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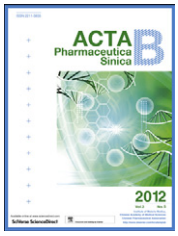

REVIEW

Preclinical experimental models of drug metabolism and disposition in drug discovery and development

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KEY WORDS

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Abstract Drug discovery and development involve the utilization of *in vitro* and *in vivo* experimental models. Different models, ranging from test tube experiments to cell cultures, animals, healthy human subjects, and even small numbers of patients that are involved in clinical trials, are used at different stages of drug discovery and development for determination of efficacy and safety. The proper selection and applications of correct models, as well as appropriate data

Abbreviations: ADME, absorption, distribution, metabolism and excretion; AO, aldehyde oxidases; AUC, area under the plasma concentration–time course curve; BCRP, breast cancer resistance protein; BDC, bile-duct cannulation; BSEP, bile salt export pump; CYP, cytochrome P450; DDI, drug–drug interaction; DMPK, drug metabolism and pharmacokinetics; FDA, the US Food and Drug Administration; FMO, flavin-containing monooxygenase; GST, glutathione transferase; hERG, human ether-à-go-go-related gene; HLMs, human liver microsomes; Met ID, metabolite identification; MRP, multi-drug resistance-associated protein; NAT, *N*-acetyl transferase, NTCP, sodium taurocholate cotransporting polypeptide; OAT, organic anion transporter; OATP, organic anion transporting peptide; OCT, organic cation transporter; PAMPA, parallel artificial membrane permeation assays; P-gp, P-glycoprotein; PK, pharmacokinetics; PD, phamacodynamics; PXR, pregnane X receptor; QWBA, quantitative whole body autoradiography; SAR, structure–activity relationship; SULT, sulfotransferase; TDI, time-dependent inhibition; TK, toxicokinetics; UGT, UDP-glucuronosyltransferase; XO, xanthine oxidase

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interpretation, are critically important in decision making and successful advancement of drug candidates. In this review, we discuss strategies in the applications of both *in vitro* and *in vivo* experimental models of drug metabolism and disposition.

1. Introduction

Drug metabolism and pharmacokinetics (DMPK) have improved success rates of drug discovery and development in the last two decades[1–5]. The contributions and improvement to the success rates by DMPK can be attributed to at least three reasons. First, DMPK is now routinely incorporated into early drug discovery, a trend beginning about 15–20 years ago. Second, DMPK scientists have a better understanding of drug metabolic enzymes, transporters, and differences among species and individuals. Many research tools and advanced instrumentation are now available to make relatively reliable predictions from the *in vitro* to the *in vivo* data and from animals to humans. Third, strategies for the optimization of appropriate DMPK properties have been formed, improved and applied to drug discovery and development.

As shown in Fig. 1, absorption, distribution, metabolism and excretion (ADME) studies are important for drug discovery and development. In fact, the ADME parameters obtained from *in vitro* and *in vivo* models, which aid in the prediction of drug behaviors in patients, are important for the decision to advance, hold or terminate a drug candidate. However, incomplete ADME studies or misinterpretation of ADME data may cause failures in drug development. ADME studies are conducted with *in vitro*, *in vivo* or *in silico* models. *In vitro* models generate many ADME parameters, including apparent permeability, metabolic stability, reaction phenotyping, protein binding, blood-to-plasma partitioning, drug–drug interaction potentials (*e.g.*, inhibition and induction of cytochrome P450 (CYP) and transporters), cell proliferation and cytotoxicity, and hERG inhibition. *In vivo* models of animals and healthy human subjects provide information such as drug oral bioavailability, exposures, distribution, clearance, and duration of exposure for a drug and its metabolites. Finally, *in silico* models predict drug behaviors based on physicochemical properties of drug candidates in combination with crystal structures of a protein (an enzyme or a transporter) and database of ADME properties generated in laboratories. Major model systems needed for particular studies are listed in Table 1. With the numerous models available, proper experimental model selection is essential for ADME property optimization.

A few common strategies for ADME property optimization, highlighted in this paragraph, will be described in the following sections, which include step-by-step, issue-driven, PK–PD and PK–TK considerations. During the early and middle stages of drug discovery (also called lead identification and optimization), ADME screening is conducted, usually in a high throughput mode. ADME screening usually includes, but is not limited to, determinations of human and animal liver microsomal stability, human CYP inhibition, apparent permeability (using parallel artificial membrane permeation assays (PAMPA) and/or Caco-2 cells), PK, metabolic soft spots, reactive metabolites and activation of pregnane *X* receptor (PXR) (using reporter gene assays). In the late stage of drug discovery (also called clinical candidate characterization), more comprehensive ADME properties of drug candidates will be determined. DMPK studies at this stage include, but

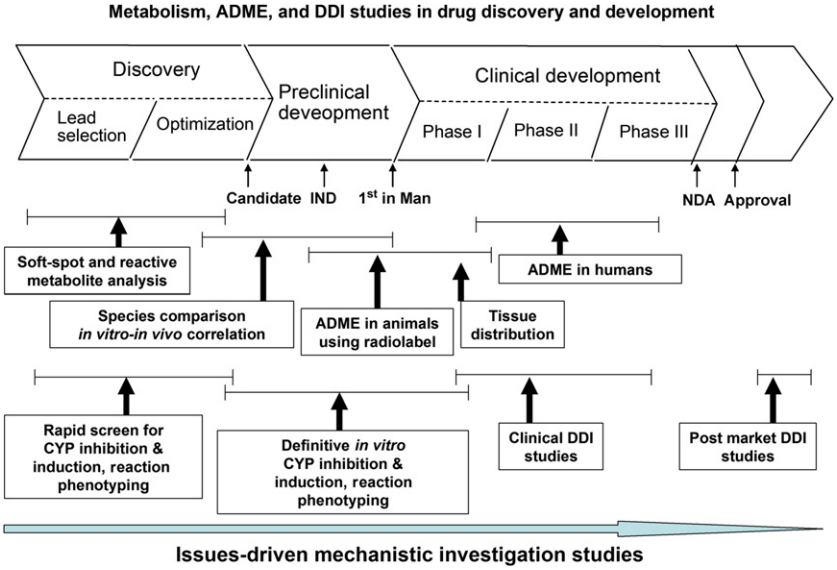

Figure 1 Typical model systems used in staged drug discovery and development.

Table 1 Model systems for particular studies.

ADME study	Test system
Metabolic stability	Liver preps, enzymes
Metabolite ID	Liver preps, bioreactors, *in vivo*
Reaction phenotyping	Microsome, hepatocyte, enzyme
CYP inhibition	Microsome, hepatocyte, enzyme
CYP induction	Microsome, *ex vivo*
Transporters	Caco-2 and other cell lines
Plasma protein binding	Plasma
Mass balance	Animals and human subjects
Metabolite profiling	Animals and human subjects
Disposition	Healthy subjects or patients
Species comparison	Animals and humans
Tissue distribution	Rats

are not limited to, protein binding (as well as blood–plasma partitioning and plasma stability), hepatocyte stability, metabolic enzyme phenotyping, human CYP inhibition, mechanism-based inactivation, ADME studies in animals (including oral and *i.v.* PK studies), mass balance, drug distribution (using quantitative whole body autoradiography) (QWBA), drug elimination pathways (using bile-duct cannulated animals), *ex vivo* induction (if needed), metabolite identification (Met ID), transporter substrate and/or inhibitor evaluation, human CYP induction (using human hepatocytes), drug–drug interaction evaluation, and DMPK modeling (which includes scaling up from *in vitro* to *in vivo*, and from preclinical animals to humans, and human dosing projection). The key transporters that need to be evaluated are efflux transporters, including P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), as well as bile salt export pump (BSEP) and uptake transporters, including organic anion transporter (OAT) 1 and 3, organic cation transporter (OCT) 2, organic anion transporting peptide (OATP) 1B1 and 1B3[6].

2. ADME experimental models

2.1. *In vitro metabolic models*

In DMPK, "drug-like" properties commonly refer to respectable absorption, adequate distribution, low metabolism, complete elimination from body and minimal toxicological risk. *In vitro* assays, which play an essential role in screening chemical entities in the discovery stage, have many unique advantages. First, these assays provide a simple, convenient and fast way to test the potency and drug-like properties of chemical entities. Second, *in vitro* assays require limited amounts of test compound, which is appealing since compound supply is limited at the early drug discovery stage and it is not always feasible to perform preclinical animal studies. Third, *in vitro* assays are often designed to answer specific questions, such as structure–activity relationship (SAR) for metabolic stability or DDI potential of the drug candidates; answers to these questions may be difficult to obtain in animal studies due to confounding factors. Fourth, human-based *in vitro* assays could provide a more accurate estimation of human clinical outcomes than could animal tests at the preclinical stage, especially for properties that are known to have species-related differences (between preclinical animals and humans) and where strong *in vitro–in vivo* correlations have already been established in humans. An example of such a property is CYP enzyme induction. It has been shown that preclinical species cannot always predict human CYP induction potential due to species related differences. However, a good *in vitro–in vivo* correlation has been observed in humans. For instance, a negative induction in human *in vitro* studies using primary hepatocyte cultures seemed to always link to a negative induction observed in humans in clinical studies. Therefore, FDA typically waives clinical DDI studies if the drug candidate is tested negative in a human *in vitro* CYP induction study.

The prediction of human efficacy and DMPK/toxicology properties from preclinical studies is perhaps the ultimate goal of drug discovery. A common practice in pharmaceutical industry is to establish *in vitro–in vivo* correlation for an ADME parameter in multi-species preclinical studies, and then find the species that correlates to humans. If the *in vitro–in vivo* correlation is established in an animal species and the *in vitro* correlation between the animal species and humans is also established, then the human *in vitro* information could be used to predict the clinical ADME outcome (Fig. 2). On the other hand, if the *in vivo* correlation for a parameter between animal and human is well understood, then animal *in vivo* data could be used to predict human outcomes. Examples of parameters often used for such predictions include human efficacy, PK properties (*e.g.*, target inhibition, absorption and clearance) and toxicological endpoints (*e.g.*, QT prolongation). Once the *in vitro–in vivo* or animal to human correlations are established, the estimations, for example, of human clearance from human *in vitro* clearance data, of human efficacy from xenograft mouse model, or of human QT prolongation risk from dog studies, are possible. In general, DMPK parameters are extrapolated from (1) comparisons of *in vitro* data across species and/or (2) *in vitro–in vivo* correlation in animals. Notably, *in vivo* models provide combined effects of permeability, distribution, metabolism and elimination. Thus, they have limitations for such applications as to pinpoint a specific factor in an observation. For example, it would be hard to rely only on differences in permeability, without considering differences in first-pass metabolism, to evaluate bioavailability among a series of drug candidates.

2.1.1. *Expressed enzymes*

A primary application of expressed enzymes is to conduct reaction-phenotyping for drug candidates. The FDA DDI guidance[7] requires conducting clinical DDI trial if a compound has greater than 25% of its clearance mediated by a particular pathway. CYP metabolism is the most common clearance pathways for the elimination of drugs. Thus, identifying the relative contributions of each CYP isoforms to the total hepatic clearance is an essential task. Additionally, when the CYP metabolism data are combined with data on other non-metabolic clearance pathways, such as renal or biliary clearance, estimations can be made on the contributions of each CYP isoform to the total body clearance of a drug. A second application of expressed enzymes is to qualitatively identify the involvement of CYPs in metabolic pathways (often referred to as CYP mapping). This assay provides important information on whether the drug candidate

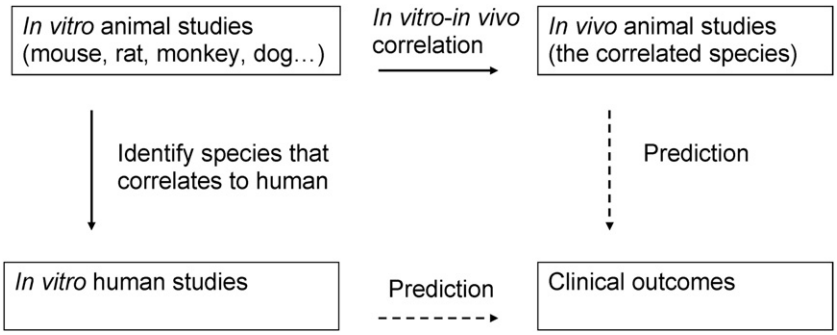

Figure 2 A model of using *in vitro–in vivo* correlation in preclinical species, combined with human *in vitro* studies, to predict human clinical outcomes.

is metabolized by a single isoform or by multiple isoforms, and furthermore, whether highly polymorphic enzymes, such as CYP2D6 and CYP2C19, are the major contributors to its metabolic clearance. It should be noted that, while in most cases hepatic metabolism are performed by the primary drug-metabolizing CYPs, other enzymes could also contribute significantly to the overall metabolism. Examples of non-CYP microsomal or cytosolic enzymes often involved in drug metabolism include flavin-containing monooxygenases (FMO), UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT) and aldehyde oxidases (AO)[8,9].

Expressed enzymes are also used for mechanistic applications. The example described by Li and colleagues[10] featured the use of expressed enzymes, either incubated separately or else in combination with each other; the results elucidated that CYP3A4 was involved in the generation of a metabolite that, in turn, was responsible for time-dependent inhibition (TDI) of CYP2D6. Additionally, since expressed CYP systems are concentrated enzymes with high activity toward a specific substrate, they are often used as "bioreactors" to generate metabolites of interest for further testing. Using the generated metabolites that are collected as chromatographic fractions, it is possible to test for pharmacological activity without the need to identify the metabolite structures. In this application, the high activity and selectivity of expressed enzymes provide a superior alternative to the use of human liver microsomes (HLMs), as HLMs tend to generate greater numbers of metabolites. The potential presence of additional metabolites adds difficulty to liquid chromatographic separation; however, the added expense of using expressed CYPs provides its own challenges.

2.1.2. *Sub-cellular fractions*

Along with the expressed enzyme systems, sub-cellular fractions prepared from drug metabolizing tissues (liver and gut), including cytosol, S9 and microsomal fractions, are used in drug metabolism studies to address various questions. The cytosol, which is isolated as a supernatant from the S9 fraction, contains a group of soluble drug-metabolizing enzymes responsible for specific routes of drug metabolism. It is the simplest system of the three. The S9 fraction contains both cytosol and microsomes, and represents a nearly complete collection of all drug metabolizing enzymes. However, the presence of so many enzymes can sometimes dilute the activities of the enzyme of interest. The microsomal fraction contains membrane-bound CYPs and primary conjugation enzymes such as UGTs. These enzymes are responsible for the metabolism of over 90% of marketed drugs, thus this is the most frequently utilized enzyme system.

The cytosol is often used to conduct mechanistic studies for identifying the soluble enzymes involved in particular metabolic pathways, such as SULT, *N*-acetyl transferase (NAT), certain glutathione transferases (GST), AO, and xanthine oxidase (XO). For example, regioselective sulfation of the phytoestrogens daidzein and genistein was investigated using human liver cytosol and purified, recombinant human SULT isoforms[11]. Additionally, cytosol assays are used to complement microsomal studies for assessing drug metabolism pathways[12].

S9, which contains both cytosol and microsomal enzymes, is the sub-cellular fraction prepared by collecting the supernatant after centrifugation of tissue homogenate at $9000 \times g$. One advantage of using S9 fractions over microsomes is its ability to capture additional metabolism mediated by non-CYP enzymes, such as sulfation and acetylation. Furthermore, S9 allows cost savings and is easier to handle when compared to primary hepatocyte incubations, which also provides a complete collection of enzymes. To predict *in vivo* clearance, the scale up factor (mg of S9 per gram of liver) from S9 incubations is scarcely available in the literature. It was determined in one of the co-authors' laboratory that there is approximately 165 mg of S9 proteins per gram of rat liver[13]. This value can be used to calculate intrinsic clearance across all species (including humans), which in turn can be used to calculate whole body clearance.

When working with sub-cellular fractions, it is important to remember that the co-factors that mediate either the oxidative metabolism (*e.g.*, NADPH) or the conjugation reactions (*e.g.*, uridine diphosphate glucuronic acid (UDPGA), 3′-phosphoadenosine-5′-phosphosulphate (PAPS) and *S*-adenosylmethionine (SAM)) are lost in the process of isolation of those fractions. Therefore, it is necessary to supplement the sub-cellular fractions with these co-factors to initiate the various enzymatic reactions. By including or excluding certain co-factors, one can pinpoint the involvement of certain metabolic pathways for a given compound. This application was well demonstrated in the study of 17α-ethinylestradiol metabolism by Li and colleagues[14]. One issue regarding the use of S9 fractions is that the enzymes in S9 are not as enriched as those in microsomes. A larger amount of S9 protein (usually 5-fold) is required to achieve a level of activity seen in a comparable microsomal incubation. This condition could result in higher protein binding of the test compound.

Microsomes are the most widely used sub-cellular fractions for drug metabolism studies, with the advantages of being inexpensive and easy to handle while containing the major drug metabolism enzymes, *e.g.*, CYPs and UGTs. Microsomal assays are the default assays for metabolism and DDI studies at the drug discovery stage. One application of microsomal assay is to determine intrinsic clearance, a useful parameter for facilitating the screening process for stable compounds and for establishing an *in vitro* correlation between animals and humans. Using microsomes from various tissues one can assess extra-hepatic metabolism and further strengthen the *in vitro* prediction of total body clearance by considering multi-organ metabolism. Microsomal assays are also preferred for determining DDI potential. Both reversible inhibition and time-dependent inhibition studies provide information on the possibility of the drug candidate being a perpetrator of DDI for a co-administered drug. On the other hand, CYP reaction phenotyping studies may answer whether the drug candidate might be a victim if co-administered with a CYP inhibitor or inducer. An additional feature of microsomes, that they contain relatively concentrated CYP enzymes, makes them useful for enzyme kinetic studies, which provide information on such parameters as the V_{max} and K_m of a metabolic pathway, the k_i or IC_{50} for reversible inhibition, and the k_{inact} and K_I for mechanism-based inhibition.

2.1.3. *Whole cell systems (hepatocytes, other cells and cell lines)*

Hepatocytes are the primary liver cells where drug metabolism occurs. Hepatocytes cell membrane contains various uptake or efflux transporters. Drugs could passively diffuse through the hepatocyte membrane or be taken up by transporters, such as OATP and sodium taurocholate cotransporting polypeptide (NTCP), to enhance the intracellular concentration[15]. Once a drug enters hepatocytes, the efflux transporters, such as P-gp, BCRP and multi-drug resistance-associated proteins (MRP), could pump the drug out, to reduce the intracellular drug concentration. The primary cultures of hepatocytes carry enzymes and co-factors at physiological concentrations and provide a drug metabolism environment that closely mimics the *in vivo* conditions. Freshly prepared hepatocytes are a good model for drug metabolism and transporter studies; however, their applications have limitations, including (1) they are not readily available from humans and other higher animals; (2) a preparation could only be used once, so it is difficult to repeat studies or compare studies between laboratories. These limitations can be overcome with cryopreserved human hepatocytes, their utility in *in vitro* systems has provided big advantages in drug discovery[16,17]. The pre-characterized cryopreserved hepatocytes serve effectively as a "reagent" and allow researchers to perform experiments at their own schedule and test drug candidates over a period of time using the same lot(s) of hepatocytes. It also allows studies to be directly compared between different laboratories. In addition, the use of cryopreserved human hepatocytes permits the choice of specific pre-characterized lots (donors) to meet one's research needs, such as studies in special populations of smokers, alcohol users, or people with poor metabolism *via* CYP2D6 or 2C19.

Hepatocytes are used in drug discovery studies as suspension or sandwich cultures. The suspension cultures are used for the study of metabolic stability or transporter-mediated uptake of drug candidates. Studies using hepatocytes suspended in plasma assist in the improvement of *in vitro–in vivo* correlations in drug clearance and DDI predictions[18–22]. The disadvantage of suspension culture is that the hepatocytes have a limited viability and typically can be used for acute dose-studies only, generally up to 4 h[15,17]. Hepatocytes cultured on a collagen coated plastic surface extend the viable period to 7–10 days. Despite decreases of CYP activities over the culture period, the hepatocyte cultures still provide insight to the effects of an increased exposure period on the test compound and to secondary metabolite formation, as might occur in *in vivo* situations. This assay format is also reported to be superior for identification of time-dependent inhibitors, which would be missed in microsomal reactions with shorter incubation time[23,24]. One major application of cultured hepatocytes is for use in CYP induction studies, because enzyme induction may involve gene transcription and translation, and therefore may require time to take effect. In fact, primary cultured human hepatocytes have become the "gold standard" for conducting CYP induction studies[7,25,26]. Cell lines, such as Fa2N-4 and HepaRG, are also used for CYP induction evaluations; however, reports suggest that some of the CYP enzymes, such as CYP2B6, may be under represented in these cell lines[27,28].

Hepatocyte culturing for longer terms has become an emerging area of research in the last decade. While there are some progress, most of the successful examples are limited at academic scale, such as the design of special formats or devices for 2-D or 3-D culturing systems, with which specialty is required to maintain and perform even a small scale study. None of these systems is ready to be practically used for commercial applications, such as industrial research for drug screen and drug development.

Liu and colleagues[29,30] have modified sandwich culture conditions by adding a thick layer of Matrigel™ on top of the hepatocytes, in order to study biliary excretion of compounds. Under these conditions, healthy bile canaliculi between hepatocytes were developed. When test compound is incubated with hepatocytes, part of the test compound is excreted into bile canaliculi and the rest remains in hepatocytes or medium. The tight junctions between the hepatocytes, which prevent the compound from escaping the bile pockets, can be opened, releasing the compound from bile pockets into the media, by treatment of the hepatocytes with a calcium-free medium (or EDTA-containing medium). The presence of the canaliculi allows the quantification of biliary excretion and subsequent calculation of the biliary excretion index and determination of clearance *via* biliary excretion, in a well designed study. This application provides a major advantage in that one could identify species similarities and find a preclinical species that can be used to predict clearance in humans *in vivo*.

2.2. *In vitro transporter models*

Transporters play important roles in drug disposition, drug–drug interactions and drug toxicity. Approximate 400 human transporters can be classified into ATP-binding cassette (ABC) transporter and solute carrier (SLC) transporter families[6]. In general, ABC transporters pump the substrates out of cells,

whereas SLC transporters take substrates into cells. Transporter substrates can be endogenous compounds or xenobiotics, including some drugs, drug candidates and their metabolites. The key efflux transporters include P-gp, BCRP and BSEP, whereas the important uptake transporters are OATP1B1, OATP1B3, OAT1, OAT3, OCT1 and OCT2[6,7,31]. Determination of a drug candidate's status as a transporter substrate and/or inhibitor requires performance of a transporter assay using one of the following *in vitro* experimental models. Readers are referred to published reviews for detailed information[6,32,33].

2.2.1. Immortalized cell lines

Human colon cancer derived Caco-2 cell line is widely used as a model of permeability and efflux transport (*via* P-gp and BCRP). This is because Caco-2 cells undergo enterocytic differentiation and become polarized in culture (usually for 21 days), resembling human intestinal epithelium in transporter expression and tight junction formation. The bi-directional permeability assay using Caco-2 is the most popular method for identification of P-gp substrates and inhibitors in drug discovery[6,34] among the various *in vitro* models.

2.2.2. Transfected cell lines

Chinese hamster ovary (CHO) cells, Mardin-Darby canine kidney (MDCK) cells, human embryonic kidney 293 cells (HEK293) and pig kidney epithelial cells (LLC-PK1) are commonly transfected to over-express a single transporter. For certain efflux transporters transfected in MDCK cells, co-expression of a corresponding uptake transporter is necessary[35,36]. The transfection can be transient or permanent. A permanent transfection cell line is usually favored over transient transfection because the former produce more reproducible data.

2.2.3. Hepatocytes

The liver plays a central role in drug metabolism and disposition through its primary metabolic enzymes (both Phase I and Phase II) and many transporters (including both uptake transporters expressed on the sinusoidal membrane and efflux transporters expressed on sinusoidal and canalicular membranes). Therefore, freshly isolated hepatocytes are regularly used to determine hepatic metabolism and clearance mediated by liver enzymes[37], as well as to assess hepatic uptake mediated by uptake transporters[38,39]. In experiments, the uptake into hepatocytes is measured in suspension by a centrifugation method, using oil-layered tubes to separate hepatocytes from incubation medium. There have also been reports that cryopreserved hepatocytes can be used for studying hepatic uptake[15]. Isolated hepatocytes cannot be directly used for efflux assay because the efflux transporters lose their function during the isolation process; however, primary cultures of hepatocytes can restore the function of efflux transporters even though the uptake transporters demonstrate decreased functionality in primary cultures.

2.2.4. Membrane vesicles

Membrane vesicles prepared from organs (such as liver, kidney and intestine) that naturally express a high concentration of transporters or from transfected cell lines (such as MDCK, HEK293 and LLC-PK1 cells) that over-express a single transporter have been used to assess the transporter mechanisms in liver, kidney and intestine[33,40]. A technical benefit of using membrane vesicles is that it allows separate preparations of the blood side of cell membranes (liver-sinusoidal, kidney and intestine-basolateral) and lumenal side of cell membranes (liver-bile canalicular, the intestine and kidney-brush-border) to fit the research needs.

2.3. In situ and ex vivo models

2.3.1. In situ models (perfusion)

The organ perfusion model most closely mimics *in vivo* drug absorption, transport, metabolism and excretion. Among various organ perfusion models, the liver perfusion model is the most studied. This model has the advantage over hepatocyte and other sub-cellular systems as it maintains the liver structure and architecture, preserves all the transporters and cell populations in addition to hepatocytes (*e.g.*, the Kupffer cells, which are important for regulation of proinflammatory cytokines), and retains hepatocyte cell-to-cell interaction and zonal differentiation. As an endpoint, the perfusate, bile and liver tissue itself can all be analyzed for the parent compound and its metabolites. This application provides information on the extent of the hepatic first-pass effect, effect of protein binding, parent uptake from the perfusate, metabolism, parent and metabolites elimination *via* canalicular transporters, as well as the potential for toxicity from reactive metabolites. The liver perfusion technique is also a useful tool for toxicological and pharmacological studies[41]. The liver perfusion technique has been standardized regarding the experimental set up[42,43] and the constituents of the perfusates usually vary depending on the purpose of the study. Liver perfusion can be performed *in situ* or in a setting where the liver is isolated. The *in situ* perfusion model requires minimal organ preparation, and therefore, the potential of organ damage is minimized. However, isolated liver preparation answers liver-specific mechanistic questions with no interference from other organs, generally making this application a more widely used model. Isolated liver perfusion systems are commercially available (*e.g.*, Harvard Apparatus, Holliston, MA). When using the liver perfusion technique, the type of perfusate used needs to be taken into account. Commonly used perfusates contain varying amounts of matrix (including albumin and/or red blood cell (RBC)), which may affect the protein binding of the test compounds and their metabolism and disposition. Heparinized whole blood from the same species is perhaps the best perfusate. However, heparinized whole blood is not always readily available and potential issues such as the formation of clots must be taken into consideration. As an alternative, bovine blood, bovine serum albumin, and bovine or human erythrocytes have been used to mimic *in vivo* conditions, because they provide hemoglobin as the oxygen carrier and ideal protein binding conditions.

2.3.2. Ex vivo models for induction and toxicity studies

Ex vivo studies refer to ones where a drug is dosed to animals and then the organ tissues are removed and processed (*e.g.*, making liver into microsomes), and used for investigations of the changes in expression levels of enzymes or transporters (or any bio-markers) upon drug treatment. In general, mRNA levels and/or activities are measured to represent the changes

in expression levels. This information can then be linked back to the toxicology or pathology findings in the animal in-life study. One of the most useful practices is to perform the *ex vivo* study in parallel with toxicokinetics (TK) studies where drugs are dosed at high levels (close to the maximum tolerable dose). Pharmacokinetic information is then collected and used to link with or explain the TK, PD or toxicity observations. Usually, at the end of the sub-chronic or chronic dosing study, organs are collected. Liver, the organ that is in most cases exposed to high drug concentrations, is often studied for the effect of drug on enzyme levels. The enzyme expression changes could in turn explain changes in PK, or could be linked to toxicity findings. Among various observations, including changes of enzyme levels, PK, and toxicity, some are directly linked to each other, whereas others may show delayed responses. PK/PD modeling allows further prediction through dissection of the response relationships. For example, in a potential perspective and under a linear PK scenario, if a decrease in drug exposure ($AUC_{(0–24),\ day\ x} < AUC_{(0-\infty),\ day\ 1}$) is observed in an animal repeat-dosing study, it is often considered to be due to an autoinduction by the drug of the enzyme(s) that are responsible for its own metabolism. By testing with CYP probe substrates in an *ex vivo* study, the induction of CYPs by a drug can be determined. On the other hand, if there is a drug accumulation ($AUC_{(0–24),\ day\ x} > AUC_{(0-\infty),\ day\ 1}$), the drug could be a mechanism-based inhibitor that inactivates the enzyme(s) responsible for its own metabolism. One advantage of the *ex vivo* application is the removal of the parent compound during the preparation of the sub-cellular fractions, thus removing potential interference from reversible inhibition by the parent compound.

2.4. *In vivo models*

The *in vitro* models discussed above have limited values, as they reflect only one particular aspect of the whole picture. Whereas, *in vivo* results are multi-factorial, provide the combined effect of permeability, distribution, metabolism and excretion, and can yield a measurable set of pharmacokinetic parameters and toxicology endpoints. Regardless the thoroughness and completeness of the *in vitro* work, animal studies are required to measure drug exposures and to determine potential toxicities. The current trend is to use rats as the first animal species for testing drug exposure because they are inexpensive and require small amount of test compound. *In vivo* rat studies can help identify ADME problems of a new chemical series, such as whether low absorption or high clearance occurs, leading to undesirable PK. Subsequently, *in vitro* models, such as Caco-2, can be used to optimize absorption of compounds from the same chemical series and microsomal stability assay can be applied to select stable compounds. It should be reiterated that human-based *in vitro* assays, relative to other assays, could provide closer estimations of human clinical outcomes, especially for properties that are known to have species differences (such as CYP induction), but have good *in vitro–in vivo* correlations in humans.

2.4.1. *Pharmacokinetic studies*

Understanding the pharmacokinetics of a compound in preclinical species with various dose routes (oral, intravenous, subcutaneous, transdermal, intraperitoneal, continuous infusion, intratracheal, as well as access ports of the portal vein) is an essential component for lead selection and optimization, and clinical candidate nomination and development. Early in the discovery process, PK screening using various dose regimens and administration routes in rodents or non-rodents allows rapid eliminations of drug candidates, which facilitate lead optimization and produce a candidate that is more likely to succeed in preclinical testing and in the clinic. Extensive PK studies are still necessary to evaluate the dose proportionality, bioavailability or food effects in single or multiple dose administration. These studies use compartmental and non-compartmental methods to determine multiple parameters, including maximum concentration (C_{max}), time of maximum concentration (T_{max}), AUC, volume distribution (V_{ss}), clearance (CL), terminal elimination half life ($T_{1/2}$), and bioavailability (F), thereby define the PK profiles of a compound. Dispositional studies also provide data on mass balance, biliary excretion and tissue distribution. Surgical animal models for dosing and sampling can be used to provide insight on biliary excretion from bile-duct cannulation and to investigate first-pass metabolism from vascular and portal vein cannulation. The applications of animal models have been extensively reviewed by Salyers[44]. Table 2 lists typical pharmacokinetic studies in drug discovery, preclinical development and safety, and clinical and bio-pharmacology studies.

2.4.2. *Preclinical ADME models*

During the discovery stage, mass balance study and ADME studies are conducted with non-radiolabeled compounds, producing limited quantitation data for parent drug and metabolites. In drug development, ADME studies are performed with either ^{14}C or ^{3}H labeled material which provides more detailed quantitative information on the circulating metabolites, the extent of metabolism, and routes of excretion of the drug and its metabolites. Zhang and Comezoglu[45] provided a detailed review on ADME study design and data presentation. Tissue distribution studies in pigmented Long–Evans rats are usually conducted first to provide dosimetry for various tissues and organs, which supports human ADME studies with radiolabeled compounds. Typically, these studies are limited to single dose by the intended route of administration (PO, IV, etc.). The radioactivity levels in various tissues at different time points are measured by QWBA, a process where whole body animals sections are exposed to a phosphorimaging screen and then scanned with a phosphor imager. Based on body surface area and body weight, tissue exposure to the radioactivity in the rat is extrapolated to human tissues[46]. For most compounds, administration of a 100-μCi radioactive dose typically exposes the human subjects to an effective dose equivalent of <1 mSiv, well below the radiation limit set by the Nuclear Regulatory Commission[47]. Tissue distribution studies have additional applications; for example, the evaluation of maternal–fetal transfers. These studies are required for the IND and NDA filings and are typically conducted in the rat strain used in the toxicological evaluation.

The preclinical species used in the mass balance studies (referred as ADME studies in this section) are based on the choice of species used in the long term safety evaluation of a compound. These studies are designed to mimic the toxicology studies as close as possible in factors such as dose and the

Table 2 Role of pharmacokinetic studies in drug discovery and development.

Stage	PK study type	Objective	PK or TK parameter
Discovery	Fast exposure and PK screening in rat	To find bioavailable compound	AUC, C_{max}
	Single/multiple/IV dose in mouse, rat, dog, or monkey	To compare PK parameters across species	AUC, C_{max}, T_{max}, $T_{1/2}$, V_d, F
	Allometric scaling from *in vivo* and *in vitro* data to predict human dose and PK	To predict human dose and PK parameters	CL, AUC, C_{max}, $T_{1/2}$ and efficacious dose projection
Preclinical develop/ safety	Formulation in animal species	To determine best formulations for drug exposures	AUC, C_{max}
	Single ascending dose TK in rat, dog, or monkey	To determine NOAEL	AUC, C_{max}, exposure multiples
	Dose-range finding in rat, dog or monkey	To determine safe doses	Same as above
	1-Month IND toxicology in rat	To determine NOAEL	Same as above
	1-Month IND toxicology in dog or monkey	To determine NOAEL	Same as above
	Long-term (0.25–1 year) toxicology and 2-year carcinogenesis in mouse and rat	To ensure safety in rat and mouse including carcinogenicity	Same as above
	Long-term (0.5–1 year) toxicology in dog or monkey	To ensure safety in animal species	Same as above
Clinical/ Bio-Pharma	Single/multiple ascending dose	To determine safety and maximum tolerability doses	AUC, C_{max}, T_{max}, $T_{1/2}$, (V_d, F)
	Radiolabeled ADME	To determine parent and metabolite profiles	Parent and metabolite exposures
	DDI with inhibitors (*e.g.*, ketoconazole)	To test DDI potential	AUC, C_{max}, T_{max}, $T_{1/2}$, (V_d, F)
	DDI with an inducer (*e.g.*, rifampin)	To test DDI potential	Same as above
	DDI with other drugs (*e.g.*, Co-meds, pH modifiers)	To test DDI potential	Same as above
	Special populations (renal/hepatic impair/age/gender, weight)	To test if dose needs be adjusted	Same as above
	Special studies (*e.g.*, food effect, bioavailability…)	To test variability	Same as above
Late clinical	Population kinetics	To determine whether physiological conditions, such as age, body weight, ethnical background, may have influence in PK	Same as above

route of administration, and compound vehicle. The amount of radioactive dose in animal species is determined by the PK properties of the parent compound. The typical administered radioactivity range of 1.5–100 μCi/kg should be sufficient to generate metabolic profiles in plasma at multiple time points with adequate radioactive sensitivity. The duration of the study is determined by the terminal half-life of the parent compound in that species and is set to ≥5-plasma half-lives. For compounds with long half-lives, the duration of the study can be based on the criteria where ≤1% of the total dose is excreted in a given 24-h interval in both urine and feces. Plasma, urine and fecal samples are collected during the duration of the study to analyze for radioactivity and for metabolite profiling. If needed, additional tissue samples are collected to determine the concentration and accumulation of drug and drug-related component in a particular tissue.

The first set of information generated from the ADME studies are the overall plasma profiles of total radioactivity (TRA) *versus* time which is compared to plasma profile of parent *versus* time measured by a validated LC/MS/MS assay. For those drugs where the parent is the major component at all time points in plasma, the total radioactivity profile usually parallels the profile of the parent. Metabolic profiles of plasma concentration over time generated by HPLC analysis, followed by radioactivity and mass spectrometric detection, provides exposure-related information for parent and metabolites in humans and animal species. Mass balance and metabolic profiles of urine and fecal samples generated in ADME studies provide information about extent of metabolism and routes of excretion for parent and metabolites. An example of administration of [^{14}C]-apixaban to healthy human volunteers shows that the compound is excreted in both urine and feces[48,49]. Based on urinary excretion and metabolites in feces, approximately 50% of the dose is absorbed when administered orally. These types of information help to understand the quantitative and qualitative differences in metabolism across species. If all the metabolites that were generated in humans *in vitro* are also observed in animals, it would suggest that the primary pathways of metabolism are similar across species. The combined metabolic profiles in plasma, urine and feces contribute to our knowledge of the complete distribution and disposition of the drug. For drugs that tend to be in the 300–700 Da range and are highly lipophilic in nature, metabolism and excretion through the bile plays a major role in their disposition. For these types of molecules, ADME studies conducted in bile-duct cannulated (BDC) animals, where bile is collected during the

duration of the study, are exceptionally valuable, particularly if conjugative pathways such as glucuronidation or sulfation are involved in the metabolic clearance of the drug. Glucuronides and sulfate conjugates can undergo hydrolysis in the large intestine when excreted through the bile into the gastrointestinal (GI) tract[50,51]. Therefore, in the absence of a bile profile, the role of conjugation in the overall metabolic clearance of the drug would be missed. BDC studies are usually run for a shorter duration than PK studies, 0–24 h in rat and 0–72 h in dog or monkey. A reasonable mass balance can be achieved in BDC animal studies for compounds that have short half-lives. Where non-clinical data shows that most of the drug is excreted in the feces through bile as conjugative metabolites, it is useful to include a panel in the human ADME where bile can be collected for a short duration. In one example, bile was collected for a short duration (3–8 h) after dosing with [^{14}C]-muraglitazar. When the bile and fecal profiles were compared, it became evident that the compound in the bile was excreted as conjugates that were hydrolyzed during their passage through the GI tract[51,52]. Data from the human ADME studies provide information about the primary pathways of metabolism for the compound. The metabolic pathways are determined through the identification of metabolites in plasma, urine and feces/bile, which in turn lead to detailed reaction phenotyping studies to identify the enzymes that generate the primary metabolites. The use of metabolism data obtained from human ADME studies in conjunction with reaction phenotyping provides critical information for decision in aid of clinical DDI studies.

2.5. *Engineered mouse models*

Engineered mouse models are increasingly used for the determination of the roles of CYP enzymes in drug metabolism and toxicity. Mouse *Cyp* gene(s) have been replaced by human *CYP* gene(s) in the mouse genome, to study the specific involvement of the given mouse and/or human CYP(s) in various aspects of ADME. Removing a cluster of *Cyp* gene allows the determination of combined functions of an entire *Cyp* gene subfamily. The composite functions of all microsomal CYP enzymes can be studied by removal or modification of the NADPH-cytochrome P450 reductase (*Cpr*) gene. All of these programmed genetic changes in a cell-type-specific or tissue-selective fashion allows specific contributions of an organ (*e.g.*, the liver or the intestine) to the metabolism and/or toxicity of a drug to be directly determined *in vivo*. The potential of these engineered mouse models for ADME applications is considerable. A number of selected mouse models are introduced below with examples of their applications in drug metabolism, pharmacokinetics, and toxicology studies briefly described, emphasizing practical advice on how to avoid potential confounding factors.

2.5.1. *Available mouse models*

A partial list of available *Cyp*-knockout mouse models and human *CYP*-transgenic mouse models are shown in Table 3. The readers are referred to recent reviews for information on additional mouse lines[53]. Notably, two knockout mouse lines, *e.g.*, *Cyp1a1*-null and *Cyp1b1*-null, can be intercrossed to produce a double knockout mouse[54], except in cases where the two targeted genes are located close to each other (*e.g.*, *Cyp1a1* and *Cyp1a2*). In those two cases, a double knockout model can be generated through genetic engineering in the embryonic stem cells. An even more challenging case is when multiple mouse *Cyp* genes occur in a gene cluster, such as the mouse *Cyp3a* gene cluster. Other mouse *Cyp* gene clusters include the *Cyp2j* cluster, the *Cyp2d* cluster, the *Cyp2c* cluster, and the *Cyp2a-b-f-g-s-t* cluster. The presence of multiple copies of structurally similar *Cyp* genes makes it difficult (and sometimes irrelevant) to identify the specific CYP isoform that is normally active in the metabolism of a given drug *in vivo*. Instead, it makes sense to knockout all members of the subfamily, and to determine the combined functions of the CYP enzymes impacted by the genetic manipulation. Indeed, this strategy has been successfully used in several cases, the first of which is the deletion of the *Cyp3a* gene cluster[55].

The human *CYP*-transgenic mouse models are most often prepared using a large gene fragment contained in a bacterial artificial chromosome (BAC) clone, although cDNA-based transgene constructs have also been used successfully (*e.g.*, for CYP2A6[56]). The tendency for two *CYP* genes in a given *CYP* gene subfamily to be located in close proximity in the genome, and potentially sharing common regulatory sequences for gene expression, has made it difficult to produce single-gene transgenic models for some CYPs, such as *CYP1A1/2*[57], *CYP2C18/19*[58], and *CYP2A13/2B6/2F1*[59]. As a result, mouse models expressing two or more closely associated *CYP* genes were produced. On the other hand, single-gene *CYP*-transgenic mice have been intercrossed, in order to produce double transgenic mouse models (*e.g.*, *CYP2D6/CYP3A4*-transgenic[60]).

A *Cyp*-knockout mouse and a human *CYP*-transgenic mouse can be crossbred, in order to produce so-called "*CYP*-humanized" mouse models, in which the human CYP is expressed, but the orthologous mouse *Cyp* gene is inactivated. For example, human *CYP2E1*-transgenic mouse was intercrossed with *Cyp2e1*-null mouse to produce *CYP2E1*-humanized mouse[61]. More recently, *CYP3A4* transgenic mouse with either hepatic or intestinal CYP3A4 expression

Table 3 A list of selected *Cyp*-knockout and human *CYP*-transgenic mouse models.

Cyp-knockout mouse	Reference(s)	Human *CYP*-transgenic mouse	Reference(s)
Cyp1a1	Dalton et al.[78]	*CYP1A1/2*	Jiang et al.[57]
Cyp1a2	Liang et al.[79]	*CYP2A6*	Zhang et al.[56]
Cyp1b1	Buters et al.[80]	*CYP2C18/2C19*	Löfgren et al.[58]
Cyp2a5	Zhou et al.[69]	*CYP2D6*	Corchero et al.[73]
Cyp2f2	Li et al.[77]	*CYP2A13/2B6/2F1*	Wei et al.[59]
Cyp2e1	Lee et al.[70]	*CYP2E1*	Cheung et al.[61]
Cyp3a (gene cluster)	van Herwaarden et al.[55]	*CYP3A4*	Cheung et al.[76], Granvil et al.[82], van Herwaarden et al.[55,81]

was intercrossed with *Cyp3a*-null mouse, yielding *CYP3A4*-humanized mouse models[55].

A unique group of knockout mouse models, in which the *Cpr* gene is targeted, is worth noting. CPR is the obligate redox partner for microsomal P450 enzymes; therefore, the deletion of the *Cpr* gene causes the inactivation of all microsomal P450 enzymes in targeted cells. Several mouse models, in which the *Cpr* gene has been deleted in a tissue-specific fashion, are available, such as liver-specific *Cpr*-null mice[62,63] or intestinal epithelium-specific *Cpr*-null mice[64]. A transgenic mouse (known as "*Cpr*-low" mouse), in which *Cpr* expression was globally down-regulated[65], as well as an extra-hepatic *Cpr*-low mouse model, in which *Cpr* expression is normal in hepatocytes, but low elsewhere[66], are also available.

2.5.2. *Utility of engineered mouse models*

Drug metabolism can play an essential role in the extent of tissue exposure to either the parent compound or, in the case of a prodrug, the active metabolite(s). Numerous mechanistic questions related to drug metabolism or toxicity can be answered through the use of a proper engineered mouse model, thus facilitating preclinical drug safety and pharmacokinetics studies. Some examples of the types of questions that can be addressed are shown below.

(1) Is *in vivo* metabolism mediated mainly by P450 or by other drug-metabolism enzymes? Examples of this type of application include the use of the *Cpr*-low mouse for demonstration of the role of P450 enzymes in the clearance of nifedipine[67], and the use of the liver-*Cpr*-null mouse for demonstration of the lack of a significant contribution by P450 enzymes to systemic acetaminophen clearance[68].

(2) Which P450 enzyme(s) are critical for *in vivo* metabolism? For example, the *Cyp2a5*-null mouse was used for demonstration of the role of CYP2A5 in the *in vivo* clearance of nicotine; the deletion of the *Cyp2a5* gene led to substantial increases in the exposure of nicotine[69].

(3) Is a given drug-metabolism enzyme responsible for bioactivation and consequent toxicity? For example, the critical role of CYP2E1 in acetaminophen-induced hepatotoxicity[70], and the lack of an essential role by CYP1A2 in acetaminophen-induced olfactory toxicity[71], was demonstrated through the use of the respective *Cyp*-knockout mouse models.

(4) Does the small intestine play a major role in first-pass clearance of a given oral drug? For example, the IE-*Cpr*-null mouse was used to show that intestinal P450 enzymes play a major role in the first-pass clearance of oral nifedipine[64]. The small intestinal *CYP3A4*-humanized mouse model (SI-selective expression of CYP3A4 and whole body knockout of all *Cyp3a* genes) was used to demonstrate the capability of SI CYP3A4 to reduce the bioavailability of oral docetaxel[55].

(5) For compounds metabolized through multiple pathways, which pathway leads to active (or reactive) metabolites *in vivo*? For example, CPR/P450 of the cardiomyocyte was found not to be essential for the cardiotoxicity of doxorubicin, an anticancer drug, which can be metabolized by multiple enzymes, including CPR and carbonyl reductase, in a study that used a cardiomyocyte-specific *Cpr*-null mouse[72]. On the other hand, hepatic CPR/P450 enzymes were found to be essential for the hepatotoxicity and renal toxicity of acetaminophen, which can be metabolized by both phase I and phase II enzymes[68].

(6) What is the capability of a human P450 enzyme to produce a given metabolite (including reactive intermediates) *in vivo*? For examples, the *in vivo* capabilities of human CYP2A6 to generate 7-hydroxycoumarin from coumarin[56]; of human CYP2D6 to produce 4-hydroxydebrisoquine from debrisoquine[73]; and of human CYP3A4 to produce various metabolites from docetaxel[55], or to produce 1′-hydroxymidazolam from midazolam[73], have been assessed using the respective *CYP*-transgenic mouse models.

(7) What is the impact of genetic polymorphisms in drug-metabolism genes on drug clearance? Few transgenic mouse models that express allelic variants of P450 genes have been reported; however, a wild-type mouse model, as compared to a human *CYP*-transgenic mouse model, or a *Cyp*-knockout mouse model, as compared to a *CYP*-humanized mouse model, can represent a mouse model containing a null allele for the human P450 gene. Furthermore, hemizygotes and homozygotes human *CYP*-transgenic mouse models can be compared for studying effects of losing one *CYP* allele on systemic drug clearance.

The extent to which each of the available mouse models has been characterized varies considerably; but it is essential that the user is aware of the properties and limitations of each model. It is beyond the scope of this review to provide detailed information about each of the available mouse models. It should also be noted that engineered mouse models are also available for a number of drug transporters (*e.g.*, see Kitamura et al.[74], for a recent review) and phase II biotransformation enzyme, such as UGT1[75].

3. Summary

Drug discovery and development remain as a complicated models-based experimental scientific exploration. These models provide a variety of data, resulting from studies on *in vitro* systems, *in vivo* animal species, and healthy human subjects, for predicting the behavior of a drug in patients. These data either address a particular aspect of drug metabolism, such as permeability and transporter properties, as derived from Caco-2 models or reveal entire distributional and dispositional properties of a drug, as obtained from a human ADME study using ^{14}C-labeled compounds. Fig. 3 contains a summary of the mechanistic input and relevance of major experimental models that are used in drug discovery and development. The selection of an appropriate model, followed by the application of the specific model(s) with a specific strategy and correct data interpretation, is critical. This review has described some of the more prevalent experimental models used in drug metabolism and disposition. Most modern drugs are discovered and developed with the timely applications of the appropriate experimental models. Certainly, there will be additional, newer, and better modified models developed in the future to support an efficient drug discovery and development process.

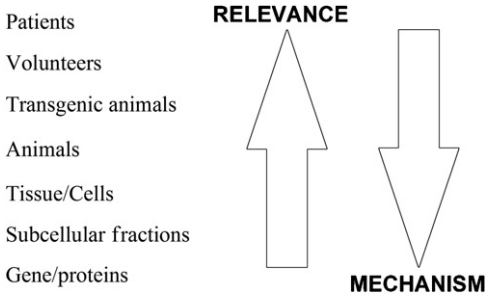

Figure 3 The relevance and significance of ADME models in drug metabolism and disposition for human clinical prediction.

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