive. The drug-protein binding also will effect the drug metabolism and elimination processes. Hence, it is very critical to accurately measure the unbound drug in plasma.

The protein binding of the drug can be measured *in vitro* by several methods. The most common method in the pharmaceutical industry is equilibrium dialysis, which uses a semipermeable memberane that separates the protein and protein-bound drug from the free or unbound drug (fu) [60]. Another method is ultracentrifugation that separates molecules based on the different molecular weights. Currently, it is possible to use HT technology to measure drug-protein binding in drug discovery combining 96-well plate and mass spectrometry technologies [61].

The fraction of the bound drug can be as high as >99.9% or as low as < 20%. Drug-protein binding is influenced by several factors, such as drug concentrations. Therefore, it will be important to test the binding potential at the pharmacological and toxicological relevant concentrations.

Brain Penetration

The brain penetration of a drug is different from other organs in several aspects. One of the most important features

is that the brain is completely separated from the blood by the blood-brain barrier (BBB) [62]. Drug must cross the BBB to enter the brain for simple diffusion. Therefore, the ability of a drug to penetrate the BBB is of fundamental importance in drug design. High penetration is needed for CNS (central nervous system)-active drugs, while negligible penetration may be desirable in order to minimize CNS-related side-effects of drugs with a peripheral site of action [63]. Some drugs may cross the BBB by passive diffusion, so physicochemical properties such as charge, lipophilicity and molecule weight are important factors for CNS discovery compounds [64]. On the other hand, the BBB penetration also can be mediated by a complex biochemical interface containing many physiological functions, such as the efflux transporters, especially PgP, located on the apical surface of the endothelial cells of the brain capillaries toward the vascular lumen [65].

Several *in silico* models for prediction of the blood-brain distribution are extensively reviewed [63]. The *in vitro* cell lines, such as Caco-2 and MDCK, are also popular methods in pharmacuticla industry at the drug discovery stage to predict the blood-brain distribution. The *in vivo* animal models are also available to assess the drug penetration to the brain, which include brain-blood partitioning, brain perfusion, the indicator dilution technique, brain uptake index, the capillary depletion technique, and intracerebral microdialysis [66].

Volume of Distribution

Distribution of a drug can be inferred from systemic exposure, or by actual tissue measurement. In general, most data are obtained by the former method. The apparent volume of distribution at steady state (Vdss) of a molecule is a theoretical volume to estimate the extent of drug distribution in the body. In the pharmacokinetic concepts, steady state has often been referred as that the rate of drug entry into the tissue compartment (contains tissues in which the drug equilibrates slowly) from the central compartment (represents the blood, extracellular fluid and highly perfused tissues) is equal to the rate of drug exit from tissue compartment into the central compartment [67]. Although Vdss has no physiological meaning, it is a primary pharmacokinetic parameter, which directly relates to the elimination half-life ($T_{1/2}$) and protein binding and is independent of total clearance (CL) of a drug (Equation 4) [67].

$$T_{1/2} = 0.693*Vdss / CL \text{ -- } (4)$$

Vdss is often expressed in unit of liter per kg body weight (L/kg). If Vdss is found to be a large number – i.e., > 1 L/kg (> 100% of body weight), it may be assumed that the drug is concentrated in certain tissue compartments [67]. Thus, Vdss is a useful parameter in considering the relative amount of drug outside the central compartment or in the tissues. However, the drug concentration in a particular target tissue (receptor) will still need to be measured directly.

To date, there are no reliable *in vitro* HT methods available to predict *in vivo* Vdss. Many experimental and computational physico-chemical parameters (*in silico*) have been used to predict preclinical and human Vdss [68]. However, the correlation between the *in vivo* and the calculated values needs to be more carefully evaluated. The most commonly used laboratory method to obtain Vdss is experimental, even in the drug discovery stage. To predict human Vdss, several prediction methods are described below.

Predicting Human Vdss

(1). Allometry Scaling

Interspecies allometry scaling is a method of interpolation and extrapolation of the underlying anatomical, physiological, and biochemical similarities in mammals [69]. In its original form, allometry was a technique to explain observed relationship between organ size and body weight of mammals [70].

Allometry scaling is the one of the best described technique to predict human Vdss [16]. In this method, plots are constructed of Vdss (or free Vdss - the protein binding factor is considered) in preclinical species (e.g., rat, rabbit, monkey, dog and etc.) vs. their body weights (Table **2**) [71] as described in Equation 5 and plotted in Fig. **2** [72]. In the plot, human Vdss will be extrapolated at the responding body weight (70 kg).

$$\text{LogVdss} = a^* \log(\text{body weight}) \text{ (kg)} + b \text{ -- } (5)$$

The values of a and b will be obtained by linear regression of the data points (slope and intercept).

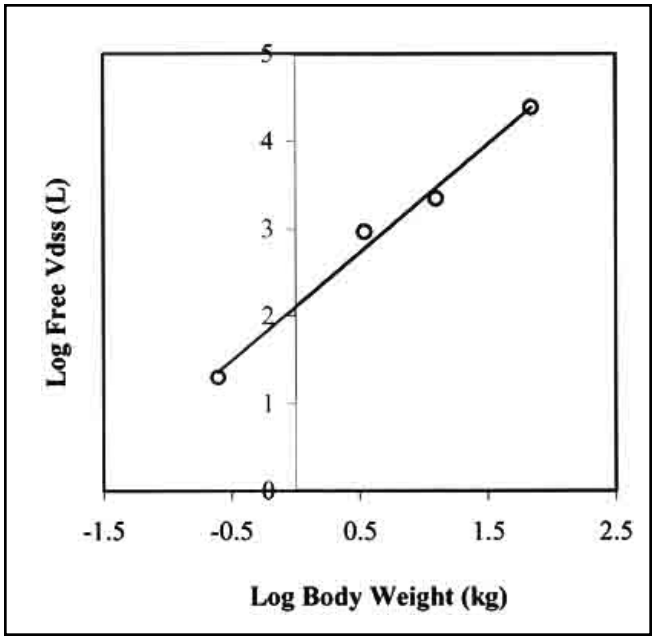


Fig. (2). Allometric scaling to predict human Vdss using free Vdss in rat, monkey and dog versus body weights of rat, dog, monkey and human.

(2). Proportionality Method

This method simply describes that the proportionality could be established between the free-fraction of drug in plasma in dog and human and the volume of distribution in these two species. This relationship yields Equation 6 [16].

$$\text{Vdss (human prediction)} / \text{fu (human)} = \text{Vdss (dog)} / \text{fu (dog)} \quad (6)$$

where fu (human) and fu (dog) represent unbound drug fraction in human and dog plasma, respectively; Vdss is in units of L/kg.

(3). Average Fraction Unbound in Tissues Method

Based on the Oie-Tozer equation [72], the fraction of unbound drug in tissues (fut) for each preclinical species can be calculated. After fut is calculated from each preclinical species, all values for a given compound will be averaged, fut (avg). Then human fut will be assumed equal to fut (avg). Therefore, human Vdss can be calculated based on equation 7 [72].

$$\text{Vdss (human)} = Vp + (fu*Ve) + [(1- fu)* Re/I*Vp] + Vr* fu/\text{fut (avg)} \quad (7)$$

where fu is unbound drug fraction in human plasma; Vp is plasma volume; Ve is the extracellular fluids volume minus plasma volume; Re/I is the ratio of drug binding proteins in extracellular fluids vs. plasma and is assumed to be 1.4 for all species and all binding proteins; and Vr is the physical volume in which the drug distributes minus the extracellular volume. All these parameters in various preclinical species and human are summarize in Table **2**.

METABOLISM / METABOLIC CLEARANCE

Drug clearance is an irreversible process to remove the drug from the body by all the routes. The drug clearance can be divided into two major pathways, excretion and metabolism (biotransformation). Excretion of the drug is the process of removing the intact drug and its metabolites from the body, which will be discussed in the later section. Metabolism involves enzymes to biotransform a drug, and is the process by which the drug is chemically converted in the body to a metabolite. Xenobiotic compounds that are too lipophilic to be excreted by the kidney are directly metabolized by the body to more hydrophilic compounds, which can undergo renal elimination [73] or be reabsorbed back to the circulating system. Enzymes involved in the biotransformation are mostly located in the liver, which makes hepatic metabolism an important topic in drug discovery [74]. Therefore, this section will focus on hepatic metabolism. However, other organs (such as lung, kidney and intestine) also contain metabolizing enzymes.

The reactions catalyzed by metabolizing enzymes can be divided into two categories, Phase I and Phase II. Phase I metabolic reactions are functional reactions, which usually introduce a polar functional group to a parent molecule to form a metabolite. The most common Phase I functional reactions include, but are not limited to, hydrolysis, reduction, and oxidation. The most important enzyme family in phase I metabolism is the cytochrome P450 family [75]. Phase II reactions are usually conjugating reactions, which conjugate a polar moiety to the parent compound or its phase I metabolite. Therefore, the metabolites resulting from Phase II are usually much more polar than the parent molecule, and are readily excreted from urine or/and bile. The common conjugation reactions for drugs are glucuronidation and sulfation.

Phase I Metabolic Reactions

The Phase I metabolic reactions commonly encountered in drug discovery are hydrolysis and oxidation. Hydrolysis usually is a spontaneous enzymatic biotransformation of esters, amides, epoxides to corresponding carboxylic acids, amines and alcohols in the tissues and plasma (Fig. **3**) [76], which can be used as prodrug approaches for the drug delivery to enhance the solubility and bioavailability.

Enzymatic oxidations usually include hydroxylation of aromatic and aliphatic carbons and heteroatoms (N-, S-, O-)

Table 2. Summary of Several Physiological Parameters in Selected Preclinical Species and Humans

Species	Body weight (kg)	liver weight / body weight (g/kg)	Hepatic Blood Flow (Q, mL/min/kg)	plasma volume Vp (L/kg)	Ve[2] (L/kg)	Vr[3] (L/kg)
Mouse	0.030	50	90	N.A.[1]	N.A.	N.A.
Rat	0.25	40	70	0.0313	0.265	0.364
Rabbit	2.75	48	45	0.0314	0.179	0.322
Dog	15.0	32	40	0.0515	0.216	0.450
Monkey	5.0	32	44	0.0448	0.208	0.485
Human	70	21	20	0.0436	0.151	0.380

[1] N.A.: not available.
[2] Ve: extracellular fluids volume minus plasma volume;
[3] Vr: physical volume in which the drug distributes minus the extracellular volume.

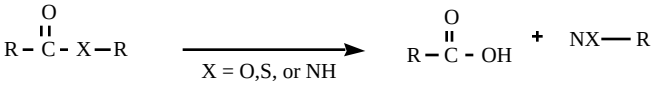


Fig. (3). Hydrolysis of an ester, a thioester or an amide to a carboxylic acid and alcohol, a thiol or an amine, respectively

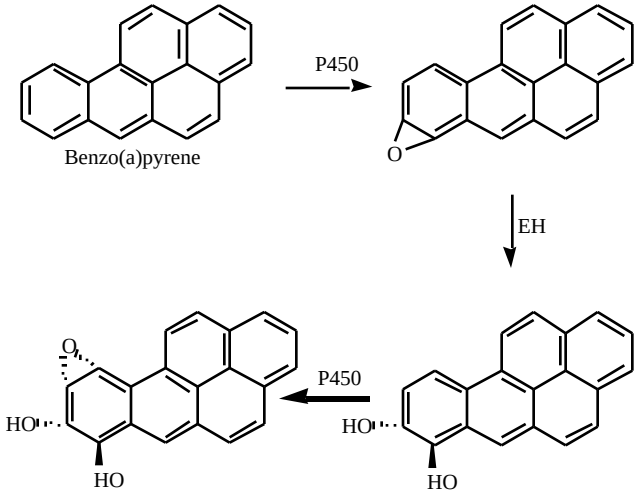
dealkylations or carbons and heteroatoms (N-, S-) oxidations [74]. The enzymes involved in these oxidations are mainly aldehyde oxidase (AO), xanthine oxidase (XO), monoamine oxidases (MAOs), flavin-containing monooxygenases (FMOs) and cytochrome P450s (P450s).

(1). Esterases

Esterases have long been studied with regard to their importance in prodrug design and detoxification of drugs [76]. Esterases are abundant in animal and human plasma and tissues.

(2). Microsomal Epoxide Hydrolases (mEHs)

mEHs are a family of enzymes that function to hydrate simple epoxides to vicinal diols and arene oxides to *trans*-diols by hydrolytic cleavage (Fig. **4**) [77, 78]. These enzymes represent one category of the broader group of hydrolytic enzymes and have been associated historically with the metabolism of xenobiotic chemicals (Table **3**) [78]. mEHs are abundant in animal and human livers.



Benzo(a)pyrene 7,8-dihydrodiol 9,10-epoxide

Fig. (4). Schematic representation of the metabolism of benzo(a)pyrene into the carcinogenic 7,8-dihydrodiol 9,10-epoxide by mEH and P450s.

Table 3. Selected Specific Substrates, Inhibitors and Inducers of Oxidative Enzymes

Enzymes	Isoforms	Substrates/Probes	Inhibitors	Inducers	Ref.
mEH		epoxides, arene oxides	1,1,1-trichloropropene oxide, cyclohexene oxide	NA[1]	78
AO		substituted pyrines, pteridines pyrimidines and 5, 6-condensed pyrimidines	menadione, 9-anilinoacridine	NA	82-84
XO			Allopurinol	NA	85, 86
MAO	MAO-A	Amines	clorgyline (nM), 1-deprenyl (μM)	NA	91
	MAO-B	Amines	clorgyline (μM),1-deprenyl (nM)	NA	91
FMOs		nicotine	methimazole, thiourea, n-octylamine	Not inducible	103-106
P450s	1A2	caffeine, phenacetin	Furafylline[2]	omeprazole	117, 118
	2A6	coumarin, nicotine	8-methoxypsoralen[2]	Barbiturates	119
	2B6	cylcophosphamide, bupropion	orphenadrine	phenobarbital	119
	2C9	diclofenac, warfarin, Coumarin	sulfaphenazole	rifampin	120
	2C19	diazepam, S-mephenytoin	iansoprazole	rifampin	121
	2D6	bufarolol, dextromethorphan	quindine	not known	122
	2E1	chlorzoxazone, acetaminophen	dihydrocapsaicin	ethanol	120
	3A4	midazolam, erythromycin, taxol, testosterone, terfenadine	ketoconazole, troleandomycin[2]	phenobarbital, rifampin, dexamethasone	16, 123

[1] not available.
[2] mechanism-based inhibitors.

(3). Aldehyde Oxidase (AO) / Xanthine Oxidase (XO)

In addition to the microsomal enzymes, liver also contains cytosolic enzymes, such as aldehyde oxidases (AO) and xanthine oxidases (XO) [79], which play a complementary role as the mixed function oxidases in drug metabolism [80, 81]. Since AO and XO are related in molecular properties and catalytic function, many compounds are common substrates for both enzymes (Table **3**) [81-86]. The mechanism of catalytic reaction by AO and XO is electron transfer via water to converting RH (e.g. xanthine) to ROH (e.g., uric acid) (Fig. **5**) [87, 88]. Liver cytosol exhibits highest activity of AO and duodenal homogenates show highest XO activity. Species difference in tissue distribution and in isoforms of AO and XO have been observed [89].

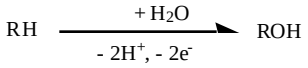


Fig. (5). Scheme for the AO and XO catalyzed reactions.

(4). Monoamine Oxidases (MAOs)

Among the amine oxidases, MAOs are the most common oxidative enzymes involved in the biotransformation of drugs [90]. In contrast to the large number of CYP isozymes, only two forms of MAO, named MAO-A and MAO-B, are presently known to exist in mammalian tissues. These MAOs follow the same chemical mechanism to oxidize primary, secondary and tertiary amines (Fig. **6**) [90], but differ in their substrate specificities and inhibitor sensitivity (Table **3**) [91]. In mammals, both MAO-A and -B are present in most tissues, such as CNS and peripheral organs, particularly in livers [92]. Species differences and genetic polymorphism regarding MAO activities are exhibited [93-95].

(5). Flavin-Containing Monooxygenases (FMOs)

FMOs are a gene family of five enzymes (FMO1 to FMO5) involved in the metabolism of numerous nucleophilic heteroatom-containing xenobiotics (e.g., N-, S-, P-) [96]. Like cytochrome P450s, the FMOs are localized primary to the endoplasmic reticulum, require the co-enzyme, NADPH and molecular oxygen for activity, and are found in all mammalian species and nearly all tissues [97], but primary in livers. Species differences and genetic polymorphism in FMO activities are exhibited [98]. Among the five FMO isoforms, FMO3 is found primarily in human liver [99, 100]. FMO3 particularly oxidizes primary, secondary and tertiary amines to oximes and *N*-oxides with stereospecificity (Fig. **7**) [101, 102], such as nicotine [103].

Since cytochrome P450s have the similar capability to metabolize nucleophilic heteroatom-containing molecules, the differentiation of the oxidation activity between FMOs and P450s can be achieved by chemical inhibitors (Table **3**) [103-106], heat sensitivity [106], and recombinant isoforms.

(6). Cytochrome P450s (P450s)

P450s constitute a superfamily of b-type heme-containing monooxygenases and are the most important drug metabolizing enzymes for numerous xenobiotics (e.g., drugs, agricultants, pollutants and dietary components) as well as some endogenous substrates (e.g., bile acids, steroids, and cholesterol) in preclinical species and human. Cytochrome P450 enzymes are present in many mammalian organs, such as kidneys, lungs and intestines with the majority in the liver. The common reactions catalyzed by

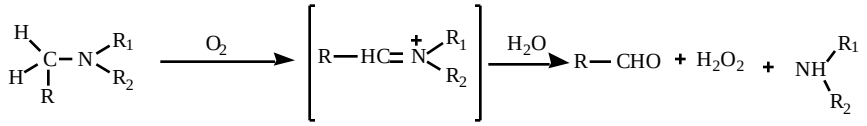


Fig. (6). Scheme for the MAO catalyzed reactions.

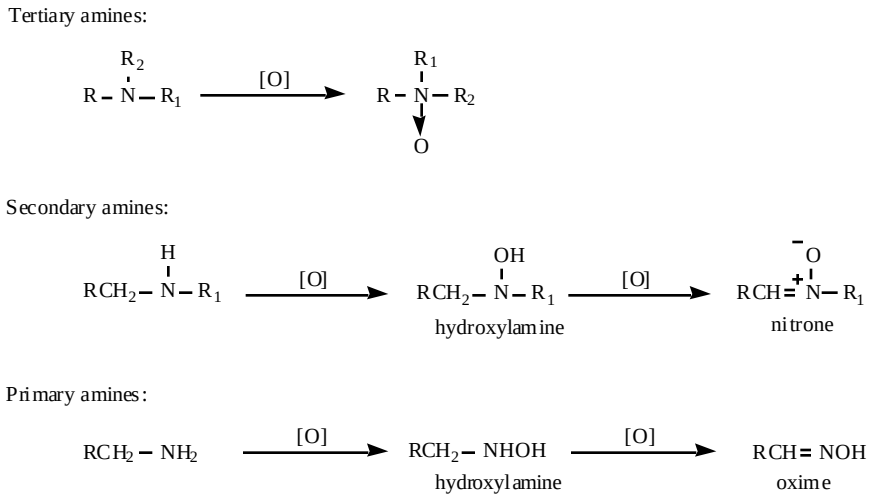


Fig. (7). Schematic representations of the N-oxygenation reactions that catalyzed by FMOs.

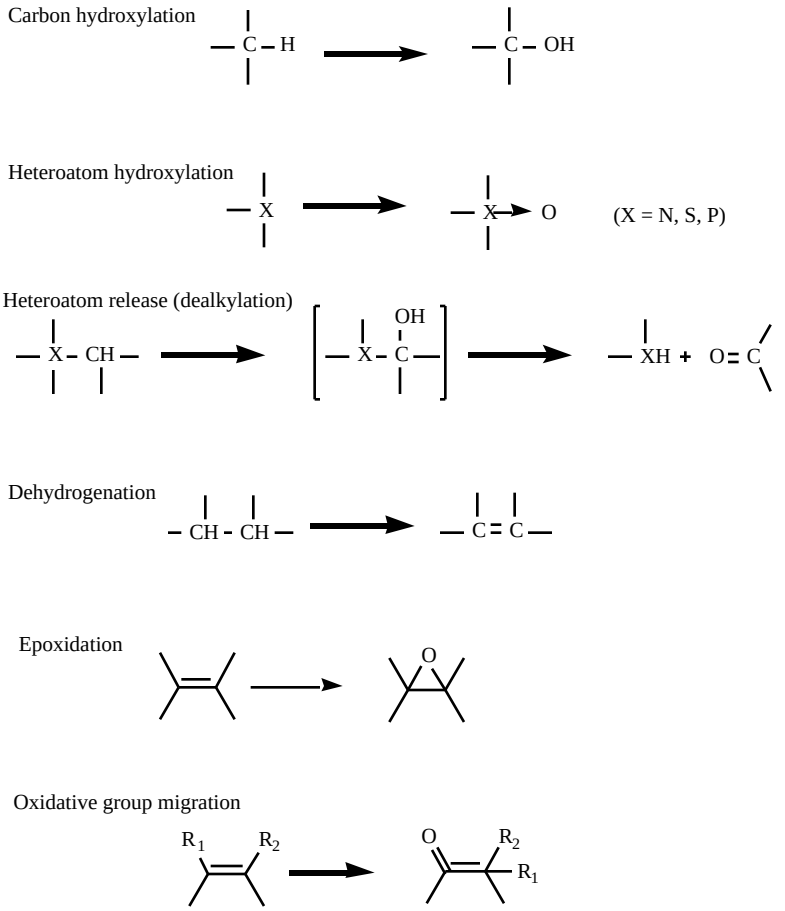


Fig. (8). Schematic representation of the common reactions that catalyzed by P450s.

P450s are listed in the Fig. **8** [75]. Many extensive reviews are available regarding the structures, mechanisms, biochemistry and drug metabolism of P450 enzyme and the cited references herein [75, 107-114].

Based on amino acid sequence homology, CYP superfamily members are divided into various subfamilies, which are further divided into various isoforms [115]. More than 25 P450 subfamilies are currently identified and only four subfamilies (i.e., 1, 2, 3 and 4) are defined as drug metabolizing enzymes. Major isoforms involved in the biotransformation of drugs in human are CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Among these isoforms, CYP3A4 is the most abundantly isoform and account for approximately 30 to 40% of the total CYP content in human liver and small intestine [116]. CYP3A4 is estimated to metabolize between 50-70% of the currently used drugs [113]. P450 isoforms exhibit significant substrate specificity during the biotransformation process, such as regio- and stereo- selectivity (Fig. **9**) [73, 74]. P450 enzymes can be inhibited, as well as induced by several specific xenobiotics (Table **3**) [16, 117-123], which possess the potential for drug-drug interactions.

Based on immunochemical cross-reactivity, protein sequence similarity, DNA sequence and substrate specificity, human and preclinical species have similar subfamilies of cytochrome P450s, despite different nomenclatures [75, 108].

It is important to identify the CYP isoform(s) responsible for metabolizing a drug, especially polymorphically expressed isoforms (i.e., 2C9, 2C19 and 2D6), which could cause inter-subject variability in human clinical PK. The identification can be established by several methods, such as chemical and antibody inhibition, correlation analysis, and recombinant P450 isoforms, which have been described in detail previously [119]. These four *in vitro* approaches to identify reaction phenotype all have their advantages and disadvantages [119]. A combination of approaches is usually suggested to identify which human P450 enzyme is responsible for metabolizing a xenobiotic.

Phase II Metabolic Enzymes

Phase II reactions are usually conjugations, which add polar moiety into either the parent molecule or its sequential, Phase I metabolites. These highly polar conjugated metabolites are readily excreted from the body. Important drug metabolizing enzymes involved in phase II reactions include sulfotransferases and UDP-glucuronosyltransferases, which are highly expressed in liver.

(1). Sulfotransferases (STs)

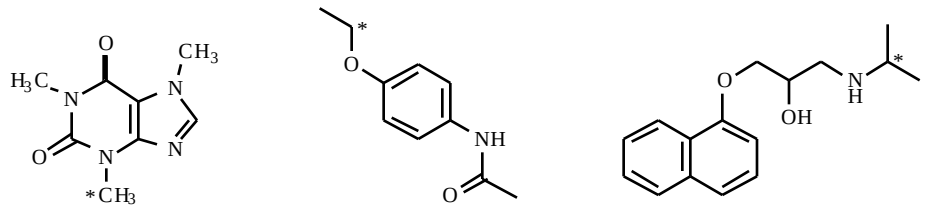
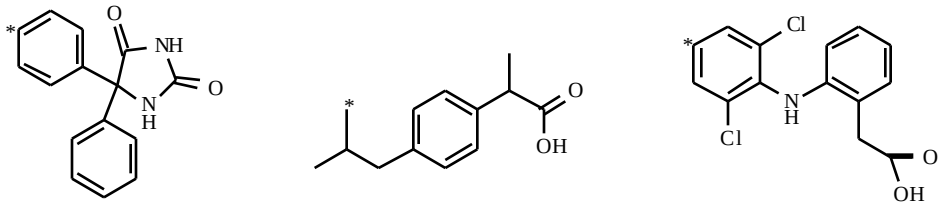
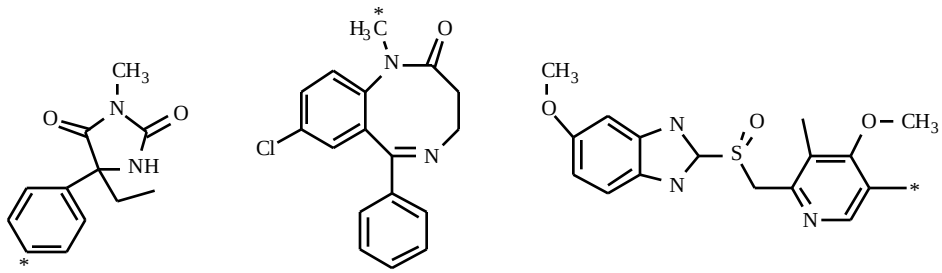
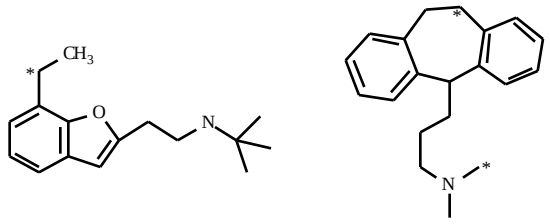
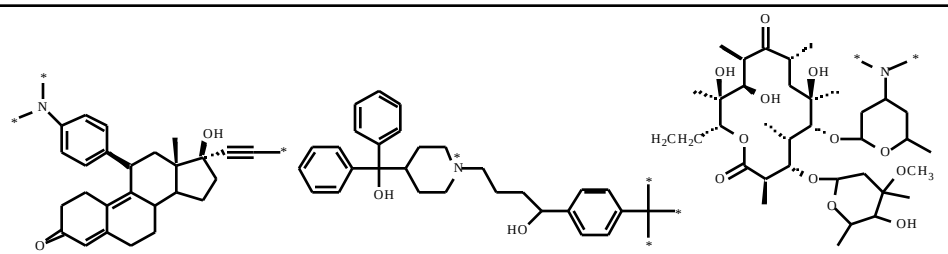
CYPs	Substrates (*indicated the site of biotransformation)	Substrate properties
1A2	 Caffeine Phenacetin Propranolol	Planar, neutral to moderate basic
2C9	 Phenytoln Ibuprofen Diclofenac	Weakly acids, ionized at physiological pH, lipophilic, H-bond donor
2C19	 (S)-Mephenytoln Diazepam Omeprazole	Weakly acid to strong basic, H-bond donor
2D6	 Buforolol Amitriptyline	Basic, relatively hydrophilic, a H-bond donor
3A4	 Mitepristone Terfenadine Erythromycin	Liphophilic, structurally diverse, H-bond donor /acceptors

Fig. (9). Substrate specificity of cytochrome P450s (compiled on basis of the reference 73 and 74).

STs are dimeric enzymes that biotransform many drugs and xenobiotics by sulfation to more hydrophilic metabolites readily excreted from the body (Fig. **10**) [124]. PAPS (3'-Phosphoadenosine 5'-phosphosulfate) is the "sulfate donor" for STs during the biotransformation. STs are either cytosolic or microsomal, however, the most common STs involving phase II metabolism for drugs and xenobiotics are located in cytosol. The catalyzing STs are expressed in a variety of tissues in human, including liver (as major), jejunum, kidney, lung, brain and - of importance for pharmacogenetic studies - the blood platelet [125]. Some of the ST subfamilies express significant genetic polymorphisms in human tissues [126].

The most common functional groups for sulfation are phenols, alcohols (catechols), arylamines and *N*-hydroxyl amines, such as acetaminophen, droloxifene and minoxidil [119]. Many other drugs are sulfated after initially

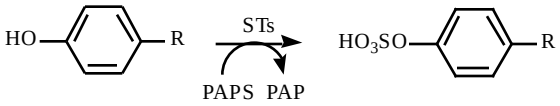


Fig. (10). Sulfation catalyzed by STs.

undergoing hydroxylation catalyzed by Phase I enzymes such as the cytochrome P450s [127]. However, compared to glucuronidation, sulfation conjugation is less common.

(2). UDP-Glucuronosyltransferases (UGTs).

UGTs are a superfamily of member-associated enzymes that catalyze the transfer of D-glucuronic acid to a large number of endogenous compounds and xenobiotics to form glucuronide metabolites (Fig. **11**) [76]. The UGTs are found primarily in hepatic tissues, with some activity in intestine [128]. Species differences in the extent of glucuronidation have been documented [129]. Multiple members of these subfamilies exist in human, and the nomenclature is directly analogous to the CYP nomenclature [130]. The human metabolizing UGTs are mainly two subfamilies, namely 1A and 2B [75]. UGTs can be inhibited or induced by several xenobiotics [131], which could cause potential drug-drug interactions.

The glucuronides generally possess different physiochemical properties compared to those of the parent compounds, e.g., very water soluble, less pharmacologically active, less toxic and rapidly excreted in the urine or bile. However, glucuronides can also re-enter the liver cell and undergo enterhepatic recirculation. Glucuronides can be hydrolyzed back to the parents by hydrolases, particular β-glucuronidase. Therefore, D-saccharic acid 1,4-lactone, a known β-glucuronidase inhibitor, may need to be included in the incubation system [132, 133].

Recently, *in vitro* UGTs-catalyzed reaction rates in liver microsomes have been used in the rank ordering of drug candidates for selection of a new drug entity in drug discovery [134]. Since the active site of the UGTs resides in the lumen of the endoplasmic reticulum (ER), the lack of the catalytic activity occurs due to the ER membrane barrier [135]. Therefore, the reagent for breakdown of this barrier is required to obtain the maximum *in vitro* enzyme activity. The most popular reagents are Brij 58 [136] and alamethicin [137]. Besides, the cofactor UDPGA is also needed in the microsomal incubations. However, it should be cautious when *in vitro* glucuronidation rates in liver microsomes are used to predict *in vivo* clearance, simply because these reagents may artificially enhance the catalytic activity in the *in vitro* system.

In Vitro Tools for Predicting Metabolism

Since the metabolizing enzymes are mainly located in the liver, the liver matrix will be emphasized in this section. *In vitro* system possesses many practical advantages and scientific accuracy. First, it is quick and cheap compared to the *in vivo* system. Second, it enables scientists to access human reagents to understand the metabolic fate and direct predict human metabolic clearance. Some current *in vitro* systems are listed in Table **4**.

(1). Identifying the Metabolic Clearance Pathways

It is important to understand the metabolic pathways in order to accurately predict human clearance. To understand the metabolic pathways, it will be critical to identify the metabolites. *In vitro* systems can be used to generate large quantity of metabolites by selective use of various reagents and cofactors (Table **4**). The metabolite structures can be elucidated by LC/MS, LC/MS/MS or NMR, which will help to confirm the metabolic pathways (Phase I vs. Phase II). One also can use specific chemical inhibitors/antibodies to identify the responsible enzymes for the metabolite formation (Table **3**).

(2). Predicting Hepatic Metabolic Clearance

The intrinsic hepatic metabolic clearance (CL'_{int}) of human and preclinical species can be predicted by measuring enzyme kinetics parameters, Km (Michaelis-Menton constant) and Vmax (maximum velocity) (CL'_{int} = Vmax / Km) [138]. However, it is time consuming to measure Km and Vmax. In drug discovery, one can use a single substrate concentration (<< Km) to determine the parent drug disappearance half-life ($T_{1/2}$). The intrinsic hepatic metabolic clearance (CL'_{int}) can be calculated based on scaling factors from microsomes (Equation 8) as well as hepatocytes (Equation 9) [139]. Then, the hepatic metabolic clearance (CL_h) and hepatic extraction ratio (Eh) can be calculated based on Equations 10, 11 (plasma and microsomal protein

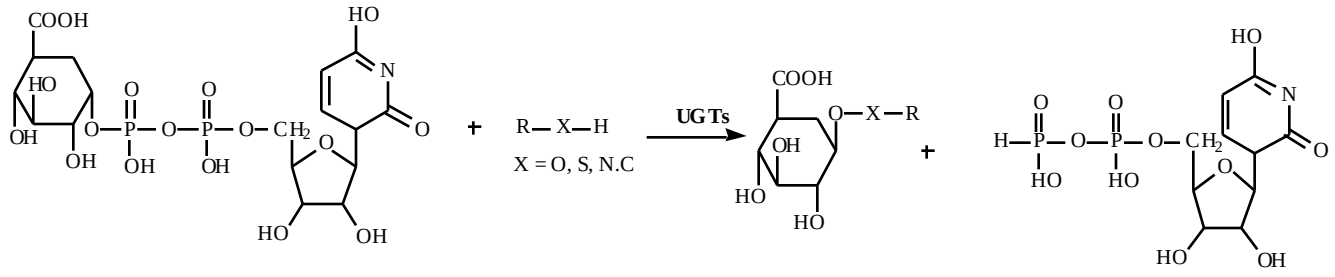


Fig. (11). Conjugation of a nucleophile (i.e. R-X-H) with uridine-5'-diphospho-a-D-glucuroinc acid (UDPGA) to form the glucuronidation metabolites.

Table 4. Available *In Vitro* Systems to Predict Clearance and Identify Metabolic Pathways

In Vitro reagents	Functional enzymes	Co-factors (co-enzymes)	Able to Predicting Clearance by scale-up
Microsomes	P450s, reductases	NADPH	yes
	UGTs	UDPGA and detergent	no, only in vitro $T_{1/2}$
	GSTs	GSH	no, only in vitro $T_{1/2}$
	STs	PAPS	no, only in vitro $T_{1/2}$
	mEH, esterases, AO	no	no, only in vitro $T_{1/2}$
Hepatocytes	All the activities	no	yes
S-9	P450s, reductases	NADPH	uncertain
	UGTs	UDPGA and detergent	uncertain
	mEH, esterases	no	no, only in vitro $T_{1/2}$
Cytosol	STs	PAPS	no, only in vitro $T_{1/2}$
	AO, XO, esterases,	no	no, only in vitro $T_{1/2}$

binding are considered) and 12, respectively [140].

CL'_{int} = [(0.693/$T_{1/2}$)*(45 mg microsomes/gm liver)*(gm liver/kg b.w.)] / (mg microsomes/mL incubation) -- (8)

where $T_{1/2}$ (minute) obtained from microsomal incubation; (mg liver/kg body weight) for preclinical species and human are listed in Table 2. The unit for clearance is mL/min/kg.

$$CL'_{int} = [(0.693/T_{1/2}) * (\text{cells/g liver})* (\text{gm liver/kg b.w.})] / (\text{viable cells/mL incubation}) \text{ -- (9)}$$

where $T_{1/2}$ (minute) obtained from hepatocytes incubation; (mg liver/kg body weight) for preclinical species and human are listed in Table 2; cells/g liver: 135, 240 and 120 million cells/g liver for rat, dog and monkey/human, respectively. The unit for clearance is mL/min/kg.

$$CL_h = (Q * CL'_{int}) / (Q + CL'_{int}) \text{ -- (10)}$$

where Q is the hepatic blood flow listed at Table 2.

$$CL_h = [(Q*fub*(CL'_{int}/fut)] / [Q + fub*(CL'_{int}/fut)] \text{ -- (11)}$$

where fub and fut are the unbound drug fraction in plasma and tissue (i.e., microsomes or hepatocytes), respectively.

$$E_h = CL_h / Q \text{ -- (12)}$$

where $E_h < 0.3$, or $0.3 < E_h < 0.7$, or $E_h > 0.7$ indicates low, moderate or high hepatic metabolic clearance.

In Silico Methods for Predicting Metabolism

The prediction of the metabolic fate of a xenobiotic is a fascinating and complex issue, and the existing domain of knowledge is vast, such as *in vitro* technologies described above. In addition to the development of *in vitro* experimental assays with greater throughput, there has been considerable effort applied to the conception and validation of computational methods (*in silico*) for predicting the potential metabolic activity of xenobiotics [141]. There are several commercial available products for the prediction of xenobiotic metabolism. These products broadly fall into three categories. First, there are enzyme-modeling systems based on theoretical and mechanistic considerations, such as COMPACT (computer-optimised molecular parametric analysis of chemical toxicity) [142, 143] and Camitro (www.camitro.com). Second, there are empirically based expert systems which rely on expert rules as the basis of their predictions, such as META [144, 145], MetabolExpert (www.compudrug.com) and METEOR [146, 147]. Last, there are database systems, such as MDLI metabolite database (www.mdli.com) and Accelrys' Biotransformations Database (www.accelrys.com).

EXCRETION

As discussed in the previous section, metabolism is an important parameter in predicting drug clearance. There are, however, many drugs that are cleared from the body without being metabolized. This concept had been widely accepted for many years for renal clearance of drugs, but only recently the understanding of hepatic processes of clearing unchanged drugs is increasing and gaining acceptance. Good reviews of examples of transporters in drug excretion can be found [148, 149]. This section will discuss the active, energy-dependent transport processes that govern the clearance of drugs by the two major clearance organs: liver and kidney. This area has been gaining significant interest in the pharmaceutical industry and attempts are being made at incorporating theses processes in lead optimization and other discovery and development stages. This review will utilize both the current and the recommended nomenclature (in backets) for active transporters. More information also is

available at http://www.med.rug.nl/mdl/english/tab3.htm, which contains the recommended nomenclature for transporters.

Hepatic Excretion

The liver has evolved several mechanisms to take up nutrients and endogenous compounds important for its function and for overall homeostasis. Mechanisms to detoxify and eliminate byproducts or metabolites by efflux have also evolved and are present in both the canalicular and sinusoidal (circulation) surfaces of the hepatocytes (Fig. **12**).

There are numerous transporters responsible for uptake of drugs into the hepatocytes via the sinusoidal (basolateral) membrane [150, 151]. Endogenous compounds, such as thyroid hormones and bile acids are transported by the OATP (SLC21 family) transporters. These transporters have been shown to transport drugs such as pravastatin, fexofenadine, rifampicin and rifamycin. Other uptake transporters include the OATs and OCTs (SLC22), NTCP (SLC10A1) and PGT (SLC21A2) [148]. These transporters can be studied using uptake into hepatocytes [152, 153], or uptake into cell lines expressing the recombinant transporter of interest [154]. Some attempts have been made into extrapolating the values obtained from *in vitro* studies to *in vivo*, but this area is still in its infancy [155]. One important parameter, the role of serum protein binding and whether extrapolations should use the free or total fraction is still poorly understood. Experiments using bilirubin [154] indicate a minor role for protein binding for OATP-C (SLC21A6), but not OATP8 (SLC21A8), however these experiments need to be explored using physiological concentrations of albumin. It is difficult to study the role of uptake transporters in animal models, because scientists cannot isolate them from the effect of efflux transporters. Another difficulty is the lack of understanding of orthologs of human isoforms in other species.

There are numerous efflux transporters in the bile canalicular side of the hepatocytes. These include P-glycoprotein (MDR-1, ABCB1), several MRPs (ABCCs), BCRP (ABCG) and BSEP (ABCB11) [156]. Numerous examples exist of drugs that are substrates for these transporters [148]. Drug-drug interactions also are possible by drugs causing inhibition of these transporters, as discussed in the toxicology section of this review.

These processes can be studied using inside-out canalicular membranes vesicles [157], and vesicles or whole cell assays from immortal cells expressing the transporter of interest [158], or long term primary hepatocyte cultures [159]. *In vivo* studies can involve bile cannulated rats or dogs and measurement of excretion of compound into bile [160]. Isolated perfused rat liver has also been used [161].

Studies designed to measure biliary clearance in rats and dogs should take into account that compounds need first to be taken up or transferred from the plasma to the liver cell, either by passive diffusion or an active transporter; and then be effluxed by one or several transporters in the canalicular side. By measuring bile flow, and drug concentration in plasma and bile, biliary clearance can be calculated as Equation 13.

$$CL_B = (\text{concentration in bile}) * \text{bile flow} / \text{concentration in plasma} \quad (13)$$

Biliary clearance is of significance when drug concentration in bile is much higher than that in the plasma, resulting in a clearance value higher than bile flow (0.5-0.8 mL/min).

Species differences for these transporters are, in general, better understood than for the uptake transporters, and data

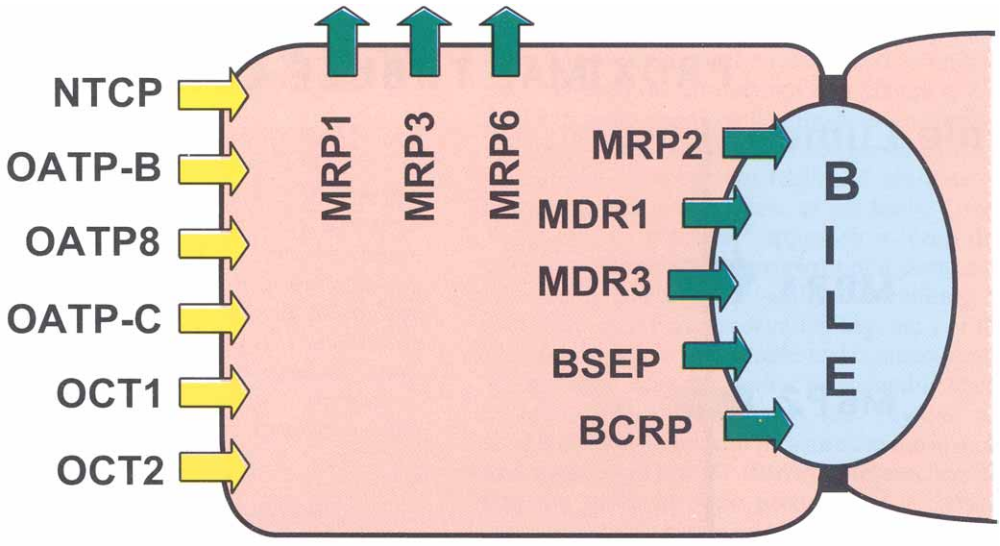


Fig. (12). Drug transporters located in the hepatocytes showing both uptake transporters (yellow arrows) and efflux transporters (green arrows). Only transporters that have been shown to transport drugs are shown; there are additional transporters that transport endogenous compounds, but these are outside of the scope of this review. NTCP, sodium taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; MRP, multidrug resistance related protein; MDR, multidrug resistance protein; BSEP, bile salt export pump; BCRP, breast cancer related protein.

from one species can be used to predict the role in other species [162].

Renal Excretion

The kidneys are responsible for the elimination of many endogenous byproducts and, through the same mechanisms, many drugs are cleared from the body. There are three major processes for drug elimination in the kidney: glomerular filtration, tubular secretion and tubular reabsorption [163]. Tubular secretion and reabsorption involve both passive and active transport processes across the membranes. Drug filtration is a diffusional passive process. The tubular secretion of drugs is mediated by many active transporters, which take up compounds from the kidney interstitium and efflux them into the tubular lumen (Fig. **13**). Organic anion (OATs) and organic cation transporters (OCTs) are the two major classes of uptake transporters, but OCTNs and OATPs can also be found in the kidney. Efflux transporters such as P-glycoprotein and MRPs are also found in the kidney tubule. A comprehensive review of these transporters, their substrate specificities, and tissue distribution has been published recently [164].

Renal excretion of drugs can be expressed as the sum of rate of filtration plus secretion and then subtract rate of reabsorption. Renal clearance can be calculated by measuring the amount of drug excreted during a time period and dividing by the time interval and average plasma concentration (Equation 14).

$$CL_R = \text{(excreted amount) /time interval / mean plasma concentration} \quad \text{-- (14)}$$

Active transport in the kidney can be studied by the preparation of vesicles from brush border membranes or tubules, by using uptake in kidney derived cell lines like MDCK and HEK cells, or vesicles prepared from these cells; as well as using isolated perfused rat kidney. A comprehensive review of *in vitro* and *in vivo* methodologies to study renal clearance including their clinical significance is available [165]. *In vitro* studies can be useful to predict a potential mechanism of clearance or a potential drug-drug interaction, and allow for well-designed, focused clinical trials to address these questions.

Predicting Human Clearance

As described in this section, if the clearance for a drug is mediated by extra-hepatic clearance (e.g., biliary or renal excretion by transporters, and kidney or intestinal metabolism), the *in vitro* hepatic tools described in the metabolism section could underestimate the total clearance. Therefore, in the lead seeking stage of drug discovery, pharmacokinetic properties in preclinical species are important to evaluate potential extra-hepatic clearance.

The total systemic clearance for preclinical species can be evaluated via bolus intravenous administration. By comparing *in vivo* systemic clearance with the clearance predicted from *in vitro* hepatic metabolism in the corresponding species, an *in vivo-in vitro* correlation can be established. If the *in vivo-in vitro* clearance is well correlated in preclinical species, the clearance is probably mainly due to hepatic metabolism and hence the confidence is increased for extrapolation to human as well. Then, *in vitro* human liver microsomes and hepatocytes can be directly used to assess human *in vivo* clearance.

If *in vivo-in vitro* clearance correlation in several preclinical species is poor, and human clearance mechanism is also found to be extra-hepatic by using described *in vitro* methods, allometry scaling has to be applied for human clearance prediction. The prediction method is very similar to that described for prediction of human Vdss in this review based on the body weights [16]. At least, three preclinical

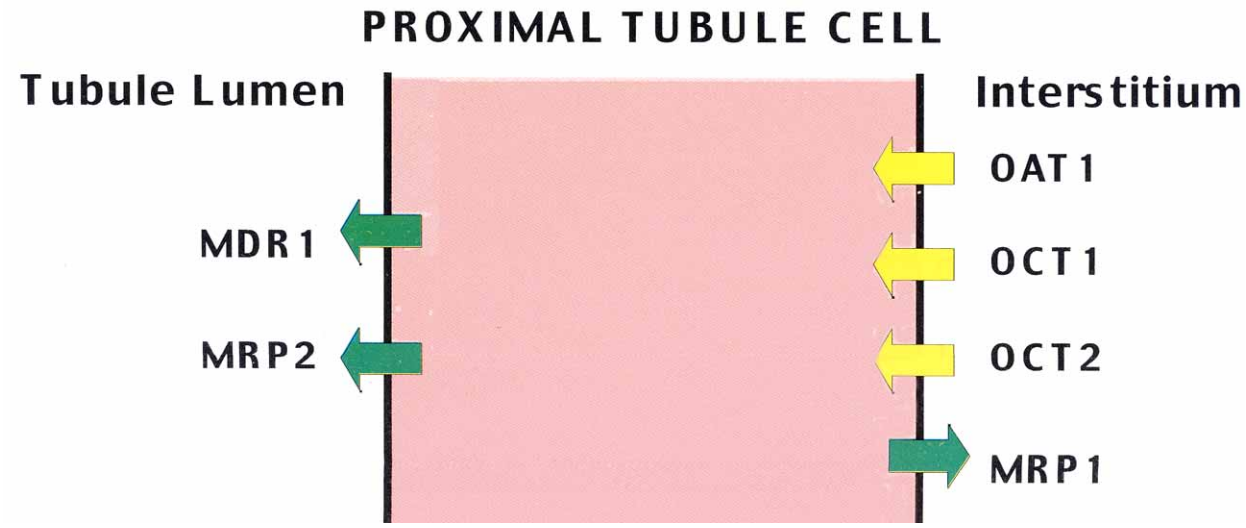


Fig. (13). Drug transporters located in the renal tubule cells showing both uptake transporters (yellow arrows) and efflux transporters (green arrows). Only transporters that have been shown to transport drugs are shown; there are additional transporters that transport endogenous compounds, but these are outside of the scope of this review. OAT, organic anion transporter; OCT, organic cation transporter; MRP, multidrug resistance related protein; MDR, multidrug resistance protein.

Table 5. A Summary of Drug Toxicity Screens Reviewed in This Article

Category	Assay Name	What is being measured?
Mutagenecity	VITOTOX®	Increase in bioluminescence
Clastogenecity	Yeast DEL assay	Color change of the pH dye
Steatosis	Inhibition of beta-oxidation	Decrease in radioactive product formation
Cholestasis	Inhibition of Bsep	Decrease in radioactive substrate accumulation
Reactive metabolites	Formation of GSH adducts	Increase in GSH adducts or decrease in free GSH
Phospholipidosis	NBD-PE or NBD-PC	Increase in fluorescence
Long QT syndrome	Dofetilide-binding to IKr	Decrease in [3H]Dofetilide binding
CYP inhibition	Inhibition of CYP product formation	Decrease in fluorescent product formation
PgP inhibition	Calcein accumulation	Increase in calcein fluorescence

species need to be used and protein binding factors also may need to be considered [16].

TOXICOLOGY SCREEN

As described earlier, drug-induced toxicity has been reported to the major cause of attrition in drug discovery and development [166]. Traditional pre-clinical drug safety evaluation relies heavily on bulk chemical synthesis and step-wise toxicity testing in laboratory animals. With major advances in combinatorial library production and increased automation in chemical synthesis and purification, the number of high quality compounds generated by medicinal chemists has far surpassed the capacity for traditional drug safety evaluation of NCEs. The rational implementation of toxicity screening strategies, preferably in the lead optimization stage, is a critical need in drug discovery.

To meet this demand, several emerging approaches have been developed or proposed. They include: 1) *in silico* or computational approaches, 2) a battery of *in vitro* screens, and 3) limited *in vivo* animal tests. To implement a rational plan, these three approaches need to be combined synergistically. For example, to build a computational model or derive an *in silico* "rule", one needs to analyze structure-activity relationships (SAR) obtained from both *in vitro* and *in vivo* tests. Likewise, one may need to "spot-check" an *in silico* alert with *in vitro* and *in vivo* tests to build confidence in computational predictions of *in vivo* outcomes.

The severity of chemical-induced organ toxicity is a function of three major determinants: 1) the intrinsic toxic property of a chemical, 2) its local concentration at a particular organ, and 3) the capability of host defense systems to detoxify the chemical and cope with chemical injury. The first determinant is embedded in the chemical structure, therefore can be viewed as fixed once its structure is conceived. It can be experimentally measured by studying the compound's effect on a specific cellular function (break down of lipids, transport of bile acids, etc.). The second is determined by dose, formulation, and ADME-PK in an individual exposed to such a chemical or drug. Local concentrations can vary widely, either because of dosing regimes, tissue specificities, individual polymorphisms in ADME pathways, or environmental factors including concomitant administration of other drugs, food, or herbs. The third determinant (capability of host defense systems) can also vary widely among individuals, due to genetic polymorphisms or environmental factors that either induce or suppress major host defense mechanisms. Therefore, to confidently predict chemical-induced toxicity in human beings is a very complex problem requiring multidisciplinary research endeavors. Initially, one needs to assess the intrinsic toxic properties of a chemical. This is followed by a series of *in vivo* toxicity tests, usually in animal models. Next, there is a need to understand the key pathway(s) in the elimination of a chemical and ask what is the likely outcome if such pathway(s) is compromised. Even so, some of the variables in categories 2) and 3) may very well exceed our current technical abilities to adequately predict results in humans at an early Discovery stage. Therefore, a "practical" approach in drug discovery is to minimize intrinsic toxic property of a chemical "as much as reasonably achievable" while maintaining "drugability" (efficacy, specificity, bioavailability, etc.) of the compound. This is already a formidable task considering the SAR for "drugability" may not always be separable from the SAR for toxicity. The remainder of this review will focus on screening assays that can measure the intrinsic toxic property of a chemical (Table **5**). However, researchers are forewarned that the intrinsic toxic property of a chemical does not automatically translate into organ toxicity *in vivo*. Factors in categories 2) and 3) play important roles in outcomes of frank organ toxicity, or lack thereof. The rational integration of screening data with ADME-PK prediction and scientific risk assessment are key to the ultimate success of minimizing drug toxicity risks to humans.

Medicinal toxicology, defined as the study of the adverse effects of medicines on biological systems (esp. the human

body), is a very broad topic encompassing many areas. There is the toxicology of various biological systems of increasing complexity: from molecular, to cellular, to organs, to whole body toxicology. Then there is the toxicology of practically all organs in the human body: liver, heart, brain, reproductive organs, etc. For the purpose of this review, we will only focus on some of the major toxicity factors contributing to drug attrition. Emphases are placed on *in vitro* assays that only need low bulk testing materials and are amenable to rapid throughput, both criteria important in drug discovery. Table **5** summarizes the drug toxicity screens reviewed in this article.

Genetic Toxicity

Carcinogens, or cancer-inducing agents, can be divided into mutagenic and non-mutagenic carcinogens. Mutagenic carcinogens are agents that induce mutations or DNA sequence changes. The goal of genetic toxicity screening in drug discovery is to identify potential mutagenic and non-mutagenic carcinogens. Since the introduction of the "classical" Ames test [167], many *in vitro* tests have been proposed to identify mutagenic carcinogens. Some of these tests can be adapted to higher throughput screens to suit drug discovery needs.

The VITOTOX® assay is based on the principle that a bacterial genotoxic response would result in an increase in bioluminescence. This was achieved by fusion of the *Escherichia coli rec*N promoter with the *Vibrio fischeri lux*CDABE operon, and introduction of such a fusion plasmid into *Salmonella typhimurium* [168]. Verschaeve *et al.* compared the VITOTOX® assay and Ames test on twelve newly synthesized pharmaceutical compounds and intermediates synthesized at the Janssen Research Foundation [169]. The VITOTOX® assay agreed with Ames results well, except a false negative finding for one of the compounds. Eight of these twelve compounds were tested in the assay at both VITO and the Janssen Research Foundation. The results were identical between the two laboratories when expressed as positives (genotoxic) or negatives (not genotoxic). Several other compounds were evaluated and compared in these test systems, but due to confidentiality, the results were not disclosed. VITO is using a 384-well plate format in a luminometer, enabling 30-60 compounds to be tested per day [169].

While Ames or Ames-like tests are capable of detecting mutagenic carcinogens, other *in vitro* tests were proposed to identify non-mutagenic carcinogens. One such test is the yeast DEL assay. It is based on the measurement of frequencies of intrachromosomal recombination in yeast *Saccharomyces cerevisiae*, both with and without chemical treatment [170]. This was achieved by integration of a plasmid containing an internal fragment of the His3 gene into its chromosomal location. This resulted in two copies of the His3 gene, one with a terminal deletion at the 3' end and the other with a terminal deletion at the 5' end. Homologous intrachromosomal recombination between the his3 duplications regenerates an intact His3+ allele, which enabled the resulting yeasts to survive in His- medium [170]. Ten chemicals (eight carcinogens and two noncarcinogens) that are difficult to detect with the Ames assay were tested in this assay. Five of the eight carcinogens reproducibly gave a strong positive response and the noncarcinogens were negative in the DEL assay. In contrast, only one of the eight carcinogens was identified by the Ames assay. The three carcinogens that were not identified by either the DEL and Ames assays were: phenobarbital, diethylhexylphthalate, and diethylstilbestrol [171]. Later, scientists at Xenometrix, Inc. converted the assay from the traditional agar plate assay to the microtiter plate format, which greatly increased its ease of use and quantitative accuracy. Results were obtained in 2-3 days. The recombinant yeast colonies were scored by color change of the pH indicator dye, which is amenable to automation [172].

One emerging technology in the genetic toxicity screening area is to use gene expression responses to predict genotoxicity outcomes. This can be applied *in vitro* to both human and bacterial cells using reporter genes to enable rapid screening throughput. In one such assay, seven different reporter constructs stably integrated into the RKO cell line (a human colon carcinoma cell line) was assembled in a 96-well microtiter plate format, using choramphenicol acetyl transferase (CAT) as a reporter gene [173]. In another, bacterial cells (both *Escherichia coli* strains and *Salmonella typhimurium* strains), each containing a different stress promoter fused to a promoterless *lacZ* structural gene, were assembled on 96 well microtiter plates for use in a modified ß galactosidase protocol. In this format, large numbers of compounds can be screened for their ability to induce any of the promoter:lacZ expression [174]. These reporter gene constructs were shown to be responsive to several genotoxic chemicals of different classes including DNA cross-linkers (mitomycin C, UVC irradiation), DNA alkylators (methylmethane sulfonate, ethylmethane sulfonate , *N*-methyl-*N*-nitro-*N*-nitrosoguanine, and dimethylnitrosamine), DNA intercalators (actinomycin D) and nonintercalators (hydroxyurea). While these "proof of concept" studies demonstrated the promise of this approach, more compounds synthesized by pharmaceutical companies will need to be tested before this method sees widespread usage.

Hepatic Toxicity

Drug-induced liver damage has emerged as a major cause of post-market withdrawal of medications [175]. Although the incidence of such liver damage represents a small percentage of patients undergoing drug therapy, the severity of the liver damage could be high, resulting in liver transplant and/or death. It is thus highly desirable to devise a strategy to detect and therefore minimize such toxicity in drug discovery.

Hepatic toxicity can be caused by a variety of mechanisms. Therefore, a variety of *in vitro* assays have been used by researchers to study potential hepatic effects of drugs, each addressing a specific mechanism (steatosis, cholestasis, phospholipidosis, reactive intermediates, etc.). Steatosis, or accumulation of fatty acid (i.e., "fatty liver"), can be caused by alcohol, aspirin, tetracycline, amiodarone, valproic acid, and several antiviral nucleoside analogues, the

most prominent of which is fialuridine. These adverse effects have led to the recall of diethyaminoethoxyhexestrol (DEAEH), the abrupt interruption of clinical trials with fialuridine, essential abandonment of perhexiline, and therapeutic guidelines for the use of tetracycline and valproic acid [176]. One of the major mechanisms involved in steatosis is the inhibition of beta-oxidation of long-chain fatty acids, either by direct inhibition or indirect inhibition such as CoA sequestration or mitochondria DNA damage [177]. The resulting fatty acid accumulation can be detected and quantified by staining primary hepatocytes with neutral lipid stains such as Oil red O [178]. In addition, DEAEH, amiodarone, and perhexiline also block the transfer of electrons in the respiratory chain, produce superoxide anion, and cause lipid peroxidation [179]. The aldehyde products of lipid peroxidation, 4-hydroxynonenal and malondialdehyde (MDA), are known activators of hepatic stellate cells, the principal collagen-producing cells within the liver. MDA is also known to stimulate inflammatory responses. Therefore, the combination of decreased beta-oxidation (resulting in lipid accumulation) and reactive oxygen generation (resulting in lipid peroxidation) represents an important mechanism of drug-induced steatohepatitis. Both of these biochemical endpoints can be measured relatively rapidly using either hepatocytes [180] or isolated liver mitochondria [181], thereby offering opportunities to study potential steatohepatitic effect of drug candidates in drug discovery.

Intrahepatic cholestasis, defined as impairment in bile formation and/or bile flow, is another common manifestation of drug-induced liver disease. In humans, intrahepatic cholestasis most often results as a side-effect of drug therapy and the clinical manifestation of this condition, jaundice, has been estimated to account for hospitalization in 2 to 5% of the cases for the general population and approaches as much as 20% in the elderly [182]. As the population ages and the occurrence of multiple drug therapy in geriatric patients increases, it is to be expected that jaundice and/or drug-induced intrahepatic cholestasis will become even more prevalent. Bile formation is dependant on the specific transporter proteins in hepatocytes. As expected, inhibition of important hepatobiliary transporters can result in cholestasis. The functions of these hepatobiliary transporters, including the bile salt export pump (BSEP), multidrug-resistance-3 (MDR3), multidrug-resistance-1 (MDR1), and multiresistance-protein-2 (MRP2), have recently been elucidated [183]. BSEP transports bile salts into the bile canalicular space. MDR3 transports phospholipids into the bile canalicular space. Inside bile canalicular spaces, the phospholipids form mixed micelles with the bile salts to minimize the cytotoxic effects of free bile salts on cholangiocytes (cells lining the bile canalicular space). It is therefore expected that disruption/inhibition of the activity of these transporters can have toxic consequences. Indeed, human mutations in BSEP and MDR3 result in familial intrahepatic cholestasis types 2 and 3, respectively [183]. In addition, women with heterozygosity for a nonsense mutation of the MDR3 transporter are susceptible to cholestasis of pregnancy as a result of the high circulating levels of estrogens [184]. Recently, several chemicals that have intrahepatic cholestasis side effects were found to inhibit BSEP. These drugs include cyclosporin A, rifamycin SV, rifampicin, glibenclamide (or glyburide), the cholestatic estrogen metabolite (estradiol-17-beta-glucuronide) [185], troglitazone, troglitazone sulfate [186], Bosentan and its metabolites [187]. In these studies, inhibition of rat Bsep was successfully shown using isolated canalicular rat liver plasma membrane vesicles, or rat Bsep-expressing Sf9 cell vesicles. It is therefore possible to utilize these rapid *in vitro* assays to study the inhibition of rat Bsep or even human BSEP, in order to identify the intrinsic cholestatic property of new chemical entities.

It is well known that many hepatotoxic agents can be metabolized to reactive metabolites that can either be detoxified or react with glutathione, enzymes, nucleic acids, lipids, or proteins [188, 189]. These intermediates are electrophilic metabolites or free radicals that are generated during the metabolism of a broad range of functional groups. Therefore on a molar basis, they are likely to be more reactive to biological nucleophiles (peptides, proteins, nucleic acids, etc.) than their parent compound or non-electrophilic metabolites. However, in practice, since the non-electrophilic metabolites are usually in molar excess over the electrophilic metabolites, it can be difficult to differentiate the toxic contribution of the parent and non-electrophilic metabolites vs. the electrophilic metabolites. With regard to covalent binding of electrophilic metabolites to proteins, it has been reported to correlate with cytotoxicity [190, 191], although exceptions were also reported [192]. Reactive metabolite formation is also considered an important factor in immune-mediated idiosyncratic drug hypersensitivity [193]. However, it remains to be seen whether all covalently modified proteins or only a subset of modified proteins are capable of initiating an immune response [193]. These uncertainties aside, there exist several rapid *in vitro* methods to detect and measure the generation of such reactive intermediates. For example, Chen *et al.* developed a high-throughput assay for identifying drug candidates that produce reactive metabolites that contribute to toxicity of the drug products [194]. The method involves incubating drug candidates with a liver microsomal drug metabolizing enzyme system in the presence of glutathione and detecting glutathione conjugates via tandem mass spectrometry [194]. In cells, reduced glutathione (GSH) is known to interact with electrophilic compounds/metabolites and free radicals to play a key role in detoxification of such molecules. Depletion of reduced glutathione was reported to be a marker of hepatotoxicity (e.g., [195]), suggesting its evaluation is important in toxicological studies. For example, monochlorobimane and chloromethylfluorescein diacetate (CMFDA) have been successfully used to monitor cellular GSH levels by epifluorescence [196, 197]. These cell-based assays are amenable to high throughput evaluation of chemical compounds and their reactive metabolites in drug discovery.

Phospholipidosis, defined as the accumulation of excess phospholipids in cells, is often accompanied with various associated or coincidental toxicities, especially the lung and the liver [198-200]. Cationic amphiphilic drugs can often induce this phenomenon *in vivo*. While phospholipidosis per se does not constitute frank toxicity [201], it is reportedly predictive of drug or metabolite accumulation in affected tissues [202], and as such, possibly associated with

toxicities that may warrant further investigation. The accumulation of phospholipids in cells can be monitored by staining cells with fluorescent phospholipid analogs such as NBD-PE [203] or NBD-PC [204, 205]. Using this method, the phospholipidosis observed for a pharmaceutical compound was attributed to a cationic metabolite [203]. Structural changes of the parent compound to alter the property of its metabolite, diminished phospholipidosis effect both *in vitro* and *in vivo* [203]. This example highlights the usefulness of the cell-based phospholipidosis assay in the lead optimization stage of drug discovery.

In addition to these *in vitro* methods to study specific hepatic side effects, several *in vivo* approaches with short-term and low bulk requirement have also been proposed. Toxicogenomics, or examination of altered liver gene expression in rats exposed to prototypical hepatic toxicants suggests that this is a promising approach (e.g., [206]). Obviously more chemicals need to be tested to ensure that reliable gene expression "fingerprints" can be identified that correlates with known hepatotoxic outcome.

Recently, 'Metabonomics' has become an emerging approach to evaluate *in vivo* toxicities. 'Metabonomics' is defined as 'the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification' [207]. By using NMR spectroscopy to study the multicomponent composition of biofluids such as urine of animals or humans treated with a chemical, metabonomics promises to provide early biomarkers of whole body toxicity [207].

Cardiotoxicity: Long QT Syndrome

During a cardiac cycle, the repolarization phase of the ventricular cells is represented by the QT interval of the surface electrocardiogram. Two histamine H1 receptor antagonists (astemizole, terfenadine) were shown to induce QT interval prolongation both *in vitro* and *in vivo* [208]. In rare cases, the long QT syndrome can result in abnormal ECG morphology termed torsade de pointes, which, in its severe form, can result in sudden cardiac death. Torsade de pointes have recently been implicated with several drugs including terfenadine and astemizole [209]. This cardiac side-effect has been interpreted as a consequence of the interference of these drugs with cardiac K+ channels involved in action potential repolarization, and in particular with the Ikr channel. Both terfenadine and astemizole have been shown to block HERG K+ channels in a concentration range similar to that found in the plasma of subjects with cardiotoxic symptoms [208]. Rapid *in vitro* assays to evaluate compounds for possible blockade of HERG K+ channels have been reported [210], including a high throughput assay using competitive inhibition of [^{3}H]Dofetilide-binding to IKr channel as the detection method [211]. These assay have been adopted by several pharmaceutical companies in drug discovery or early development phases to screen novel compounds for potential cardiotoxicity.

Drug-drug Interactions Due to Inhibition of P450s and PgP

(1). Cytochrome P450 Inhibitions

In the clinic, many drugs are metabolized by cytochrome P450s, mainly by CYP3A4, such as theophylline, erythromycin, saquinavir, terfenadine, midazolam and many more. When these drugs are co-administratered with CYP3A4 inhibitors, such as ketoconazole, ritonavir and cimetidine, a drug concentration in plasma will be increased and could cause toxicity, especially for drugs with narrow therapeutic indexes (TI) [212-214]. Therefore, these drug-drug interactions (DDIs) should be avoided. Numerous reviews are available regarding DDIs [212, 215-218].

Considerable effort has been made to predict the magnitude of *in vivo* metabolic drug-drug interactions using *in vitro* data, which can be conducted using human liver microsomes and recombinant P450 isoforms [215, 219]. Mechanism-based DDIs also have been extensively studied [73]. A mechanism-based inhibitor is a metabolic-intermediate that covalently binds to the metabolizing enzyme, which will lead to most likely irreversible inactivation of the enzyme. It will be more difficult to use *in vitro* data to predict mechanism-based drug-drug interaction in the clinic [215].

(2). PgP Drug-drug Interactions

It has been increasingly recognized that inhibition of drug transporters such as the P-glycoprotein (PgP) can be an important mechanism of drug-drug interactions [220]. Because of its wide substrate specificity and tissue distribution, PgP plays a critical role in excreting drugs and their metabolites into the intestinal lumen, bile, urine, and in limiting their exposure to the brain and uterus. Clinically important PgP-mediated drug interactions have been reported and include: digoxin cardiotoxicity when coadministered with PgP inhibitors such as verapamil, quinidine, or cyclosporin A (CsA) [221, 222]; vincristine neurotoxicity when co-administered with CsA [223]; and loperamide neurotoxicity when co-administered with quinidine [224].

As digoxin is one of the most frequently prescribed drugs to elderly patients, avoidance of potent PgP inhibitors minimizes the potential drug interaction with this common medication. Using polarized cell lines expressing PgP, both transport [225, 226] and inhibition [227-229] of PgP can be studied. High throughput assays to measure PgP inhibition using calcein fluorometric readout have been reported [226, 230-232]. These assays make it possible to screen for potent PgP inhibitors in drug discovery.

In Silico Approaches

With regard *to in silico* approaches to toxicity, predictions of mutagenecity will likely be one of the first success stories in the near future [233]. This is due to the fact that predictions of mutagenecity come from considerations of how likely a compound will interact with largely a single target - DNA. With organ toxicity, not only does one have to take into account the multitude of targets within one organ, one need also consider the organ exposure/concentration of a particular compound (a function of pharmacokinetics), and the metabolism of a compound in

the liver and/or the target organs (a function of metabolism). Thus, it is unlikely that virtual toxicity testing will be a reality in the near term [234]. Since SAR ultimately draws its validity from linkage to mechanisms, a mechanism-based in silico/vitro/vivo approach must be produced from an understanding of the interaction of chemicals within human bodies at the molecular, cellular and whole body level. Before and following the advent of this reality, refined *in vitro* and *in vivo* assays will still be needed to maintain the validity of both in silico and *in vitro* approaches in their ability to predict the likelihood of toxic liabilities for any novel compounds.

In Vivo Approaches

In addition to the *in vitro* and *in silico* methods to study specific drug-induced side effects, *in vivo* approaches will always be needed to define critical toxicity parameters such as "dose-limiting toxicity", "therapeutic index", "species differences", etc. Species difference is an important point to address at this stage, because *in vivo* animal studies in two or more species will serve to provide a more completed picture of a drug's potential side effects before it can be ethically tested in human bodies. If observed species differences are due to ADME differences between species, it is critical to define key ADME parameters so that a more physiologically-based human risk assessment can be made.

To detect undesirable drug effects earlier, more sensitive and reliable biomarkers of drug toxicity will be needed to monitor and measure drug-induced side effects in animals and obviously humans. The traditional "gold standards" such as the measurement of released liver enzyme levels in serum is not very sensitive for certain damage (e.g., liver steatosis). Therefore, new biomarkers will need to be developed and validated. Recently, metabonomic, genomic, and proteomic tools have been used to identify novel biomarkers of drug toxicity [235-237]. These emerging tools may one day prove their usefulness in detecting organ toxicity in relatively short-term *in vivo* experiments.

SUMMARY

ADMET properties of a drug candidate are crucial to its commercial success or future. In this review, basic ADMET principles, common reagents, "drug-like" criteria, screening tools and human prediction have been discussed. However, there is no a single screening paradigm will fit for all discovery project needs. A successful drug discovery team must carefully evaluate the ADMET screening tools and incorporate / balance with other key criteria, such as drug action potency and selectivity.

Recent technologies in robotics and bioanalytical tools have made HTS for ADMET in drug discovery a reality. As industry accumulates ADMET data from these screens, one can expect to see knowledge, databases towards *in silico* drug design, testing, perhaps even directing synthesis.

In summary, unfavorable ADME properties and drug-induced side effects are the major causes of drug attrition. As we understand more about the underlying mechanisms of ADME and drug toxicity, more "predictive" *in vitro* assays can be devised to support the discovery screening process. As any *in vitro* assays only detect the inherent properties of a chemical, one must also integrate these results with predictions/measurements of many other factors to ensure a rational prediction of the ADMET properties of a drug candidate *in vivo*.

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