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A PERSPECTIVE ON THE PREDICTION OF DRUG PHARMACOKINETICS AND
DISPOSITION IN DRUG RESEARCH AND DEVELOPMENT

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List of Abbreviations:

ADME = Absorption, distribution, metabolism and excretion

AUC = Area Under the Curve

BCS = Biopharmaceutics classification system

BDDCS = Biopharmaceutical Drug Disposition Classification System

CYP = Cytochrome P450

CL = Clearance

CL_{int,u} = Unbound intrinsic clearance

DDI = Drug-drug interaction

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DMPK = Drug metabolism and pharmacokinetics

EHC = Entero-hepatic circulation

F_a = Fraction absorbed

F_g = Fraction passing through the intestine

f_u = fraction unbound in blood

f_{ut} = fraction unbound in tissues

GI = Gastrointestinal

ISEF = Intersystem Extrapolation Factor

IVIVC = In vitro-in vivo correlation

MRT = Mean residence time

NCE = New chemical entity

PBPK = Physiologically-based pharmacokinetic

PK = Pharmacokinetics

PSA = Polar surface area

QSAR = Quantitative structure-activity relationship

τ = dosing interval

$t_{1/2}$ = Half-life

VD = Volume of distribution

VD_{ss} = Volume of distribution at steady state

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Abstract

Prediction of human pharmacokinetics of new drugs, as well as other disposition attributes has become a routine practice in drug research and development. Prior to the 1990s, drug disposition science was used in a mostly descriptive manner in the drug development phase. With the advent of in vitro methods and availability of human-derived reagents for in vitro studies, drug disposition scientists became engaged in the compound design phase of drug discovery in order to optimize and predict human disposition properties prior to nomination of candidate compounds into the drug development phase. This has reaped benefits in that the attrition rate of new drug candidates in drug development for reasons of unacceptable pharmacokinetics has greatly decreased. Attributes that are predicted include clearance, volume of distribution, half-life, absorption, and drug-drug interactions. In this article, we offer our experience-based perspectives on the tools and methods of predicting human drug disposition using in vitro and animal data.

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Introduction and History

The prediction of human pharmacokinetic and disposition attributes of new chemical entities from preclinical data has become a mainstay of DMPK organizations within pharmaceutical research and development operations. In the vast majority of large R&D groups, the nomination of new compounds into the development phase requires a prediction of what the human pharmacokinetics will be. Tools have been developed to assess important human disposition attributes during the drug design phase so that medicinal chemists can simultaneously optimize ADME properties and pharmacological potency. These tools and methods are standard in modern drug discovery, and are a critical element to successful drug discovery. But this was not always the case.

Prior to the 1990s, the focus of the ADME scientists in the pharmaceutical industry was compound characterization. Most work was descriptive rather than mechanistic. Drug candidates were generally not studied until the development phase, and the focus was on gathering pharmacokinetic and toxicokinetic data, and offering a description of the metabolism and excretion of new compounds in humans and laboratory animals. The value that a study of ADME properties of new chemicals could bring to the drug design phase was well-recognized by scientists at Pfizer's Sandwich, UK research facility (Smith, 2012), and this was enhanced by an organizational structure wherein the Sandwich Drug Metabolism Department reported into the leadership of the local Drug Discovery organization. The contrast with the sister Drug Metabolism Department at the Pfizer Groton, CT research facility was notable in that the latter group was under the Toxicology Department, an organization that was very oriented to descriptive data gathering in the drug development phase. Nevertheless, the incredible value that drug

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metabolism scientists could bring to the discovery phase was recognized in the early 1990s because there were many compounds that failed in phase 1 first-in-human studies due to poor pharmacokinetics (i.e. very short half-life and/or very high oral clearance) (Kola and Landis, 2004). This led to a marked expansion of DMPK groups across the industry, as the remit evolved from one of providing descriptive data in the drug development phase to also include working with drug discovery project teams to leverage ADME data in the optimization of drug molecules.

A critical contribution that DMPK scientists make to discovery project teams is offering a data-driven opinion as to what the human pharmacokinetics would be prior to nomination of the drug candidate into the more expensive early development phase. In the early days of this contribution, the only approach available was to gather pharmacokinetic data in laboratory animal species and make suppositions from that data (e.g. if clearance was low in rats and dogs, then it would be low in humans, etc.). Allometric scaling (discussed in greater detail below) offered a use for animal pharmacokinetic data that was one level of sophistication greater than merely stating that PK characteristics in humans would be like those in animals. In allometry, there is a fundamental assumption that differences in pharmacokinetic processes will mirror differences in other attributes across species (i.e. weight, life-span, other), and thereby a quantitative projection of human pharmacokinetic parameters could be made (Boxenbaum, 1982). In the early 1990s, the increasing availability and characterization of human-derived reagents that could be used for in vitro drug metabolism studies revolutionized the approaches to applying ADME to drug discovery. These included subcellular fractions from human liver, like human liver microsomes, as well as cloned

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and heterologously expressed human drug metabolizing enzymes. Thus, methods that had been described previously for scaling in vitro data to animal pharmacokinetic data (Rane et al., 1977; Houston, 1994) could be tried with human-derived reagents.

Back in the early 1990s, in our company there was some disbelief among some leaders in the research organization that techniques such as in vitro-in vivo scaling and allometric scaling could really offer quality predictions of human pharmacokinetics. There was an opinion that compounds should be nominated into the development phase and that phase 1 studies would be the ones to sort out compounds that had appropriate or inappropriate human pharmacokinetics. Thus, the challenge was laid down to DMPK scientists to prove whether preclinical data could really be useful for quantitative predictions of human pharmacokinetics. The response to the challenge was a retrospective analysis of human pharmacokinetic data and whether various prediction techniques (in vitro and in vivo) would have been adequately predictive. (“Adequate” prediction methods are those such that the accuracy is good enough for correct and confident decision-making when selecting candidate compounds for the early development phase of drug research. Predicted half-lives need to permit the desired dosing regimen frequency. Predicted oral bioavailability has to permit a dose level that is not higher than what can be absorbed and economically manufactured.) The results of this analysis were promising and were reported in 1997 (Obach et al., 1997). Since that time, scientists at Pfizer have reassessed these techniques as human pharmacokinetic data for new drug candidates became available (Hosea et al., 2009). Around this time, other groups were also pursuing predictions of human pharmacokinetics using in vitro scaling

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(Iwatsubo et al., 1997) and allometric scaling with in vitro intrinsic clearance corrections (Lave et al., 1997).

Thus, the prediction of human pharmacokinetics prior to nomination of new drug candidates into drug development has become an expectation of drug discovery project teams. Furthermore, human liver microsomal liability data is gathered using high throughput methods for testing of every new chemical made by medicinal chemists, for use in building structure-metabolism relationships and driving compound design to low rates of cytochrome P450 metabolism. Other types of in vitro data to address drug disposition attributes (e.g. membrane penetrability, P450 inhibition, others) are also gathered early using high throughput methods. We have progressed far from the early times of merely striving for predicting human half-life. We now seek to predict many aspects of drug disposition in humans, including clearance, volume of distribution, half-life, oral absorption, oral bioavailability, drug-drug interactions, impact of genetic polymorphisms on drug disposition, penetration into target organs, efficacious dose, and even complete concentration vs. time curves with accompanying interindividual variability. Prediction algorithms have evolved from simple equations to complex physiologically-based pharmacokinetic (PBPK) modeling. Some parameters, like volume of distribution, can be reasonably be predicted from computational methods alone. Other properties that we desire to predict, such as target tissue free concentrations, are dependent on emerging areas of ADME science (e.g. drug transporters) and our ability to predict such properties are still immature. It is also important to note that methods used to predict human pharmacokinetics and other disposition properties have a level of inaccuracy beyond which distinctions among compounds cannot be made

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(Beaumont and Smith, 2009). For example, if two compounds are predicted to have pharmacokinetic parameters within 2-fold of each other, these cannot be truly distinguished from each other, and other non-pharmacokinetic attributes may be more important for drawing any distinction.

In this commentary, we offer a perspective of predictions of human pharmacokinetic/disposition attributes from a pharmaceutical industry research and development viewpoint. We describe each of the major attributes that we currently seek to predict using preclinical data, an overall assessment of the advantages and shortcomings of various methods that have been used, and a desire for what is needed to increase success in the future. While the advances that started in the early 1990s are well-established, it is clear that several areas remain to be explored with basic scientific research so that the development and application of methods can be accomplished.

Predicting Half-Life

Half-life, in reference to a xenobiotic, is the oldest pharmacokinetic parameter and is used extensively in pharmacokinetic and pharmaceutical literature. It is an important component to help define the dosing frequency (along with knowledge of the PK/PD relationship). Convenient dosing regimens are strived for in drug discovery, so the half-life in humans is one of the most important parameters to predict. Half-life is a function of the clearance and the volume of distribution, two fundamental parameters that will be discussed in the following sections.

A major utility for predicted human half-life-values is to identify or predict the maximum dosing interval (τ_{\max}) to maintain the drug concentration within the therapeutic

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range ($C_{\text{upper}}/C_{\text{lower}}$). This value is estimated with consideration of the therapeutic index and can be expressed with the following equation (Rowland and Tozer, 1995):

$$\tau_{\text{max}} = 1.44 \cdot t_{1/2} \cdot \ln\left(\frac{C_{\text{upper}}}{C_{\text{lower}}}\right) \quad (\text{Eq. 1})$$

In addition, the predicted human half-life values are used to rank order chemical series, identify the most ideal drug candidate, assess clinical feasibility, and to retrospectively determine how well pre-clinical tools and reagents were applied in predicting the actual human half-life (Hosea et al., 2009). Half-life is frequently the only human pharmacokinetic parameter that can be used to retrospectively evaluate performance of human pharmacokinetic prediction methods that use preclinical tools and reagents. Without corresponding intravenous data, accuracy in CL and VD predictions cannot be assessed individually (Hosea et al., 2009).

To be practical about predicted human half-lives, the accuracy in prediction should be considered within ranges of half-life values (Obach et al., 1997). Nevertheless, half-life predictions should be as accurate as possible. To this extent, two approaches are predominantly being used to predict human half-life. The most used method employs the above mentioned approach using predicted human VD_{ss} and clearance values to predict half-life according to equation 2 (Hosea et al., 2009). Various prediction methods for VD_{ss} and CL are described in other sections within this paper. But, as expected, the accuracy of the half-life prediction is more a function of the accurate prediction of the independent parameters, CL and VD_{ss} .

$$t_{1/2} = \ln(2) \cdot \frac{VD_{\text{ss}}}{CL} \quad (\text{eq 2})$$

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However, all these reported approaches are retrospective analyses, and the forward predictiveness of the various approaches is not always clear. In one example, it was determined that simple allometry can accurately predict the elimination rate constant for irbesartan from animal data (Pavan and Srinivas, 2008). In another study, it was concluded that human half-life values can adequately be predicted from rat half-life values using either linear regression or allometric scaling between human and rat (Caldwell et al., 2004). About 76% and 77% of the values were predicted within an average fold error of less than 3 for the regression and allometric method respectively. The authors also concluded that deriving half-life values from VD_{ss} and CL values were not more accurate than predicting half-life directly. However, 77% predictability within an average fold error of less than 3 is on par with other reported successes in predicting half-life from VD_{ss} and CL-values (Hosea et al., 2009). Regardless of the reported success using simple animal to human correlations, using VD_{ss} and CL to predict effective half-life should preferentially be used. While human VD_{ss} values can be reasonably well-predicted from animal data (see below), CL can vary considerably across species which can thus confound predictions of half-life from animal data alone. With contemporary drug chemical space more frequently involving transporters as a major CL mechanism, it is likely that a half-life predicted from CL (and VD_{ss}) separately could be more accurate.

Predicting Volume of Distribution

Volume of distribution (VD) is a critical PK parameter and is relatively predictable compared to the other more challenging PK variables, such as clearance.

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Many comprehensive reviews have been written on this topic (Obach, 2007; Berry et al., 2011; Zou et al., 2012; Lombardo et al., 2013b). Over 30 different in vivo, in vitro and in silico methodologies are available to predict human VD. The literature information is controversial on what the best approach is and significant validation and refinements of the methodologies are still on-going in the field. Here we discuss a few common methods and their strengths and limitations. It is important to understand that these various methods find their greatest applicability at different times during the drug discovery-development timeline. In general, interspecies scaling with in vivo animal data is more accurate than other approaches and should be used for later stages of drug discovery when accurate prediction of human PK is essential. In silico methods can be effective for early stages of drug discovery, where large number of compounds need to be profiled (or even predicted even prior to synthesis). Mechanistic based PBPK models are particularly useful for whole body PBPK modeling. In vitro approaches tend to be less frequently employed, since in silico models are predictive without any added experimental cost. It is important to note that VD itself has little impact on compound selection (except in extreme instances such as very high VD values that may indicate excessive tissue partitioning). Compounds should not be designed to manipulate VD; it is free clearance that should be the focus. Rather, the importance of VD is in its contribution to half-life and MRT, which in turn is used to help predict the dosing interval needed for chronically used medication (see above).

There are several different VD terms that describe distribution in different ways. The two most commonly described are the VD_{ss} and VD_{β} terms. VD_{ss} is a more informative value for dosing regimen, as it contributes to mean residence time, a value

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that better reflects the extent of accumulation that will occur with multiple dosing. VD_{β} contributes to the terminal elimination phase half-life, which may not have as much impact on dosing regimen if there is only a small percentage of the total exposure that occurs during this phase. Thus, prediction of VD_{ss} is generally more valuable than VD_{β} .

Key Determinants of VD

VD is a function of the extent to which the drug binds to plasma components ($f_{b,p}$) versus the extent to which the drug binds non-specifically to tissue components ($f_{b,T}$).

$$VD \propto \frac{f_{b,T}}{f_{b,p}} \quad (\text{Eq. 6})$$

Because VD is mostly determined by nonspecific binding to plasma and tissue components, key determinants of VD are physicochemical properties rather than specific pharmacophores. Lipophilicity and charge state of a molecule at physiological pH (dependent on the pK_a) are the most important descriptors for prediction of VD. Generally, VD increases with increasing lipophilicity or cationic fraction, and it decreases with increasing anionic fraction at physiological pH (Lombardo et al., 2006). Transporters have also been shown to have significant impact on VD (Grover and Benet, 2009; Shugarts and Benet, 2009).

Prediction of VD Using in Vivo Data from Preclinical Species

Despite the empirical nature of interspecies scaling and the criticisms of this approach for predicting clearance and other PK parameters, it is quite effective in predicting human VD from in vivo animal data. Several methodologies are commonly used by DMPK scientists for interspecies scaling of VD (Lombardo et al., 2013b): (a) single species scaling, (b) allometric scaling with multiple species, (c) equivalency or proportionality approach, (d) Øie-Tozer model (Oie and Tozer, 1979), (d) Wajima method (Wajima et

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al., 2004), and (e) multiple linear regression approach. There are divergent points of view on interspecies scaling of human VD from preclinical animal data (Obach, 1997; Ward and Smith, 2004; Obach, 2007; Hosea et al., 2009; Sui et al., 2010; Berry et al., 2011; Jones et al., 2011b): (1) Which method gives the most accurate prediction? (2) Which species or species combination is the best approach? (3) Does correction of plasma protein binding yield better or worse estimation of human VD? Depending on the animals used in the experiments, the diversity of the compounds and the number of the compounds in the studies, the conclusions can be different (Obach, 1997; Ward and Smith, 2004; Obach, 2007; Hosea et al., 2009; Sui et al., 2010; Berry et al., 2011; Jones et al., 2011b). A recent study with a large set of well-characterized structurally diverse compounds concluded that monkey was superior to dog or/and rat in all the methods tested, presumably because monkey is evolutionarily closest to human (Lombardo et al., 2013b). Both uptake and efflux transporters in monkey have been shown to be more predictive of human than rodents and dogs (Syvaenen et al., 2009; Shen et al., 2013). The Øie-Tozer model gave the best prediction compared to the other methods. However, for some compounds, aberrant f_{ut} values were calculated (either < 0 or > 1), potentially due to transporter involvement (Waters and Lombardo, 2010). The abnormal f_{ut} values from Øie-Tozer equation can be used as a diagnostic criterion on whether the method is suitable for a particular compound. Correction for plasma protein binding (PPB) yielded significantly lower performance than those without PPB correction in all the approaches evaluated. This could potentially be due to inaccuracy in PPB data, since they were generated from different labs using various devices. In practice, when monkey data is not available, the recommendation is to use dog single species scaling, Øie-Tozer, or Wajima

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models with rat and dog data to predict human VD_{ss} (Oie and Tozer, 1979; Wajima et al., 2004). Overall, prediction of human VD_{ss} from preclinical species is reliable and accurate. For most compounds, 70-80% of the predictions are within 2 to 3 fold of the measured human values (Lombardo et al., 2013b).

Prediction of VD Using in Vitro Assays

Prediction of human VD from in vitro tissue binding data has been reported over several years (Obach, 2007; Berry et al., 2011; Berezhkovskiy, 2012). The assumption is that in vitro surrogate measurements of tissue homogenate binding are representative of in vivo tissue binding. The in vitro experiment measures drug binding to tissue homogenates (e.g., adipose, muscle, lung, brain, liver, etc.) using equilibrium dialysis, ultrafiltration or ultracentrifugation methods. The fraction of tissue homogenate binding was found to correlate with VD_{ss} . In vitro tissue binding approach for VD determination is not very commonly used, mainly due to the intense resources needed experimentally and the predictability is comparable with in silico models.

Prediction of human VD has also been shown to be successful using chromatography methods (Hollosoy et al., 2006; Valko et al., 2011). Plasma protein binding [Log K (HSA)] and phosphate lipid binding [Log K (IAM)] were derived from gradient HPLC retention time using HSA and phosphatidyl-choline IAM (immobilized artificial membrane) columns. The method is based on the assumption that the sum of the albumin and phospholipid binding has the most significant impact on VD. By using two biomimetic HPLC columns, the method provides a high throughput reliable approach to estimate VD in early drug discovery.

Prediction of VD Using In Silico Methods

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VD is reasonably well predicted using *in silico* approaches and the methodologies have improved in recent years. The two most commonly used mechanistic PBPK models are based on tissue composition equations. They are the Poulin and Theil (Poulin and Theil, 2002) method enhanced by Berezhkovskiy (Berezhkovskiy, 2004) and the Rodgers and Rowland method (Rodgers et al., 2005; Rodgers and Rowland, 2006; Rodgers and Rowland, 2007). Both models only require *in vitro* input or computed physicochemical properties of lipophilicity, plasma protein binding and blood-to-plasma ratio. Although these methods are generally slightly less accurate than interspecies scaling, mechanistic based PBPK models do not need *in vivo* data, which is a significant time and resource saving. In addition, these models can be incorporated into whole body PBPK models (e.g., Simcyp,) and they provide a powerful approach to individualized human PK and PD prediction. There are several assumptions for the tissue composition based PBPK VD models: a) no saturation of binding processes; b) membrane permeation is *via* passive diffusion only; c) the binding constituents within the tissues are plasma proteins and lipids; and d) each tissue has a well-stirred distribution model limited by blood perfusion. The models predict reasonably well for most compounds with some exceptions (~65% within 3 fold of *in vivo* values) (Berry et al., 2011). For highly lipophilic compounds ($\log P > 3.5$), $VD_{ss,u}$ tends to be over-predicted by Rodgers and Rowland method. In some cases when the assumptions do not hold true, e.g., there are extensive involvement of active transporters or binding to tissue constituents beyond plasma proteins and lipids, the prediction accuracy will be significantly compromised (Rodgers et al., 2012). Other factors contributing to the inaccuracy of the methods are inaccuracies in calculating or

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measuring physicochemical properties, and Log P values between octanol and water might not reflect the partition into tissues with various lipids.

Several QSAR in silico models have been developed to predict human and rat VD_{ss} using various computed descriptors based solely on inputs of molecular structure (Ghafourian et al., 2004; Gleeson et al., 2006; Lombardo et al., 2006; Berellini et al., 2009). The assumption is that binding to tissues is nonspecific and the models rely heavily on physicochemical properties. The prediction accuracy of the QSAR models decrease when binding is specific or when transporters are involved. The models typically include a large number of compounds in the training set to cover diverse chemical space and prediction is, in general, reasonably accurate with the majority of the compounds within 2-3 fold of the measured values. The performance of the models is highly dependent on whether similar molecular structures are in the training set. For completely novel structures, QSAR models are less accurate. Even though the QSAR in silico models are inferior to interspecies scaling in human VD_{ss} prediction, they do not require animal data, and thus save time and experimental cost. QSAR models are particularly useful to estimate VD_{ss} prior to synthesis, to prioritize chemical series and guide SAR.

Overall, prediction of VD_{ss} is important for feeding into half-life predictions. VD_{ss} itself is not necessarily a parameter upon which drug design should be based. Among the parameters described in this commentary, VD_{ss} appears to be the most amenable to in silico methods at the present time, although the use of animal models will also provide good predictions.

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Predicting Clearance

Among the human pharmacokinetic parameters to predict, clearance is the most important and challenging. For any given eliminating organ (e.g. liver, kidney), clearance is determined by three main factors: the rate at which the drug is delivered to the organ (i.e. blood flow), the extent of drug binding in blood that reduces the ability of the organ to extract the drug, and the intrinsic capability of the organ to clear the drug (via metabolism or transport). Clearing organ blood flows and size are allometrically scalable across species, and there has been considerable focus on allometric scaling as a clearance prediction method (Mordenti, 1986; Mahmood, 1999; Tang and Mayersohn, 2006; Lombardo et al., 2013a). However, intrinsic clearance is reliant upon the activities of specific drug metabolizing enzymes and transporters. These differ between humans and laboratory animal species (in both substrate specificities and expression levels) thus making it more difficult to reliably predict human clearance from allometric scaling (i.e. “vertical” allometry described in (Tang and Mayersohn, 2006) and favoring the use of in vitro methods based on human-derived reagents to make predictions. In our research, allometry is rarely used for human clearance prediction, with the exception of drugs undergoing simple renal filtration as the clearance mechanism, or when intrinsic clearance is so high that the overall clearance will be limited by organ blood flow, which is allometrically scalable. One of the most important insights that a drug metabolism scientist can bring to the process of predicting human clearance from preclinical data is a notion of what the clearance mechanism for the new compound will be in humans. Clearance mechanisms can be viewed to be largely driven by the polar surface area (PSA) of the molecule (Figure 1): hydrophilic, high PSA compounds tend to be less

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membrane permeable and thus removed by drug transport into excretory fluids (urine, bile). Hydrophobic, low PSA compounds require biotransformation to more hydrophilic membrane-impermeable metabolites that can then be transported into excretory fluids. This is a fundamental theme of drug disposition, and has been nicely summarized in the development of the Biopharmaceutical Drug Disposition Classification System (BDDCS) (Wu and Benet, 2005). Insight into the most likely clearance mechanism will assist in the selection of which methods to use to make clearance predictions (Figure 2).

Metabolic Clearance.

In the 1970s, the well-stirred model of hepatic clearance was first described (Gillette, 1971; Rowland, et al., 1973; Wilkinson and Shand, 1975, Pang and Rowland, 1977), and while other models of extraction have also been proposed (e.g. parallel tube model, dispersion model), it is the well-stirred model that has been relied upon the most when predicting clearance in drug research, mostly due to its simplicity:

$$CL_h = \frac{Q_h \cdot f_u \cdot CL_{int,u}}{Q_h + f_u \cdot CL_{int,u}} \quad (\text{Eq 7})$$

The terms Q_h , f_u , and $CL_{int,u}$ refer to the hepatic blood flow, fraction unbound in blood, and hepatic unbound intrinsic clearance, respectively. The liver is generally the focus in drug discovery, and thus three in vitro measurements are needed to make a prediction of human clearance: free fraction in human plasma, blood/plasma ratio, and a measurement of intrinsic clearance. For cytochrome P450 mediated metabolic clearance, the use of pooled human liver microsomes is commonplace in early drug research. In fact, many research organizations use a human liver microsomal lability assay as one of the first assays used to characterize new compounds. Intrinsic clearance is defined by the enzyme kinetic parameters K_M and V_{max} :

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$$CL_{int} = \frac{V_{max}}{K_M + [S]} \quad \text{if } [S] \ll K_M, \text{ then } CL_{int} = \frac{V_{max}}{K_M} \quad (\text{eq 8})$$

However, determination of K_M and V_{max} generally requires the measurement of metabolite formation, and while this can typically be done in later drug development (when either authentic standards of metabolites are available for development of bioanalytical methods, or radiolabelled drug is available for use in radiometric HPLC), in drug discovery this is more challenging. To accomplish this, microsomal lability assays are run by monitoring parent compound depletion at a low substrate concentration (usually 1 μM or less) to determine the in vitro $t_{1/2}$ which can be converted to CL_{int} and scaled to represent the entire organ (Obach, 1999). Values for the content of microsomal protein in the liver, an essential parameter for scaling, have been proposed to range between 30 and 50 mg/gm tissue. (Our preferred value is 45 mg/gm liver that was proposed by Houston (Houston, 1994).) Some have proposed using empirical scaling factors when estimating in vivo from in vitro, but in our current experience this has not been necessary.

While screening for human liver microsomal lability is a useful approach to prioritize compounds for further consideration and to develop SAR for overall CYP metabolism, it is typically desirable to increase the confidence in the prediction of human pharmacokinetics for compounds about to enter the development phase of drug research. The development of cross-species in vitro-in vivo correlations (IVIVC) is a powerful means to enhance the confidence in the human pharmacokinetic prediction. Laboratory animal species (e.g. mice, rats, dogs, monkeys, or other) are dosed with the compound of interest and the clearance is determined. Plasma free fraction, blood/plasma partitioning, and in vitro CL_{int} are determined in these species and the scaled clearance values are

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plotted against the actual clearance values (Figure 3). A linear relationship is developed and the predicted human in vivo value is extrapolated from this relationship using the value predicted from the in vitro data alone. A further enhancement of this approach was developed by investigators at Hoffman-LaRoche wherein in vitro data was combined with allometric scaling principles (Lave et al., 1999).

Measurements of non-specific binding to liver microsomes at concentrations used in the in vitro assays can be important for some types of compounds (especially hydrophobic, cationic compounds) and the CL_{int} values must be corrected by the free fraction value to yield reliable prediction of clearance (Obach, 1997). Also, in order to ensure that CL_{int} is not saturable at anticipated doses, measurement of in vitro $t_{1/2}$ at a lower concentration than 1 μ M is typically done. If the in vitro $t_{1/2}$ at a lower concentration is shorter than at a higher concentration, the potential for a supraproportional dose-exposure relationship is more likely.

These approaches have been available and applied in drug research for well-over a decade. Throughout that time, advances have been made to expand to other metabolic clearance enzymes, the use of other in vitro systems, and to predict other attributes related to clearance. In drug discovery, these other systems and methods are not *routinely* applied, but the astute ADME scientist will recognize the situations in which alternate systems and experiments are needed to appropriately predict human clearance. Prediction of human clearance for compounds that are glucuronidated has been accomplished using alamethicin-treated liver microsomes (Kilford et al., 2009). Examples of the use of in vitro approaches to predict human clearance for compounds metabolized by sulfotransferase, aldehyde oxidase and monoamine oxidase have also

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been reported (Sawant et al., 2010; Zientek et al., 2010; Cubitt et al., 2011; Akabane et al., 2012). These new approaches are now being applied to drug discovery, as the instance merits.

Besides liver microsomes, the use of other in vitro systems from liver and other drug-metabolizing tissues is made when appropriate. Human liver cytosol serves as a source for aldehyde oxidase. Whole blood can be used in the prediction of human clearance for drugs conjugated by GST enzymes. Also, recombinant heterologously expressed P450 enzymes are used in predicting clearance (Stringer et al., 2009; Chen et al., 2011). However, the most compelling of all in vitro systems to predict clearance is human hepatocytes. The development of the capability to cryopreserve human hepatocytes has led to the more routine use of this reagent in human clearance prediction. The advantages are obvious in that human hepatocytes are a closer representation of the in vivo situation, since the entire complement of hepatic drug metabolizing enzymes are present in appropriate proportions, and drug uptake transporters that may actually be the rate determining step in clearance for some drugs are operational. Reports of the utility of human hepatocytes for prediction of clearance have been made (Chiba, et al., 2009; Hallifax, et al., 2010; Poulin and Haddad, 2013). For highly permeable compounds, hepatocytes perform similarly to the enzyme systems (e.g., microsomes, cytosols) with the aforementioned advantage of a complete complement of metabolizing enzymes and cofactors. The scaling factor typically applied to intrinsic clearance is 120 million cells/gm of liver for humans (Barter, et al, 2007). Preclinical species have higher scaling factors than human with the exception of monkey (same as human). Hepatocytes are particularly useful when non-CYP mediated pathways are involved (UGT, AO, SULT,

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MAO, etc.). For compounds that have low passive permeability and/or are efflux transporter substrates, apparent intrinsic clearance in hepatocytes is usually lower than that in enzyme systems, due to lower free hepatocyte concentrations compared to the medium concentrations ($K_{p,uu} < 1$). For compounds that are hepatic uptake transporter substrates, intracellular free drug concentration is higher than medium and apparent intrinsic clearance in hepatocytes is usually higher than that in enzyme systems (Shitara et al., 2006; Kusuvara and Sugiyama, 2009; Di et al., 2012a). Therefore, it is important to understand the interplay between transporter – metabolizing enzymes when interpreting hepatocyte intrinsic clearance data.

Metabolism studies with hepatocytes have also been extended to address low clearance challenges by using the relay approach (Di et al., 2012b). In some cases, substrate consumption is too slow to provide an accurate measurement of CL_{int} . A typical suspension hepatocyte incubation will be kinetically competent for 4 hrs—with the relay protocol, extended incubation times can be accomplished and scaled in vivo intrinsic clearance values as low as 2 mL/min/kg cells can be reliably measured. Alternatively, low CL_{int} compounds can be evaluated by measuring metabolite formation, however, as stated earlier, this requires the availability of authentic standards of metabolites for quantitation or the use of radiolabelled substrate (but are rarely used in the early discovery phase). Methods whereby metabolites are biosynthesized, isolated, and concentrations established through the use of quantitative NMR spectroscopy are being increasingly utilized (Walker, et al., 2010).

Transporter-Mediated Hepatobiliary Clearance

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Drug transporters are expressed in a variety of organs including the intestine, liver, kidney and brain, and play a key role in the drug disposition, adverse reactions and therapeutic efficacy of drugs (Giacomini et al., 2010). The clinical importance of transporters has been recognized, especially through transporter genetic polymorphism and drug interaction studies, where significant changes in the pharmacokinetics of some drugs and consequent clinical responses are observed (Niemi et al., 2005; Shitara et al., 2006; Shitara and Sugiyama, 2006; Giacomini et al., 2010; Niemi et al., 2011; Elsby et al., 2012; Lai et al., 2012). A number of studies suggested that hepatic uptake could be a rate-determining step in hepatic clearance, not only for the compounds that are metabolically stable such as rosuvastatin (Bergman et al., 2006) and pravastatin (Yamazaki et al., 1996; Watanabe et al., 2009a; Varma et al., 2012c), but also for the compounds that are extensively metabolized, e.g. repaglinide (Kalliokoski et al., 2008; Varma et al., 2013b) and atorvastatin (Maeda et al., 2011).

Characterization of hepatobiliary transport proteins was well advanced by the mid-1990s (Keppler and Arias, 1997; Muller and Jansen, 1997). Such investigations were aided by advancement of transporter molecular biology that enabled experiments to be conducted *in vitro* to investigate active hepatic uptake and biliary excretion via the study of individual transporters. Many drugs are actively taken up into hepatocytes via specific uptake transporters that include organic anion transporting polypeptides OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), and OATP2B1 (SLCO2B1), the organic anion transporter OAT2 (SLC22A7), and the organic cation transporter OCT1 (SLC22A1). ATP binding cassette (ABC) efflux transporters localized on the canalicular membrane of hepatocytes, such as P-gp (ABCB1), MRP2 (ABCC2), BCRP (ABCG2), and BSEP

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(ABCB11), are mainly responsible for canalicular secretion (Ishikawa et al., 1995; Keppler et al., 1997; Muller and Jansen, 1997; Suzuki and Sugiyama, 1999; Chandra and Brouwer, 2004). Based on the analysis of physicochemical property space, ionization state, size, and polarity were noted to be important determinants in the biliary elimination and these properties are also closely associated with molecular interaction with the hepatic uptake transporters (Yang et al., 2009; Varma et al., 2012a).

The extended clearance equations (Eq. 9 and 10) can be applied to get an estimate of the effect of transporter involvement in the hepatic disposition and further to predict the overall hepatic clearance (Webborn, et al., 2007; Liu and Pang, 2005; Shitara et al., 2006; Barton et al., 2013).

$$CL_h = \frac{K_{puu} \bullet CL_{int\,efflux+metab} \bullet f_{ub} \bullet Q_h}{(K_{puu} \bullet CL_{int\,efflux+metab} \bullet f_{ub}) + Q_h} \quad (\text{Eq. 9})$$

where CL_h is hepatic clearance, $CL_{int,efflux+metab}$ is the sum of biliary intrinsic clearance and metabolic intrinsic clearance, f_{ub} is fraction unbound in blood, Q_h is hepatic blood flow and K_{puu} represents the unbound concentration in liver relative to the unbound concentration in plasma at steady state, given as

$$K_{puu} = \frac{CL_{int\,active} + CL_{int\,passive}}{CL_{int\,passive} + CL_{int\,efflux} + CL_{int\,metab}} \quad (\text{Eq. 10})$$

The uptake parameters in these equations ($CL_{int,active}$ and $CL_{int,passive}$) are obtained from *in vitro* uptake experiments utilizing hepatocyte or recombinant cell line systems. The efflux parameter can be derived from sandwich culture hepatocyte or transporter vesicle

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experiments and the $CL_{int,metab}$ from conventional *in vitro* stability assays (e.g. microsomes). All these parameters are appropriately scaled to *in vivo* CL_{int} values before being entered into Eq. 9 and 10 using hepatocellularity and liver weight factors (Umehara and Camenisch, 2012; Varma et al., 2013c). Success of mechanistic-based prediction of transporter-mediated disposition and DDIs depends upon: 1) the adaptation of experimental tools and study design to obtain *in vitro* parameters that are relevant *in vivo*, 2) bridging the functional and protein expression difference between *in vitro* and *in vivo* systems, and 3) understanding the uncertainty associated with the parameters obtained from *in vitro* tools (Barton et al., 2013).

Despite many limitations and a lack of a comprehensive demonstration of quantitative IVIVE, *in vitro* experimental tools have significantly improved the ability to generate relevant transporter affinity parameters to predict transporter-mediated disposition and further the associated drug interactions. Currently available *in vitro* tools increase in complexity from single gene overexpressing immortalized cell lines to isolated hepatocytes and three-dimension cultured hepatocyte systems. Cells expressing individual uptake transporters have been extensively utilized to determine if a test compound is an inhibitor of a transporter and/or a substrate (Sharma et al., 2012; Soars et al., 2012; Varma et al., 2012a). The studies are readily amenable for obtaining various kinetic parameters including K_m , V_{max} and IC_{50} of substrates or inhibitors. Experiments may also be designed to obtain the uptake rate by estimating accumulated amount of compounds in the cells over time. When information on relative expression of transporter proteins between *in vitro* tools and in the liver becomes available, the *in vitro* transport kinetics can be directly used in mechanistic models. On the other hand, primary isolated

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hepatocytes express transporters and phase I/II enzymes, and are generally recognized as the closest *in vitro* surrogate of the liver for CYP metabolism (Watanabe et al., 2009b). This tool is commonly accepted as a holistic and inexpensive method to assess *in vivo* liver clearance involving hepatic uptake transport (Lam and Benet, 2004; Li et al., 2008; Soars et al., 2009; Yabe et al., 2011; Menochet et al., 2012). In addition, the suspension system facilitates assessing the impact of P450 metabolic activity, the intra-individual variations caused by genetic polymorphisms and other pathophysiological factors (Guguen-Guillouzo and Guillouzo, 2010). However, membrane leakage in the suspension hepatocyte model was also shown to negatively impact the active uptake estimates (Kimoto et al., 2012b). A media-loss assay has been developed to measure uptake rate by influx transporters using hepatocyte suspension with very short time points (Soars et al., 2007; Soars et al., 2009). The method was found to give better prediction of hepatic clearance of uptake transporter substrates, particularly for acidic compounds due to OATP uptake into the hepatocytes. However, the media-loss assay was found to be unsuitable for compounds with high passive permeability or weak uptake transporter substrates (Jigorel and Houston, 2012). Overall, for definitive uptake rate determination, an oil spin assay or hepatocyte sandwich culture experiment is recommended to generate reliable data.

To investigate the interplay between metabolic clearance, hepatic uptake and biliary efflux, a tool in which all of these processes are active is desirable. SCH involves culturing primary hepatocytes between two layers of gelled matrix in a sandwich configuration, allowing the hepatocytes to form a bile canalicular network (Bi et al., 2006). While down-regulation of uptake transporters in sandwich cultured rat

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hepatocytes has been a concern for the reliability of parameters obtained (Luttenger, et al., 2002), sandwich cultured human hepatocytes (SCHH) has proven to maintain the expression of both transporters and major CYP enzymes (Li et al., 2010; Kimoto et al., 2011; Kotani et al., 2011). This model can be used to estimate the initial hepatic uptake and biliary secretion (Bi et al., 2012). SCHH allows simultaneous assessment of multiple processes occurring in the hepatocytes in a mechanistic manner and provides context to the relevance of the processes for hepatic disposition of active transporting drugs (Jones et al., 2012). However, since uptake transporters are down-regulated, there can be a lack of an ability to derive quantitative *in vitro-in vivo* correlations across preclinical species as is done for metabolically cleared compounds.

In our laboratories, a whole PBPK model was developed by incorporating uptake and biliary clearance parameters derived from SCHH experiments together with other ADME and physicochemical properties, to assess the predictability of the clinical disposition for the compounds cleared by hepatic transporters (Jones et al., 2012). In addition to the retrospective analysis, the model was prospectively applied to predict the human PK for 4 proprietary compounds prior to clinical investigation, with averaged empirical scaling factors from literature compounds. When compared to allometric scaling approaches (Hosea et al., 2009), the approaches resulted in a favorable prediction accuracy (Jones et al., 2012). Further work is required to understand the necessity of employing large scaling factors, and it seems clear that there are factors not yet identified/understood that prevent quantitative extrapolation.

During the past decade, numerous attempts have been made to find suitable *in vitro* models for predicting human biliary secretion. Human bile samples are difficult to

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obtain in the clinic, and therefore, the extent of biliary secretion of drugs and/or their metabolites in human is commonly measured by collecting feces (Ghibellini et al., 2004). The measurement is confounded by the presence of unabsorbed drugs, or under-estimated due to EHC. As a result, bile clearance in humans is mostly assessed based on *in vitro* or *in vivo* preclinical models. Bile duct cannulated preclinical species have been widely used in practice for many years to characterize biliary clearance. Although biliary clearance in rat often overestimates clearance in human, partly because of the higher expression levels of the hepatobiliary transporters and bile flow in rats (Lai, 2009), assessing drug eliminated from bile duct cannulated rats remains as a useful approach to collect qualitative information about biliary excretion (Varma et al., 2012a). The *in vitro* biliary clearance values obtained from SCH was shown to be linearly correlated to *in vivo* biliary clearance in rat to a certain extent (Liu et al., 1999; Abe et al., 2008; Fukuda et al., 2008), although considerable misprediction of human biliary clearance were also noted (Jones et al., 2012). Certain studies demonstrated the correlation of clearance between SCHH and human biliary clearance (Ghibellini, et al, 2007). In addition, by incorporating the differential expression of hepatobiliary transport (Li et al., 2010), or the use of *in vitro* ADME data including biliary clearance from SCH within PBPK models (Jones et al., 2012), the biliary clearance prediction from *in vitro* SCH model can be further improved. In the *in vitro* hepatocyte systems, the intrinsic transport parameters are derived based on the drug concentration in the incubation medium. This is relevant for calculating uptake transport parameters, but the lack of reliable tools to estimate free intracellular concentration limits estimation of accurate efflux parameters. Nevertheless, given these challenges in predicting and validation of biliary clearance, there are concerted efforts in

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the industry towards better characterization of hepatobiliary elimination in relation to physiochemical assessment, transporters affinity and IVIVE in early discovery. This include: 1) further understanding the molecular biology, quantitative expression and functional activity of hepatobiliary transporters; 2) improving preclinical models to better predict hepatobiliary clearance in humans; 3) managing the capacity for high throughput *in vitro* testing and 4) better understanding of hepatobiliary transporter IVIVE to predict drug disposition and DDIs.

Renal Clearance

Renal clearance (CL_r) is determined by passive glomerular filtration, active tubular secretion, and passive as well as active reabsorption. Methods for prediction of renal clearance in humans and an understanding of the renal clearance mechanisms are needed in order to predict plasma concentration–time profiles and the potential for renal DDIs. This is particularly important for compounds that have low or negligible metabolic clearance.

Interspecies allometric scaling is widely accepted for extrapolating the pharmacokinetic parameters obtained from animal studies to successfully predict human pharmacokinetic parameters (Lin, 1995). As mentioned above, interspecies scaling is based on empirically observed relationships between physiological parameters and body weights among animals. Simple allometric scaling of the renal clearance is undoubtedly useful for drugs that are eliminated in the urine by glomerular filtration because the glomerular filtration rate depends on the molecular size and conforms to allometric scaling across species. In our experience, when renal clearance by glomerular filtration

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of parent compound is the major clearance pathway, allometric scaling was pursued as the best method to predict renal clearance in human. However, when species differences exist in the mechanism of elimination, simple allometry will likely not accurately predict clearance from animal pharmacokinetic parameters. Specifically, allometry performs more poorly when active processes are primarily involved. Mahmood (1998) used allometry to predict human CL_r for 10 renally actively excreted compounds from at least three animal species data and found that the prediction errors ranged from -85% to $+72\%$ (Mahmood, 1998). However, it is interesting to note that allometry worked well for prediction of the CL_r of creatinine and fluconazole, which involve tubular secretion in humans (Dedrick, 1973; Jezequel, 1994). It is likely allometric scaling works for compounds which are eliminated mainly via glomerular filtration as the main renal clearance mechanism, with minor active secretion and mainly passive reabsorption.

Transporters expressed on basolateral and apical sides of renal proximal tubule cells mediate the active renal secretion and reabsorption of endogenous compounds and xenobiotics. It is known that organic cation transporter 2 (OCT2) and organic anion transporters 1 and 3 (OAT1 and OAT3) are major transporters on the basolateral membrane, whereas MDR1 (multidrug resistance protein 1), MRP2 (multidrug resistance-associated transporter 2) as well as MATEs (multidrug and toxin extrusion protein) are major transporters on the apical membrane. Thus, transporters work together to mediate active renal secretion of compounds. Meanwhile peptide transporter 2 (PEPT2) and System L amino acid transporter (LAT1), etc. are more related with active reabsorption. Drug transporters can have large species differences in terms of transport function and transporter expression level. Additionally, transporter capacity shows

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remarkable heterogeneity along the proximal tubule (Masereeuw and Russel, 2001). Thus, it is difficult to predict renal elimination of actively secreted compounds from in vitro data.

Compared with metabolism, the frequency of drug candidates in our research organization that have renal as the main clearance pathway is much lower. We studied 10 structurally related, renally excreted amino acid-like compounds including gabapentin, pregabalin, and eight of their structural analogues. These compounds are not significantly metabolized in humans, and are predominantly excreted unchanged in urine with a wide range of net renal clearance values ranging from significant net secretion to extensive net reabsorption. Since these compounds were significantly metabolized by dog and to a lesser extent by rats, these animal species were not considered to be good preclinical models to predict human PK. Similar to humans, monkeys showed minimal to no metabolism for these compounds. The studies indicated human and monkey clearance can be described using allometric scaling based on body weight; and both had the same allometric coefficient for the net clearance. Importantly, the high correlation of renal clearance between monkeys and humans was observed regardless of whether or not the net renal excretion processes resulted in net secretory, reabsorptive, or neutral profiles relative to GFR. These findings demonstrated that renal active transport mechanisms and compound disposition of these compounds are similar in monkey and humans. Based on these studies, monkey was a good species for predicting human renal clearance with net secretion, reabsorption or filtration clearance of this series of compounds. However, it should be noted that the compounds used in this study are amino acid-like and differ from typical small molecular weight drugs.

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Tahara and co-workers (2006) reported that renal and renal tubular secretion clearances of famotidine in humans were successfully estimated by simple allometric scaling using data from rats, dogs, and monkeys (Tahara et al., 2006), although the contribution of the renal transporters differs depending on the species. In this instance monkeys were more appropriate animal species for quantitatively predicting the renal DDI in humans. Regarding basolateral transporters in renal proximal tubule cells, the monkey OATs and OCTs have similar characteristics to the human orthologs. Additionally, the conservation of net secretory processes between monkeys and humans for OAT3 was demonstrated *in vitro* and *in vivo* (Tahara et al., 2006), whereas the correlation of the transport activities between rat and human OAT3 was poor. The same group also reported that in terms of substrate specificity and localization in the kidney, there was minimal species difference between monkey OAT1 and OAT3, compared with human orthologs (Tahara et al., 2005).

Recently, Paine and co-workers (Paine et al., 2011) compared three animal scaling methods, including direct correlations between renal clearance in humans and each of the two main preclinical species (rat and dog); simple allometry, and Mahmood's renal clearance scaling method, to predict human renal clearance of 36 diverse drugs that show active secretion or net reabsorption. The data suggested the most accurate predictions were obtained by using a direct correlation with the dog renal clearance after correcting for differences in f_u and kidney blood flow. This suggested a good species crossover between the transporters involved in any active process for this diverse set of compounds.

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Overall, prediction confidence of transporter-mediated renal clearance in humans is not high. Since cynomolgus monkey is a closer species to human in the evolution than rats or dogs, it is likely that cynomolgus monkey is a suitable animal model for the prediction of renal clearance of compounds with significant active renal secretion. However, other species as well as allometric scaling could be valuable for predicting certain compounds with active renal secretion. Additionally, human renal proximal tubule model and human kidney slice could be very valuable for predicting renal clearance, although it is a big challenge to maintain the renal transporter functions in these in vitro models. Long term goal is to develop PBPK models by incorporating renal uptake and efflux clearance using transporter-transfected cell lines together with other ADME and physicochemical properties, to assess the predictability of the transporter-mediated renal clearance in humans.

In drug discovery and development, it would be ideal for compounds to have multiple clearance pathways, including a renal clearance component. Renal clearance can be desirable because of the relatively low potential for drug-drug interactions. However, drugs cleared exclusively by the kidney can be subject to high exposure in renal impaired patients. Furthermore, since kidney function decreases naturally with aging, a drug cleared exclusively through the renal route can attain higher exposures in the elderly population.

Overall, clearance is probably the most important pharmacokinetic parameter to predict, since it is a contributing property to $t_{1/2}$, oral bioavailability (via first-pass extraction), and DDI. ADME scientists have achieved a reasonably reliable standard for predicting clearance for P450 metabolized compounds, but other clearance mechanisms

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require more work for method development. In drug design, optimizing unbound intrinsic clearance should be the primary focus when optimizing ADME, since this is the parameter that will reflect the maximum potential free drug concentrations to which the receptor will be exposed. Intrinsic receptor potency, target tissue penetration, and free intrinsic clearance are the three parameters with which medicinal chemists and their ADME scientist partners must be most concerned when optimizing drug properties through design.

Predicting Oral Bioavailability

Oral bioavailability (F) is one of the most important determinants of the dosing regimen. The extent to which a drug fails to be absorbed or is removed by first-pass extraction before it can reach the systemic circulation and consequently the pharmacological target compartment will dictate how large a dose must be administered. Thus, during the new drug design phase, considerable effort is expended to optimize oral bioavailability. Solubility and permeability are critical determinants of drug absorption following oral administration. Based on these fundamental properties, Amidon *et al.* proposed a biopharmaceutics classification system (BCS), which is extensively used for regulatory and industrial purposes, particularly to waive conducting expensive bioequivalence clinical studies (BE) for high solubility-high permeability (Class I) drugs. (Amidon *et al.*, 1995; CDER/FDA, 2000; Yu *et al.*, 2002). Class I compounds possess no absorption limitations, and thus the goal of drug research teams is to drive the chemistry space towards the Class I behavior through compound design.

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High throughput methodologies for measuring solubility and permeability have been utilized in screening large number of compounds in early drug discovery stage (Bevan and Lloyd, 2000; Roy et al., 2001; Obata et al., 2004; Alsenz and Kansy, 2007; Alsenz et al., 2007). In several high throughput solubility assays, compounds are introduced as the dimethyl sulfoxide stock solution and the non-thermodynamic solubility is estimated typically in pH buffer at 7.4 (Obata et al., 2004). However, the solubility data generated under thermodynamic equilibrium conditions represents the best case scenario (Alsenz and Kansy, 2007), especially in simulated GI fluids adjusted to the physiologically relevant pH. Preclinical models such as the *in situ* rat intestinal perfusion and *in vitro* epithelial cell culture models, that are appropriately validated to predict the extent of drug absorption in humans, can be used for permeability assessments (Varma et al., 2004). Cell-based permeability assays utilizing Caco-2, Madin-Darby Canine Kidney (MDCK), and recently the low efflux transporters MDCK cell lines have been employed in discovery settings.(Artursson, 1990; Artursson and Magnusson, 1990; Irvine et al., 1999; Di et al., 2011) Similarly, the parallel artificial membrane permeation assay (PAMPA) has been established as an alternative to cell-based assays for predicting oral passive absorption (Avdeef, 2005; Avdeef et al., 2005).

Sigmoidal relationships, as described by the complete radial mixing (parallel tube) model (Artursson and Karlsson, 1991; Fagerholm et al., 1996; Varma and Panchagnula, 2005), were observed between apparent permeability and human F_a , using different cell-based and animal models. For example, Artursson and Karlsson showed a good relationship between the transport across Caco-2 monolayers and human F_a (Artursson and Karlsson, 1991). Irvine *et al.* showed an approximately sigmoidal

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correlation between permeability and human F_a and also demonstrated a linear correlation of MDCK and Caco-2 permeability for 55 drugs (Irvine et al., 1999). Recently, using a dataset of over 100 drugs, we showed good relationship between apparent permeability across MDCK-LE (cloned for low P-gp expression (Di et al., 2011)) and human F_a , when permeability values at apical pH 6.5 and 7.4 were used for acidic and non-acidic drugs, respectively (Figure 4) (Varma et al., 2012b). Also, a logarithmic relationship was established between the *in vitro* apparent permeability and the *in situ* human effective permeability (Figure 4), which could be used to predict rate and extent of oral absorption using dynamic models. Generally, the human effective permeability estimates are several times higher than the *in vitro* apparent permeability determined across cell monolayers, including Caco-2. A major reason for the fold differences between the estimates could be due to the larger effective absorptive area of the human intestinal perfusion segment provided by the villi and microvilli, although differences in the diffusion coefficient, membrane thickness, membrane bilayer composition, luminal content or extra cellular mucus layer affecting drug partitioning into the membrane may also contribute (Artursson and Karlsson, 1991; Fagerholm et al., 1996). Also, a major drawback of cell-based models is the associated intra- and inter-laboratory variability in the permeability features. Understanding such differences and establishing correlations for the in-house models is therefore required to accurately predict the rate and extent of oral absorption.

In situ intestinal perfusion in animals is a more reliable technique than *in vitro* models because of the intact blood supply and innervation, however, the model is not very amenable for increased throughput and is not commonly used in preclinical development. *In vivo* pharmacokinetic studies in laboratory animals provide more

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reliable estimates for oral absorption. In general, fraction of oral dose absorbed in rats and monkeys showed good linear correlations (slope near to unity) with fraction absorbed in humans (Chiou and Barve, 1998; Chiou and Buehler, 2002). However, dogs show a poor relation especially for the hydrophilic drugs, presumably due to leaky paracellular pathway (Chiou et al., 2000). Although a good correlation was observed with fraction dose absorbed in monkeys and human, a comparison of oral bioavailability values reveals considerably lower bioavailability in monkeys for several drugs, which was attributed to the greater first-pass metabolism of monkeys, taking place in the gut wall and/or liver (Takahashi et al., 2009). Thus, when using animal data to project human bioavailability, considerable attention must be paid to the potential species differences in metabolism that can occur during first pass, as well as differences in GI physiology.

One area of increasing interest is that of membrane transporters localized in the intestine. Enterocytes express several transporters, belonging to the ABC and SLC superfamilies, on the apical and basolateral membranes for the efflux or influx of endogenous substances and xenobiotics. P-gp, BCRP, MRP2 and MRP4 are localized on brush-border (apical) membrane while certain MRPs are expressed on the basolateral membrane of the enterocytes. These efflux transporters limit the enterocytic levels of their substrates. P-gp substrate screening using various *in vitro* cell-based models is now an integral part of drug discovery due to the observation that the substrate specificity of this protein is broad (Gupta et al., 2010). Discovery teams design or tailor molecules to reduce substrate specificity to P-gp in order to improve the oral bioavailability of drugs and brain penetration. Several SLC transporters such as PEPT1, OATP2B1, OCTN1/2 localized at the apical surface of enterocytes were suggested to primarily drive oral

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absorption of compounds in a particular chemical space (Varma et al., 2011; Tamai, 2012). However, lack of standardized experimental tools and limited knowledge on the *in vitro-in vivo* extrapolation of transporter kinetics has limited the quantitative prediction of oral absorption when such active mechanisms are involved.

Although the small intestine is regarded as an absorptive organ and may act as a rate-limiting barrier, it also has ability to metabolize drugs by several pathways involving both phase I and phase II reactions and may limit systemic exposure (Paine et al., 1996). CYP3A4 is the most abundant P450 present in human hepatocytes as well as the intestinal enterocytes (Paine et al., 2006; Thummel, 2007). It has also been proposed that for some compounds drug interactions involving CYP3A inhibition and induction may be largely occurring at the level of the intestine (see below). Although, the average human intestinal content of CYP3A has been estimated to be only 70 nmol, versus the average hepatic content of about 5000 nmoles, intestinal metabolism was suggested to contribute to presystemic extraction more than the hepatic metabolism for certain drugs (Paine et al., 1996; von Richter et al., 2004; Paine et al., 2006). This could be a result of better access to the enzymes in the enterocytes as a function of transcellular flux and large absorptive area. It could also be due to the notion that drug is presented to the enterocyte as free drug from the lumen while the liver only encounters the drug in plasma where it potentially can be highly protein bound. Recent studies demonstrated that efflux transporters present on the apical membrane of enterocytes, in particular P-gp, can affect the intestinal metabolism by prolonging the enterocytic transit time and consequent exposure to CYP3A enzymes (Wacher et al., 2001). A significant overlap has also been identified between substrates and inhibitors of CYP3A4 and P-glycoprotein, suggesting

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that these two proteins may act complementarily in further limiting F_g of CYP3A substrates. In a recent trend analysis, we note that ~30% of the small molecule drugs showed F_g less than 0.8, underscoring the importance of considering intestinal metabolism in predicting bioavailability and dose projections in drug discovery and development settings (Varma et al., 2010).

There exist gaps in predicting the gut extraction due to shortcomings in the *in vitro-in vivo* extrapolation, owing to the complex process. An operational model of the gut extraction, analogous to that of well-stirred liver model, was proposed (Rostami-Hodjegan and Tucker, 2002; Yang et al., 2007); and investigated for F_g prediction success (Yang et al., 2007; Gertz et al., 2010). This model utilizes a nominal flow term (Q_{Gut}), which is a hybrid function of permeability clearance (CL_{perm}) and enterocytic blood flow (Q_{Ent}). The predictability of F_g , particularly for drugs with high gut extraction was generally low, and may warrant further investigation into the physiological components to achieve reliable predictions.

$$F_g = \frac{Q_{Gut}}{Q_{Gut} + f_{uGut} \cdot CL_{int,Gut}} \quad (\text{Eq 11})$$

$$Q_{Gut} = \frac{Q_{Ent} \cdot CL_{perm}}{Q_{Ent} + CL_{perm}} \quad (\text{Eq 12})$$

Where f_{uGut} and $CL_{int,Gut}$ are the unbound fraction in enterocytes and unbound intrinsic gut clearance, respectively.

Also species differences exist where rat and monkey typically under-predicts the F_g in human (Cao et al., 2006; Nishimuta et al., 2010). Recently, a transgenic mouse model with constitutive expression of human CYP3A4 in liver or intestine that provides

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quantitative estimation of the contribution of hepatic and gut extraction to the first-pass metabolism has been generated (van Waterschoot et al., 2009). Overall, due to limited access to the sophisticated models and complexities with *in vitro-in vivo* extrapolation and species differences, intestinal metabolic disposition is far from reliably predictable. Application of mechanistic models is expected to provide improved prediction of gut-wall metabolism (Darwich et al., 2010; Fan et al., 2010).

Integrated models for predicting oral absorption from early preclinical data are now implemented in several commercial software packages. These models aim towards appropriate reconstruction of the physicochemical, anatomical and biochemical complexities in mathematical terms; and have demonstrated reasonable success in predicting oral absorption, particularly for passively permeable drugs. Yu et al. introduced the compartmental absorption transit (CAT) model comprising of seven compartments connected in series with each possessing physiological relevance to the specific intestinal segment (Yu and Amidon, 1999). This was later modified to incorporate several additional processes such as release from formulation, dissolution, precipitation, gut-wall metabolism and drug transporters in the enterocytes. The advanced CAT model is the basis of the commercial software package Gastroplus™ (Agoram et al., 2001). The segregated-flow model describes intestinal absorption and metabolism while considering drug flow to a non-absorbing layer and an absorbing enterocyte layer (Cong et al., 2000). This is further modified to segmental segregated-flow model where the intestine is divided into three segments of equal lengths and flows, describing heterogeneity in segmental transporter and metabolic functions (Tam et al., 2003; Fan et al., 2010). The Advanced Dissolution, Absorption and Metabolism

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(ADAM) module of the Simcyp Simulator® is based on the CAT model with features of segmental segregated-flow model. It divides the GI-tract into nine segments that are heterogeneous in terms of size, abundance of enzymes and transporters, transit time, pH and bile salt (Jamei et al., 2009b; Darwich et al., 2010). Several recent evaluations have demonstrated the utility of PBPK methodology for the prediction of oral absorption and human pharmacokinetics before ‘first-in-human’ studies (Jones et al., 2011a).

Early screens for kinetic solubility, permeability, P-gp lability and microsomal stability are extensively used for identifying leads and optimizing chemical matter to maximize oral bioavailability. Driving SAR based on individual components may not necessarily improve bioavailability. For example, increasing lipophilicity to increase membrane permeability may result in compounds with low solubility and/or rapid metabolism. Therefore, all the determinants of F should be integrated in the process of decision-making. To this end, discovery teams often adopt multiparametric optimization scores to rank-order compounds and candidate selection. Alternatively, the ability to accurately predict F is important in projecting the clinical dose. Existing preclinical models seem to do an acceptable job in predicting intestinal absorption. However, further work is necessary to gain confidence in the prediction of F_g , while F_h can be estimated from hepatic clearance based on the assumed clearance mechanism. Increasingly, whole-body PBPK models are used to predict oral bioavailability and to assess the sensitivity of experimental inputs.

Predicting Drug-Drug Interactions (DDI)

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Prediction of DDI liability of a new chemical entity (NCE), both as victim and perpetrator, is an important factor to consider during drug discovery and development since elicitation of clinically relevant DDIs could impact patient safety, compliance due to adverse effects, or abolish drug efficacy (Bjornsson et al., 2003; Zhang et al., 2007; Bloomer et al., 2013). Accordingly, the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) recently issued draft and final guidance for pharmaceutical industry, which provides recommendations for the *in vitro* and *in vivo* evaluation of enzyme- and transporter-based DDIs (EMA, 2012; FDA, 2012).

The primary purpose of determining clinical relevance of a DDI is to establish whether the DDI will require a dose adjustment of the NCE or co-administered drugs, requirement of additional therapeutic monitoring, or if co-administration is considered to be a contra-indication since the risk could not be adequately mitigated by the aforementioned strategies. Therefore, early in drug discovery the ADME scientist is providing a perspective on the potential clinical risk considering the uncertainty around dose predictions and efficacious plasma concentration. During drug development, these risks are defined in order to influence clinical use and labeling. An early discovery finding of, for example CYP3A4 inhibition or time-dependent inactivation, does not necessarily result in immediate discontinuation since factors around target patient population or medical need (e.g., oncology target), competitive landscape, and uncertainty in human efficacious exposure relative to inhibition potency need to be defined.

In the case of a NCE being a victim of a DDI, the most important understanding for the drug discovery scientist is a thorough elucidation of the major clearance pathways

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for the NCE. This allows mechanistic predictions of the potential impact of inhibition or induction of these pathways on NCE exposure (Einolf, 2007; Fahmi et al., 2008; Guest et al., 2011). Several *in vitro* and *in vivo* tools are required to more fully characterize NCE disposition as described below (Obach et al., 1997). Conversely, when the potential impact of a NCE is evaluated as perpetrator of DDIs, it is necessary to evaluate the potential of the NCE to result in inhibition, inactivation, or induction of clearance pathways of co-administered drugs. The relative exposure or inhibitor concentration (I) of the NCE compared to the inhibition potency (e.g., I/K_i) is crucial in predicting clinical risk (Rowland and Matin, 1973; Obach et al., 2006; Fahmi et al., 2009).

Involvement of transporter-mediated clearance mechanisms in drug disposition results in further complexity when attempting to predict DDIs, since a combination of enzyme- and transporter-mediated clearance may be impacted with the resulting extent of the DDI primarily influenced by the rate-determining process (Watanabe et al., 2010; Maeda et al., 2011). The prospective quantitative prediction of complex DDIs is particularly challenging since these efforts require expansion of current static mechanistic models or utilization of mechanistic PBPK modeling (e.g., Simcyp, Gastroplus, PK-Sim) to quantitatively predict the magnitude of DDIs in the clinic (Rowland Yeo et al., 2010; Varma et al., 2013c).

Drug-Drug Interaction Liability of a NCE as Victim

Important parameters required to predict the DDI liability of a NCE as victim requires a thorough understanding of the relative contributions of either hepatic or intestinal metabolic clearance, renal clearance, or hepato-biliary elimination in the disposition of the NCE (Figure 2). By regulatory standards, a significant contribution of

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a particular elimination pathway is generally considered when $\geq 25\%$ of the NCE's elimination is subject to metabolic clearance, renal clearance, biliary elimination, or transporter-mediated disposition (albeit it needs to be appreciated that even *complete* inhibition of a pathway contributing 25% to clearance would only yield an increase in exposure of $\sim 30\%$). During drug discovery, these estimates are obtained from a combination of *in vitro* studies utilizing human tissue preparations, evaluation of renal clearance in animals, and biliary elimination estimates obtained from studies with bile-duct cannulated (BDC) rats and/or dogs, or *in vitro* studies with sandwich-cultured human hepatocytes (SCHH) (see above) (Jones et al., 2012; Barton et al., 2013). In certain cases, transporter-mediated disposition at the level of the intestine, liver, kidney, or biliary excretion could also impact NCE disposition, particularly for compounds classified as BDDCS class III and IV (Wu and Benet, 2005). Therefore, the ability of NCE to be transported by human transporters should be evaluated *in vitro*, not only from the standpoint of clearance prediction (as above) but also for DDI. These initial estimates of drug disposition made in the early phase can be further refined during drug development when human pharmacokinetic data are obtained, including estimates of oral or intravenous clearance and renal elimination of unchanged drug (Jones et al., 2011a). Ultimately, the availability of human radiolabel disposition data obtained from mass balance studies allows further refinement of estimates on the contribution of specific metabolic pathways to overall systemic clearance. (However, estimations of unchanged drug clearance in the bile is somewhat uncertain since assumptions are required regarding the fraction of unabsorbed drug detected in feces since biliary clearance is often not studied following intravenous administration of radiolabeled drug or in post-surgical

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cholecystectomy patients with T-tube drainage of the bile duct.) Other clinical studies, specifically DDI studies and studies evaluating the impact of pharmacogenetic polymorphisms on pharmacokinetics can also yield insight into clearance pathways. Once in vivo clearance pathways are confirmed, the enzymes and/or transporters involved can be definitively established.

Drug-Drug Interactions Involving Cytochrome P450 (CYP)

The majority of small molecules developed as drug candidates are eliminated through phase I metabolism by CYP, accordingly most of the DDI prediction efforts for NCEs have focused on understanding the extent of NCE disposition impacted by CYP-mediated clearance. The magnitude of a DDI or change in exposure ratio (AUCR) following oral drug administration in the presence of an inhibiting drug is generally defined by the following equation:

$$\text{AUCR} = \frac{\text{AUC}'_{\text{po}}}{\text{AUC}_{\text{po}}} = \frac{F'_a}{F_a} \cdot \frac{F'_G}{F_G} \cdot \frac{\text{CL}_{\text{int,h}}}{\text{CL}'_{\text{int,h}}} \quad (\text{eq. 13})$$

where AUC'_{po} and AUC_{po} represent the areas under the plasma concentration-time curve (AUC) following oral dosing in the presence and absence of an inhibitor, F'_a and F_a is the fraction of drug absorbed from the intestine, F'_G and F_G is the fraction escaping gut-wall metabolism, and $\text{CL}'_{\text{int,h}}$ and $\text{CL}_{\text{int,h}}$ represent the intrinsic hepatic clearance in the presence and absence of an inhibitor, respectively.

Although the fraction of a drug available for oral absorption (F_a) may be impacted by changes in gastrointestinal pH, solubility, complex binding, or intestinal active transport, a ratio of $F'_a/F_a = 1$ is generally assumed when considering DDI involving CYP using static models. Dynamic PBPK models (e.g., Simcyp) allows evaluation of altered F_a due to changes in dosage form or intestinal transporter DDI when using the ADAM

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model (Jamei et al., 2009b; Darwich et al., 2010) (see above). In turn, the impact of F_g is generally only considered for CYP3A4 since this enzyme has a significant impact of first-pass extraction for many drugs (Fan et al., 2010; Gertz et al., 2010; Tachibana et al., 2010). Regional distribution of additional intestinal CYPs including 3A4, 3A5, 2C9, 2C19, 2D6 and 2J2 have been published (Paine et al., 2006) and is incorporated in the ADAM model.

The major hepatic CYPs currently evaluated for their contribution to NCE clearance include CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5. If a particular CYP is involved in the metabolism of a NCE, the impact of inhibition of the particular pathway is derived from the mechanistic static model using the equation modified from Rowland and Matin (Rowland and Matin, 1973), except for CYP3A4 where the inhibition of intestinal metabolism is also considered (Tachibana et al., 2010).

$$DDI = \frac{1}{\left(\frac{f_m}{1 + \frac{[I]}{K_1}} \right) + (1 - f_m)} \quad (\text{eq. 14})$$

Based on this equation, the most important parameter to estimate is the fractional metabolism (f_m) through individual CYPs. This is obtained from CL_{int} estimated using recombinant heterologously expressed enzymes scaled to human hepatic clearance by incorporating enzyme abundance and Intersystem Extrapolation Factor (ISEF) values (Venkatakrisnan et al., 2000; Proctor et al., 2004; Chen et al., 2011). Data obtained

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from specific chemical inhibitor studies in human liver microsomes (HLM) are also used to corroborate the estimates of f_m obtained with rCYPs. The CL_{int} estimate obtained in HLM is generally compared to CL_{int} determined in human hepatocytes incubated with and without aminobenzotriazole (ABT) to determine if other metabolic pathways could contribute to reduce the estimates of f_m CYP (Emoto et al., 2010; Kimoto et al., 2012a). It is important to recognize that the predicted clinical DDI (AUCR) is very sensitive when the estimates of f_m by one particular metabolic route or enzyme exceed 0.8-0.9 and is therefore important to account for all possible metabolic pathways (Figure 5). In turn, there is also a limit in the ability of *in vitro* systems to provide an accurate assessment of f_m CYP above ~0.85, which would require *in vivo* studies in human to define the clinical DDI risks. Such clinical studies are particularly important when predicting AUCR >5 in a DDI or when polymorphic CYP enzymes (e.g., 2D6) are contributing to clearance (Ito et al., 2005; Goutelle et al., 2013). The challenge is further compounded when metabolic pathways are contributing where IVIVE to human hepatic CL is not established (e.g., AO, SULT, MAO, etc.), which impacts accurate *in vitro* estimates of CYP f_m .

Drug-Drug Interactions Involving Phase II or Other Enzymes

The prediction of DDIs mediated by inhibition of UGT-mediated clearance is the most advanced among phase II metabolic processes, although clinically significant DDIs are generally less severe than described for CYPs (Williams et al., 2004; Kiang et al., 2005). A primary concern is however to determine the f_m for UGT mediated by polymorphically expressed enzymes such as UGT1A1 (FDA, 2012). The f_m CYP vs f_m UGT could initially be determined from CL_{int} estimates obtained in separate incubations of alamethicin-activated microsomes incubated with the appropriate cofactors (NADPH

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or UDPGA). Based on these data and additional *in vitro* or *in vivo* information on the relative abundance of UGT-mediated metabolites formed, the f_m UGT could be estimated. The availability of human mass balance data is not very useful to estimate f_m UGT since glucuronides cleared in the bile can be subject to hydrolysis in the gut. An alternate approach would be to estimate the f_m UGT1A1 from experiments with rUGTs. This approach requires incorporation of emerging data on the relative UGT abundance in expressed systems, HLM and human liver or intestine (Harbourt et al., 2012; Ohtsuki et al., 2012). Collectively, these data could be used to establish ISEF values for UGTs when data on selective substrate assays are incorporated although these approaches are still being developed and validated (Gibson et al., 2013). Furthermore, whether extrahepatically expressed UGTs can be involved in DDI has yet to be ascertained.

The estimation of f_m or metabolism through other CYPs (e.g., CYP2A6, CYP2J2, CYP4F2, CYP2E1), phase I pathways (e.g., MAO, FMO, XO), or phase II enzymes (e.g., sulfotransferases) should be considered during the ADME elucidation of a NCE's clearance, although estimation of f_m through these pathways is complicated by a lack of IVIVE. Clinical DDI through inhibition of NCE clearance mediated by these pathways is considered case-by-case. In fact, in many instances, clearance by enzymes other than P450 serves to blunt the maximum possible interaction that can be caused by a P450 inhibitor.

Drug-Drug Interactions Involving Transporter-Mediated Disposition

As alluded to above, the involvement of transporters in NCE disposition is considered early on in drug discovery, especially for chemical series that are generally subject to hepatobiliary and/or renal secretion (Wu and Benet, 2005; Darwich et al.,

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2010). Based on the clinical evidence, it is important to determine if NCEs are substrates for transporters, which may include P-gp, BCRP, OATP1B1, OATP1B3, OAT1, OAT3 and OCT2 (Giacomini et al., 2010). In particular, OATP1B1 and OATP1B3, selectively expressed in the human liver, cause significant DDIs as observed with an increasing number of drugs. It is evident that DDIs involving hepatic transporters are capable of altering systemic, as well as tissue-specific exposure of drug substrates, resulting in marked differences in drug safety and/or efficacy (Giacomini et al., 2010; Elsby et al., 2012; Yoshida et al., 2012)

A conservative assessment of hepatic uptake transporter-mediated DDIs for NCEs can be achieved with a simplified static model incorporating *in vitro* and *in vivo* drug parameters in order to estimate the ratio of the area under the plasma concentration-time curve in the inhibited state (AUC_i) in comparison with the control state (AUC) (Eq. 1), AUC ratio (AUC_i/AUC), also referred as R-value (Giacomini et al., 2010).

$$\frac{AUC_i}{AUC} = 1 + \left(\frac{[I_{in,u}]}{K_i} \right) \quad (\text{Eq. 15})$$

where $[I_{in,u}]$ is the liver inlet inhibitor concentration and K_i is the unbound inhibition constant. The R-value generally provides an oversimplification of the transporter-mediated DDI risk, assuming hepatic uptake of the victim drug is mediated entirely by a single transporter. Therefore, a predicted R-value >2 would generally require further assessment using mechanistic static or dynamic models for quantitative DDI evaluation.

Drugs targeting the liver generally carry the implication that these agents could be victims of transporter-mediated DDI since liver uptake transporters (e.g., OATP1B1, OATP1B3) could be inhibited by co-administered drugs (Barton et al., 2013). NCE

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disposition is studied in systems with increased complexity: transporter gene overexpressing cells, primary isolated hepatocytes and/or sandwich-cultured human hepatocytes (see above). Since transporter abundance data amongst *in vitro* systems and *in vivo* are not readily available and selective transporter probe substrates or inhibitors are lacking, it is difficult to establish comprehensive IVIVE. Nevertheless, metabolically competent hepatocyte systems provide valuable information on the relative passive and active cellular uptake rates and biliary clearance, which could be used to assess the rate-determining step in NCE disposition (Parker and Houston, 2008; Maeda et al., 2011; Kimoto et al., 2012b; Varma et al., 2013b). The availability of intravenous human PK is extremely valuable to determine the role of hepatic uptake in the overall disposition of these substrates. Intravenous PK profiles for liver-targeted drugs are generally multi-compartmental, which could be simulated from *in vitro* data using compound-specific or average empirical scaling factors to accurately capture the role of hepatic uptake in the overall disposition (Jones et al., 2012). Defining the rate-determining process in substrate disposition enables accurate simulation of the clinical DDIs for drugs subject to active liver uptake when incorporated in PBPK models (Varma et al., 2012c; Barton et al., 2013; Varma et al., 2013b).

Significant DDIs could also be elicited by several drugs (e.g., verapamil) through inhibition of the efflux transporter, P-gp, at the level of the intestine resulting in increased exposure of narrow-therapeutic index agent digoxin (Fenner et al., 2009). Less frequently, clinically relevant DDIs could be related to inhibition of renal transporter-mediated disposition and inhibition of these transporters are only studied when renal secretory CL is greater than 25% on the NCE's disposition (FDA, 2012).

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Drug-Drug Interaction Liability of a NCE as Perpetrator

The three primary mechanisms of a perpetrator DDI that result in changes in exposure of a co-administered drug are 1) reversible inhibition, 2) inactivation, and 3) induction of drug metabolizing enzymes and transporters. The extent of the clinical DDI is predicted using several *in vitro* approaches as below and although activation of substrate clearance is sometimes observed *in vitro*, there is limited clinical evidence for translation to DDI.

Considering the mathematical basis for the prediction of perpetrator DDI (Einolf, 2007; Fahmi et al., 2008; Guest et al., 2011), it is important to consider the relative NCE drug exposure [I] to the inhibition constants (e.g., K_i , K_I , or EC_{50}). A primary difference exists in the inhibitor concentration currently thought most predictive of clinical outcome in that for inhibition the unbound portal vein concentration is used while for inactivation and induction the unbound C_{max} or liver exit concentration is considered most relevant. It is challenging to understand the mechanistic basis for these differences, but it may reflect pharmacodynamic differences in that inactivation and induction result in downstream enzyme changes to achieve an altered steady-state whereas competitive inhibition is a more direct interaction requiring inhibitory drug present at the site of interaction. Current PBPK software packages such as Simcyp provides flexibility in terms of applying unbound liver inlet or outlet concentrations, although the latter is standard and considered more predictive of clinical outcome (Jamei et al., 2009a; Guest et al., 2011).

Enzyme Inhibition and Inactivation

Prediction of perpetrator interactions is currently most advanced and accurate clinical DDI predictions for inhibition is reasonably standard (Obach et al., 2006; Wang,

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2010). The inhibitory constants for inhibition (IC_{50} or K_i) or inactivation (K_I and k_{inact}) are generally measured in human liver microsomes. There are however several experimental design challenges resulting in systematic inaccuracies for measurement of these *in vitro* inhibition constants and results in considerable variability in estimates reported by different laboratories. In general, it is important to measure the free drug concentration in the incubation even when low protein concentrations are employed. Estimates of inactivation parameters usually require higher protein concentration in the initial incubation due to the dilution requirement, which could result in differences when compared to inhibition estimates (Austin et al., 2002; Obach, 2009).

Predictions of DDI caused by inactivation (time-dependent inactivation; TDI) of CYP enzymes are generally accurate except in the case of CYP3A4/5 substrates where over-prediction of the clinical DDI risk is often observed. The reasons for over-predictions are likely related to uncertainties on the relevant free inactivator concentration along the length of the intestine, the F_g for CYP3A substrates other than midazolam (e.g., simvastatin), and the natural enzyme degradation rate (k_{deg}). In addition, the relative turnover of enterocyte and enzyme is not currently incorporated in modeling and needs to be considered in a nested model. Since the natural rate of biosynthesis of enzymes are not readily measurable, several indirect measurements of k_{deg} are reported in literature ranging a half-life of 10-140 h. This has led to reductions of the half-life estimates used for *in vivo* hepatic k_{deg} (96 h vs 36 h) in DDI predictions and a recent internal evaluation indicated a half-life of 23 h may be more appropriate (Rowland Yeo et al., 2011). The free intestinal inhibitor concentration also has a significant impact on DDI predictions, especially when plasma unbound fraction (f_u) <0.2 . Current static

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models do not account for intestinal inhibitor binding while incorporation of binding is suggested for PBPK models (Rowland Yeo et al., 2011). Suboptimal prediction of TDI is likely to reflect not only uncertainty in k_{deg} but also errors or variability in estimation of K_I and k_{inact} based on the conventional experimental design (Yang et al., 2005; Yang et al., 2007). Recent efforts have incorporated improved statistical analysis to reduce improper or false estimation of K_I (Yates et al., 2012). The use of in-house PBPK models are also useful in estimating the free intestinal concentration, which results in more accurate predictions of DDI due to enzyme inactivation.

Induction of Drug Metabolizing Enzymes

Considerable advances have been made in our ability to predict clinical induction liabilities of NCEs and drugs during the last decade (Sinz et al., 2008; Almond et al., 2009; Fahmi and Ripp, 2010). Nevertheless, prediction of induction liability of a NCE is particularly challenging since the impact of the NCE on metabolizing enzymes levels occurs *via* an indirect mechanism of receptor binding resulting in transcriptional activation of the receptor's target genes. These receptors primarily include pregnane X receptor (PXR), constitutive androstane receptor (CAR), aryl hydrocarbon receptor (AhR), and others less commonly associated with the activation by drugs. Although there is some overlap in the receptors involved in the activation of target genes, current regulatory guidance recommends initially studying the induction liability of CYP1A2 (AhR), CYP2B6 (CAR and PXR) and CYP3A (mainly PXR with some CAR involvement), (EMA, 2012; FDA, 2012). Of the target enzymes studied to date, CYP3A is generally considered to be the most sensitive marker of enzyme induction and due to its important role in drug metabolism, CYP3A4 induction liability is routinely studied

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during drug discovery prior to first-in-human clinical studies. Recent results generated in our laboratories indicated that although PXR and CAR are involved in the induction of several CYPs, phase II enzymes, the increases in CYP3A4 mRNA was greater than those studied for all other enzymes, suggesting that measurement of CYP3A4 mRNA is sufficient to predict induction liabilities mediated *via* PXR and CAR activation (Fahmi et al., 2010). That is, to date there are no known examples of CAR inducers (primarily associated with CYP2B6) which do not also induce CYP3A4. A negative result with CYP3A4 induction negates the obligation for studying PXR induction of CYP2C. Although the induction of many other drug metabolizing enzymes and transporters are regulated by PXR and above mentioned transcription factors, the clinical prediction of DDI risk due to induction is not established and needs to be evaluated on a case-by-case basis.

Induction is mainly studied in cultured human hepatocytes since these are fully competent systems with regard to regulatory elements. The use of cultured human hepatocytes also allows comparison of induction ability within a chemical series since induction could be studied in the same donors, accounting for individual variability in induction response. Since both increases in mRNA and catalytic activity is generally measured, confounding results on catalytic activity for compounds exhibiting TDI could be positioned by knowledge on changes in mRNA. Several other systems have also been used to study induction including freshly prepared hepatocytes, immortalized hepatocytes (Fa2N4 cells), minimally derived hepatocyte cell lines (HepaRG, HepG2), reporter gene assays, and ligand-binding assays (e.g., PXR). The SAR for induction is not well established, which complicates the ability of drug discovery teams to limit induction

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liabilities when using a lower throughput and costly system such as cultured human hepatocytes. The use of ligand binding and reporter gene assays may allow higher throughput screening when attempting to do SAR and move away from an induction liability, but there are significant disconnects in the translation between these assays and induction in human hepatocytes (Shukla et al., 2011). It is therefore important to follow up positive results in ligand binding or reporter gene assays with studies in hepatocytes.

There has been a considerable effort to improve the quantitative prediction of clinical DDI risk from *in vitro* data utilizing static mathematical equations and PBPK models (Almond et al., 2009; Chu et al., 2009; Fahmi and Ripp, 2010). A number of these approaches (e.g., relative induction score) requires calibration of *in vitro* induction with known clinical inducers. Even more sophisticated static mathematical predictions requires the use of a calibration factor or “d” value (Fahmi et al., 2008). PBPK models such as Simcyp also requires relative induction values by expressing hepatocyte induction data relative to the rifampin positive control EC_{50} and E_{max} . Since there is a significant variability in induction parameters obtained between laboratories or external vendors, it is important to define the assay variability within a specific laboratory or for each lot of human hepatocytes by defining positive control results or generating positive control data for each experiment. Ultimately, all compounds progressing to phase 1 clinical trials and beyond should be evaluated for clinical induction potential utilizing an appropriate mathematical or PBPK model.

Complex Drug-Drug Interactions and Applications of PBPK Modeling

PBPK modeling of DDI risk during all stages of drug discovery and development is currently standard practice for most pharmaceutical research and development

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organizations due to the significant advantages these models provide compared to more traditional or static mathematical approaches, employing dynamic changes in drug or metabolite concentration as opposed to assuming static concentrations throughout the study (Huang and Rowland, 2012; Rostami-Hodjegan, 2012). PBPK modeling of drug disposition and DDI risk is particularly useful during the drug development stages and regulatory review since modeling is essential to more accurately or quantitatively predict clinical risk, or inform labeling decisions (Zhao et al., 2011). This is important since drug disposition and DDI mechanisms are generally complex and models need to be calibrated with both *in vitro* parameters (“bottom-up”) and observed *in vivo* PK or clinical DDI results (“top-down”) (Huang and Rowland, 2012; Rostami-Hodjegan, 2012; Varma et al., 2012c; Gertz et al., 2013; Varma et al., 2013a; Varma et al., 2013b). Although PBPK models are useful to predict DDI risk prior to first-in-human (FIH) studies, there is a significant level of uncertainty around the human PK and efficacious drug concentration in man, since these parameters are predicted (Chen et al., 2012; Sinha et al., 2012; Zhao et al., 2012). Therefore, the utility of PBPK models are generally fully realized once human PK have been confirmed and human ADME data obtained to calibrate PBPK models, which could be utilized to inform decisions on allowable concomitant medications or exclusion criteria during Phase II studies and beyond.

PBPK models are considered “bottom-up” since these models employ information on human physiology and the relevant biological processes, which are important when mechanistically evaluating interactions arising from altered drug absorption and disposition due to changes in drug metabolism and/or transport (Rostami-Hodjegan, 2012). This is in contrast to a classical “top-down” model constructed from

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compartmental or non-compartmental PK analysis of clinical data. PBPK models employ system parameters (e.g., human demographics and genetics, tissue volumes and blood flows, enzyme and transporter expression levels) and drug-dependent parameters (e.g., tissue partition coefficients, metabolism or transport rates), which allows prediction of outcomes across population extremes or disease variability beyond the prediction for an “average” individual. This information facilitates prediction of complex DDI situations, allowing simultaneous evaluation of competitive inhibition, time-dependent inhibition or auto-inhibition, enzyme-induction or auto-induction, and transporter-mediated DDI with multiple co-administered perpetrator drugs (Varma et al., 2013b; Varma et al., 2013c). It is also possible to simultaneously evaluate the impact of metabolites contributing to DDI or population extremes with respect to enzyme or transporter expression levels, ethnicity, or pharmacogenetic variability in enzyme or transporter expression (Inoue et al., 2006; Jamei et al., 2009; Barter et al., 2013; Dumont et al., 2013). The utility of PBPK models are further realized in the potential financial savings during drug development since appropriately calibrated models reduce development cost by avoiding the conduct of additional or unnecessary clinical DDI studies when used to simulate “what-if” scenarios.

ADME Predictions: The Future

Certainly there has been tremendous progress over the past two decades in our ability to predict human drug disposition attributes using preclinical data. This has led to the incorporation of ADME science considerations in drug design and early research, and has made the ADME scientist a critical member of modern drug discovery research teams. The general success of this has resulted in fewer failures of drug candidates in

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drug development caused by ADME flaws (e.g. low bioavailability, short $t_{1/2}$, DDI, others). Intrinsic receptor potency, target tissue penetration (and membrane permeability in general), and free intrinsic clearance are three parameters with which medicinal chemists and their ADME scientist partners must be concerned when optimizing drug properties through design. For orally administered agents, ensuring good exposure through the balance of membrane permeability and solubility, while paying attention to active efflux processes, is also important.

Despite these successes, there are still numerous areas for improvement and there are exciting technical developments ready to be investigated more deeply. This is particularly true for clearance. Our ability to screen for P450 based metabolism and our knowledge of how to design compounds more stable to P450 metabolism has yielded two challenges: (1) intrinsic clearance can be so slow that it is difficult to measure (using substrate depletion) and (2) other clearance mechanisms of lower familiarity are now more important in clearance. But not all clearance mechanisms have reliable methods whereby human in vivo clearance can be predicted from in vitro or animal data. Scaling methods for some enzyme systems are yet to be established (e.g. SULT, GST, MoCo, MAO, FMO, others) while methods for others have only recently emerged (UGT; Kilford, et al., 2009). The contributions from organs other than the liver can add a complicating factor to making human clearance predictions. And above all this lies the challenge of predicting PK for drugs cleared by transport proteins. Unlike clearance mediated by P450 enzymes, there are far fewer drugs for which transporters have been reliably assigned as the primary in vivo clearance mechanism. Progress toward mapping important drug transporters and their expression levels in various body tissues is an area

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of active pursuit. The fruits of these investigations will be realized in coming years, and the ADME toolbox will become more complete. (Artursson, 1990)

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FIGURE LEGENDS

Figure 1. Generalized diagram of clearance mechanisms as they relate to molecular properties. (It is not intended that there are discrete boundaries defining the categories of drugs, this represents a very general trend that can help frame thinking about clearance mechanisms. Exceptions exist.)

Figure 2. General Layout of Drug Clearance Mechanisms

Figure 3. Example of an In Vitro-In Vivo Correlation for Clearance for Ezlopitant (Obach, 2000)

Figure 4. A. Correlation between apparent permeability across (MDCK-LE) cell monolayer and human fraction absorbed. The apparent permeability of 5×10^{-6} cm/s represents a cut-off above which compounds show complete absorption. Inset shows the sigmoidal relationship considering complete radial mixing model, $F_a = 1 - \exp^{-2 \cdot (A \cdot P_{app}) \cdot T_{res} / r \cdot 2.8}$. B. Relationship between apparent permeability across cell monolayer and human effective permeability. MDCK-LE apparent permeability values represent transport at pH 6.5 for acids and pH 7.4 for bases, neutrals, and zwitterions. Figure modified from (Varma et al., 2012b). Key: Data points are categorized based on BCS classes I (red), II (blue), III (yellow) and IV (black).

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Figure 5. The relationship between maximum possible DDI (AUC ratio) and f_m . (Inset: X-axis scale truncated to $f_m = 0.75$.)

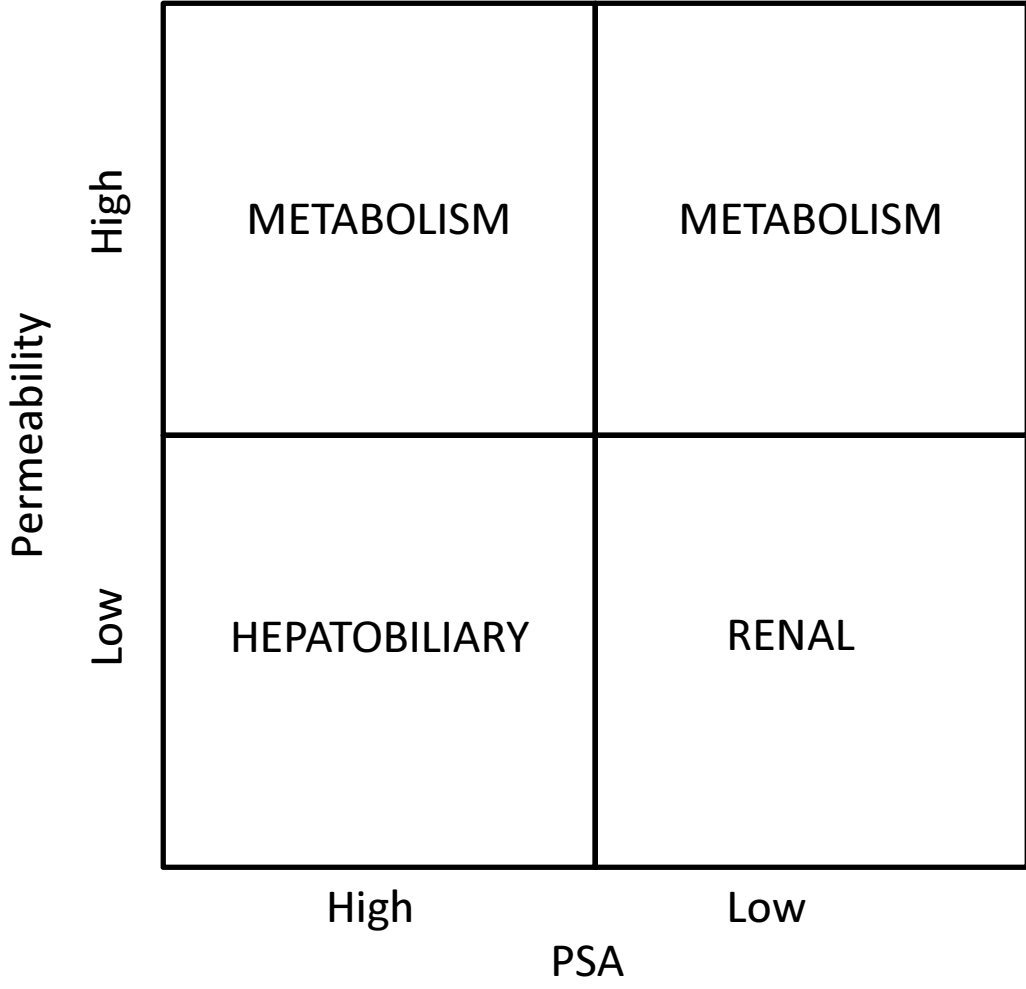


Figure 1

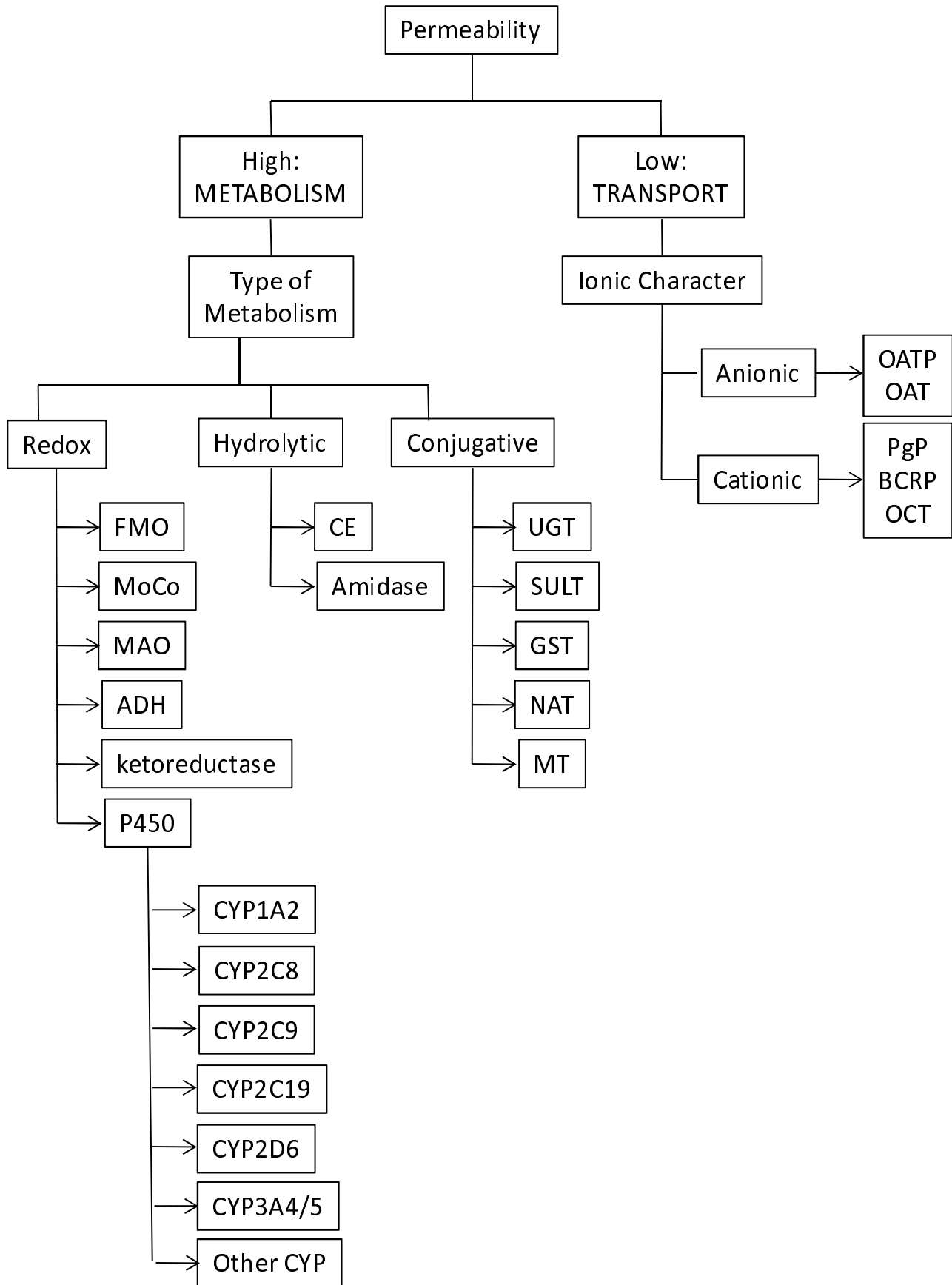


Figure 2

Species	Scaled from In Vitro	Measured in Vivo	Fold Difference
rat	44	45	1.02
dog	14	25	1.78
monkey	41	39	0.95
			Mean: 1.25
human	6.3		
human predicted	$6.3 \times 1.25 = 7.8$		
human actual		$CL_{po} = 8$	

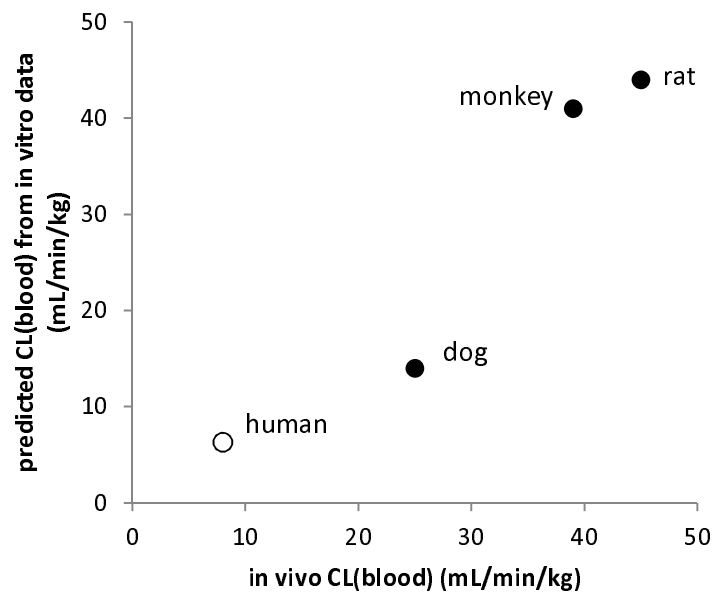


Figure 3

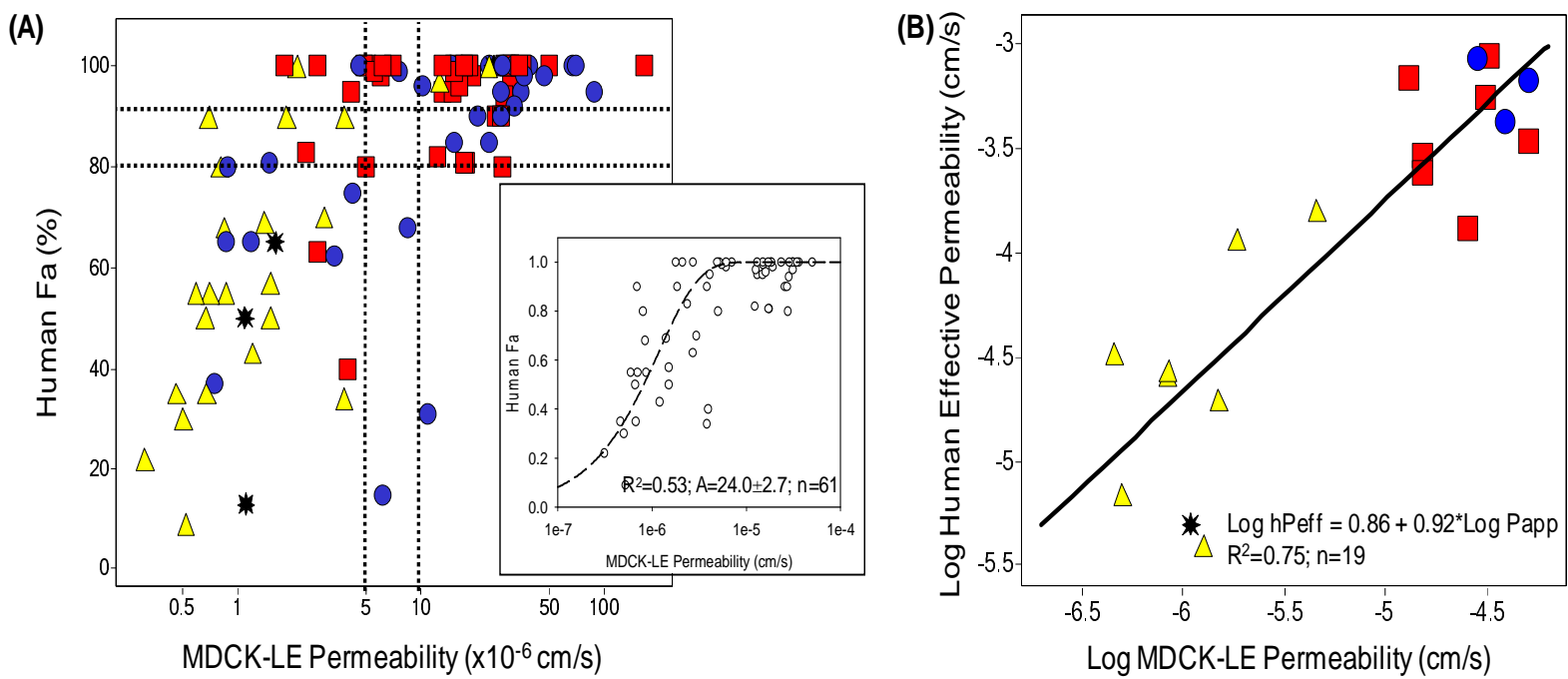


Figure 4

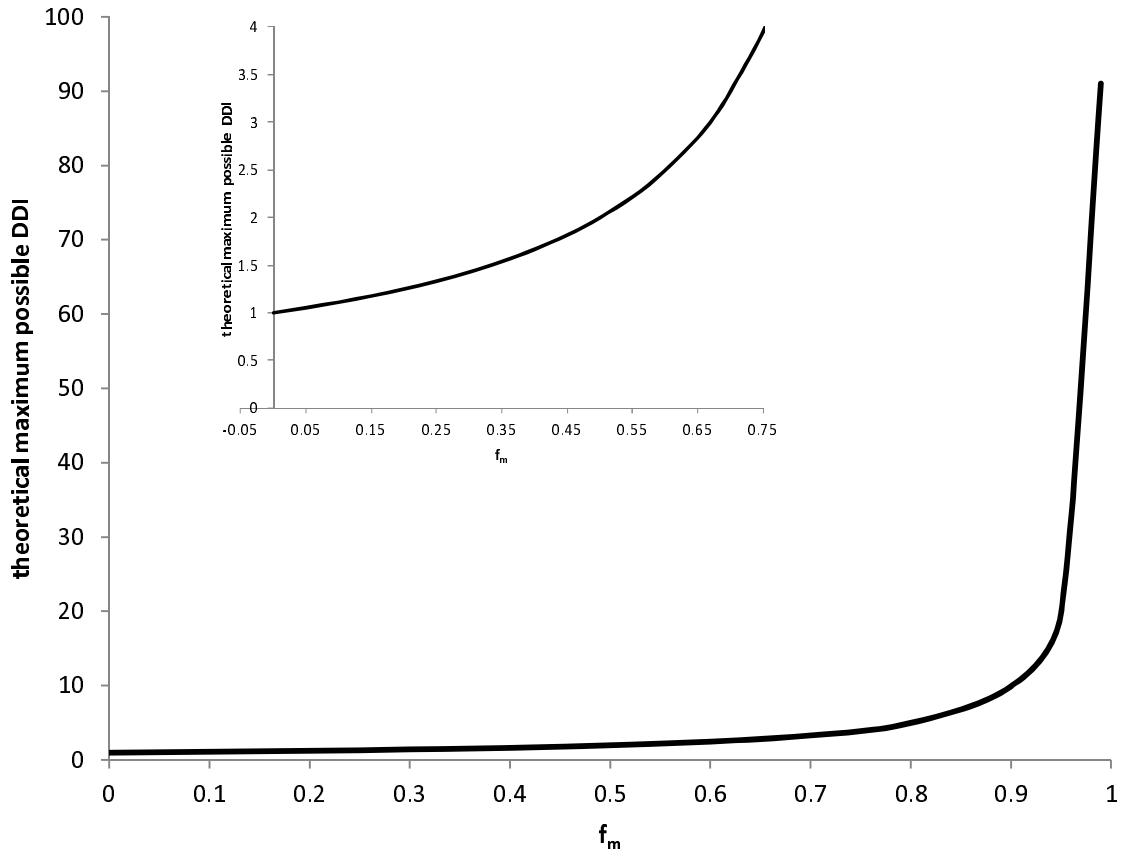


Figure 5