Drug discovery and drug metabolism

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The decision as to which chemical entity should progress to development as a drug candidate has, in the past, been based on relatively expensive preclinical efficacy and safety studies that have been performed in animals. This review presents a proposal to improve the efficiency of human drug development and increase the 'hit rate' of clinical candidates. The proposal is based on the concept that human drug metabolism strategies established early on in the drug development/drug discovery paradigm could lead to untenable candidates being discarded at an early stage in the drug development process. This strategy would tend to reduce costs and provide greater focus on those agents more likely to be successful as drug candidates.

In the USA and elsewhere, the process of developing a drug candidate has become extremely expensive and time-consuming. Possibly the most critical juncture in the drug development scheme is the decision-point at which a candidate is brought forward for sophisticated clinical testing and evaluation. In the past, the decision as to which entity should go forward was often based on relatively expensive efficacy and safety studies in animals. In this review, a proposal to improve the efficiency of human drug development and increase the 'hit rate' of clinical candidates is presented, based on the idea that human drug metabolism strategies could be put in place early on in the drug development/drug discovery paradigm so that untenable candidates could be more effectively discarded at an early stage in the process. The use of modern bioanalytical, biochemical and molecular biological approaches to human drug metabolism issues is discussed, and the advantages over the use of animal models are presented. A general strategy useful for *in vitro–in vivo* correlations in obtaining key pharmacokinetic parameters is described and may provide a basis for predicting human drug metabolism and toxicity as a key component in drug development.

Recent history of drug development

In the USA, the drug development process has become a more expensive and time-consuming endeavor than ever before. While research and development costs have doubled every 5 years since 1970, cost constraints, including competition from outside the USA and managed health care changes from within the USA, have placed an urgency on incentives to make the drug discovery process more efficient and less costly[1]. In 1990, the cost of producing a successful drug was approximately $200 million and it is likely that the cost will now rise above $400 million[2].

One of the traditional approaches to drug discovery has been the random screening of chemical libraries or natural products. So-called 'rational drug design' and, recently, combinatorial chemical approaches, are replacing traditional methods, but, even so, considerable effort is still involved in the structural modification of existing compounds or synthesizing libraries of recognized active drugs. The limited examples available suggest that the rational drug design process is less expensive, but it is possible that it is just as time-consuming as the traditional method of drug discovery. Approximately one out of 1,000 compounds identified in preclinical studies is eventually found to be suitable for human studies. The inefficiency of the drug development process necessitates advances in the drug discovery paradigm[3].

Possibly the most critical juncture in the drug development process is the decision as to which entity is to be brought forward to a more sophisticated stage for clinical testing in

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humans. It is extremely expensive to evaluate efficacy and safety in order to file an NDA with the FDA. Clinical testing of new entities can take up to 6 years. Before that, a putative drug candidate must undergo a series of sequential Phase I and Phase II testing. The post-discovery part of the drug development time period is additional to the time spent identifying, synthesizing and biologically testing the new entity in the laboratory. All told, the drug development process generally takes 12 years, including review and approval by the FDA[2].

What is needed to shorten the time period and decrease the cost required for drug development is to develop new strategies to increase the clinical 'hit rate' and more closely approximate the human situation in an *in vitro* setting when preparing drug candidates for preclinical evaluation. One way to achieve this would be to introduce human drug metabolism studies earlier into the process than is traditionally the case. In addition, *in vitro–in vivo* correlations of human metabolism studies could provide an efficient means for discerning entities that are suitable for further (much more expensive) human clinical trials. *In vitro* drug metabolism information could also aid in the selection of animal species for toxicological testing and safety evaluation[4]. The key strategy should be to discard unsuitable candidates (due to metabolic or biochemical toxicological liabilities) early in the drug development process. This would help to reduce costs and provide greater focus on those agents more likely to be successful as drug candidates.

A clear example of the need for cost effectiveness and close communication between drug discovery and drug development personnel can be seen in the biotechnology industry. The increasingly difficult financial picture in the past 5 years, coupled with investors' demands, has often propelled biotechnology management to push drug candidates through the early stages of research, and even clinical research, in an effort to stay ahead of the competition. At the same time, the widely held notion that the FDA would somehow 'go easy' on biotechnology drug candidates led the industry to a precarious situation during 1992–1994, when a number of late-stage clinical trial failures set the biotechnology industry back significantly. Part of the solution to such problems has come from the recognition that what is needed is a more fully integrated drug development capability, whereby *appropriate* drug candidates move forward in the drug development pipeline. On the other hand, the biotechnology industry has been moving towards a situation where it concentrates on what it does best (i.e. drug discovery) and then relies on established companies such as the pharmaceutical giants (or larger and successful biotechnology companies) for subsequent drug development and commercialization. Regardless of who pays for the safety and efficacy trials, as well as the production and marketing costs, it is clear that the biotechnolgy industry will still need to staff and provide preclinical and clinical drug development expertise at the early drug discovery stage.

It is recognized that advances in screening technologies and other strategies, including tissue-, cell- and enzyme-based methods involving synthetic chemical, peptide, antibody or gene combinatorial libraries, have and will continue to evolve and contribute to more rapid drug discovery processes. However, in many cases, it is the clinical testing that creates the bottleneck in development. With recent advances in computer-aided molecular design, molecular modeling, structure elucidation techniques (i.e. high field NMR, X-ray crystallography) and 3-D pharmacophore modeling, it is possible that the drug candidate pipeline will not be limited by drug discovery issues. It is reasonable to assume that advances in rational drug design and combinatorial chemical approaches, as well as structure identification, will provide a means to circumvent possible rate-limiting steps in the drug discovery activities of pharmaceutical and biotechnology companies. What is needed, however, is the elaboration of new paradigms to hasten the delivery of more cost-effective therapeutic agents to the market. The strategy outlined below uses common methods that are often used in industrial studies of human drug metabolism to improve the efficiency of the human drug development process.

Suitability of metabolic enzyme screens in human drug development

In the past, most *in vitro* studies of human drug metabolizing systems were in fact performed using animal liver preparations. Animal liver metabolic processes can, in many cases, be quite different from those in human liver – a view recognized by the FDA – hence, more studies directed at supporting human drug development are now being conducted using human metabolic screens. Traditionally, the most often used hepatic preparation has been adult human liver microsomes (vesicles of the endoplasmic reticulum formed by centrifuging hepatic preparations) or the corresponding S9 fraction (liver homogenate after removal of the nuclei and mitochondria). Now, large numbers of form-selective antibodies and a large battery of substantially pure adult human liver drug metabolism enzymes are commercially available[5,6].

In principle, there are at least three general approaches to using enzyme preparations in metabolic screens in human

drug development. One approach is to use cDNA-expressed drug metabolism enzymes to evaluate drug candidates. A second approach is to study the metabolism of a drug candidate in the presence of a bank of highly characterized human liver microsomes. Formation of the metabolite of interest can be correlated with selective functional markers of activity and immunoreactivity to identify the enzyme(s) responsible. Human liver microsomes can be treated with chemical inhibitors of drug metabolism or selective inhibitory antibodies to validate results of studies with cDNA-expressed enzymes or human liver microsomes. For a laboratory endowed with (often commercially available) enzyme preparations, the drug candidate can be evaluated and the metabolic profile can also be obtained.

Knowledge of the major human liver metabolites of a drug early in its development is useful for clinicians, toxicologists and drug metabolism chemists in evaluating whether or not the compound should be taken further in development. A clinician may re-evaluate the development of a drug candidate if the entity is an extensively metabolized compound or does not have the desired pharmacokinetic properties. From the point of view of the biochemical toxicologist, unusual or potentially reactive metabolites may eventually cause toxic conditions or untoward manifestations, and it is important to obtain such information early on[6]. A drug metabolism chemist may note that a compound or a metabolite inhibits metabolism. This may eventually have consequences for enzyme induction, drug–drug interactions or other unfavorable pharmacodynamic properties. Finally, knowledge of the human liver microsomal or reconstituted enzyme generated metabolic profile of a drug candidate is useful to enable the judicious and justifiable selection of an animal species for toxicological testing.

Human liver cytochrome P-450

Although there are numerous exceptions for extensively metabolized drugs, the cytochrome P-450 (CYP) enzymes are generally primarily responsible for metabolic clearance. Over 100 hepatic CYP enzymes are known from animal studies[7]. One of the more extensively studied forms of CYP, the CYP2B subfamily, is the form of enzyme most commonly associated with the 'phenobarbital-inducible cytochromes P-450' in experimental animals[7]. The form most analogous to CYP2B in human liver is CYP2B6, but it is unlikely that this plays a prominent role in adult human drug metabolism because of its low level of expression in adult human liver[8]. This emphasizes an important point that confounds correlative work with adult human liver and animal model testing: often the animal hepatic model possesses an entirely different complement of hepatic CYP enzymes than does the adult human liver and displays a completely distinct metabolic profile, such that direct comparison of pharmacokinetics or metabolism would be of little value. Finally, a number of examples of human CYP-mediated drug metabolism genetic polymorphisms have been described in the literature; this, of course, has significant consequences for idiosyncratic variations in human drug metabolism.

In contrast to animal liver, adult human liver appears to rely on about 10–15 prominent CYP enzyme forms for most drug metabolism[9,10]. Large differences in enzyme expression and substrate specificity have been observed between human and experimental animals. For example, the CYP3 family of enzymes is probably the most important drug-metabolizing member of the cytochrome P-450 group[7], yet it is not present to a great extent in most experimental animals without selective induction.

Table 1 lists the major forms of CYP present in adult human liver microsomes. As discussed above, most CYP are available as cDNA-expressed enzymes (or available in stably transfected mammalian cells) that can be used to verify the metabolism of a drug candidate or provide a method to enhance the formation of a minor metabolite or to evaluate the potential toxicity of a metabolite. Among the advantages of working with human liver microsomes is that a carefully preserved sample can mimic the mixture of CYP present in the membrane-containing milieu of the cell to which the drug candidate would be expected to be exposed. Among the disadvantages of using microsomes are:

- Other enzymes, such as esterases and hydrolases, are present,
- Sample availability can be problematic,
- For ethical reasons, sample pretreatment regimens (i.e. induction regimens) are limited, and
- Sometimes the enzyme-specific activity of interest is low, unstable or highly variable from sample to sample.

One way to obviate such shortcomings is to use highly characterized (or biochemically phenotyped) cytochromes P-450 present in a bank of adult human liver microsomes; such banks are now commercially available. By using selective functional markers of prominent CYP enzyme activity and form-specific antibodies, microsomes from a bank of human liver specimens can be characterized for the specific activity and amount of immunoreactive CYP present in each microsome sample[4,11–15]. It is then a simple matter to evaluate the drug candidate and compare by correlation analysis the CYP present that is presumably responsible for drug metabolism. This approach has been successfully used in a number of

Table 1. Selective functional substrate, activity and inhibitors of adult human liver cytochromes P-450[a]

Cytochrome P-450	Selective functional substrate	Activity	Inhibitor
1A1	Polycyclic aromatic hydrocarbons	Hydroxylation	7,8-Benzoflavone
1A2	Ethoxyresorufin	*O*-Deethylase	7,8-Benzoflavone
2A6	Coumarin, Nicotine	Hydroxylation	
2B6	Unknown		
2C8	Tolbutamide	Hydroxylation	
2C9/10	Tolbutamide	Hydroxylation	Sulfaphenazole
2C18	(*S*)-Mephenytoin	Hydroxylation	
2C19	(*S*)-Mephenytoin	Hydroxylation	
2D6	Dextromethorphan	*O*-Demethylase	Quinidine
2E1	*N*,*N*-Dimethylnitrosoamine	*N*-Demethylase	4-Methylpyrazole
3A3/4	Erythromycin	*N*-Demethylase	Troleandomycin
3A5	Testosterone	Hydroxylation	Troleandomycin
3A7	Dehydroepiandrosterone-3-sulfate	Hydroxylation	

[a]Adapted from Ref. 4 with permission from Marcel Dekker, Inc.

laboratories to determine a role for a particular CYP in the formation of a metabolite[16].

Adult human liver flavin-containing monooxygenases

Most human liver drug metabolism studies have implicated CYP in the oxidation of chemicals and drugs. Recently, evidence has suggested that human liver flavin-containing monooxygenases (FMO) may be a key component of the monooxygenase system that converts nucleophilic heteroatom-containing drugs and chemicals into relatively polar metabolites that are readily excreted in the urine[17,18]. Because many of the metabolites are, in principle, also capable of being formed by CYP and could also undergo futile metabolic recycling (i.e. be converted to a metabolite such as an *N*-oxide or *S*-oxide which can then be reduced back to the parent amine or sulfide, respectively), careful studies must be performed to distinguish these possibilities.

A number of studies have shown that adult human liver microsomes are capable of tertiary amine *N*-oxygenation and sulfide *S*-oxygenation[13,19–25]. Human FMO is unstable and subject to thermal inactivation in the absence of NADPH, and this may account for the few reports of FMO-mediated human drug metabolism. In contrast to human CYP, and based on animal and limited human studies, human FMO activity is probably not dependent on the nondietary treatment of the subject from which the tissue was obtained[3,19,21]. For example, it is well known that CYP1A1 and CYP1A2 are induced by cigarette smoke and other chemicals. In addition, CYP2C9/10 and CYP2C19, and 2E1 are induced by rifampicins and ethanol, respectively[4]. As new drugs are evaluated, it is likely that additional examples of drug induction of CYP will be discovered. In contrast, based on numerous animal studies and limited human studies, human FMO activity is unlikely to be increased by induction. If anything, FMO appears to be maximally induced in the 'normal' state, at least in animals. However, it is possible that it could be de-induced during various dietary treatments and this may have consequences for human drug–drug interactions for drugs efficiently detoxicated by FMO. Like human CYP, human FMO activity is not markedly dependent on the gender or age of the adult tissue donor.

The physiological role of human FMO is currently not known, but there is some evidence that FMO deficiency is related to a dietary-derived trimethylaminuria in humans[26]. The idea of a drug metabolism genetic polymorphism of human FMO has been suggested and, if true, may have consequences for drug candidates that are primarily metabolized by FMO. It is possible that FMO evolved to protect humans from the nucleophilic compounds present in foodstuffs[27]. As discussed above, human FMO may be maximally induced in adults, and xenobiotic exposure will probably not increase its activity further.

Currently, there is evidence for five forms of FMO in humans (Table 2)[28]. Human FMO1 is apparently expressed in greatest abundance in fetal liver. In adults, FMO1 is present in kidney, and possibly in other organs including the intestine. FMO2 has been associated with the lung in experimental animals, but in adult humans, FMO2 appears to be the prominent form in kidney. FMO3 is the major form present in adult human liver. Traces of FMO4 and FMO5 have been reported to be present in human tissue but, with the exception of *n*-octylamine, they have not shown activity against any substrate examined[18].

Human liver microsomes have been phenotyped for FMO activity[4,12,13]. For adult human liver tissue that was rapidly

procured and snap-frozen, a good relationship between microsomal FMO activity and FMO immunoreactivity was observed[12]. Because of the uncertainty of supplies of adult human liver samples in the future, cDNA-expressed enzymes from prokaryote sources or stably transfected mammalian cells will be useful sources for human FMO drug metabolism and mutagenicity studies.

Table 2. Selective functional substrates and products of adult human flavin-containing monooxygenase oxygenation

Flavin-containing monooxygenase (FMO)	Selective functional substrate	Major product
FMO1	(*S*)-Nicotine	*cis*-Nicotine *N*-1′-oxide
	Cimetidine	(–)-Cimetidine *S*-oxide
FMO2	(*S*)-Nicotine	*trans*-Nicotine *N*-1′-oxide
	n-Dodecylhydroxylamine	Hydroxylamine, oxime
FMO3	(*S*)-Nicotine	*trans*-Nicotine *N*-1′-oxide
	Cimetidine	(+)-Cimetidine *S*-oxide
FMO4	Unknown	
FMO5	*n*-Octylamine	Unknown

Human liver Phase II enzyme studies

In many cases, nucleophilic functionalities (e.g. carboxylic acids, alcohols, amines, hydroxylamines) of lipophilic parent drugs or metabolites are conjugated with endogenous molecules such as glucuronic acid, sulfuric acid or other amino acids, such as glycine, to form glucuronides, sulfates and hippuric acid, respectively[29]. The resulting conjugate is usually devoid of any significant biological and pharmacological activity (compared with the pharmacological activity of the parent compound). Formation of glucuronosyl conjugates of xenobiotics or drug development candidates constitutes one of the most important Phase II reactions. The formation of conjugates is an important route through which xenobiotics are detoxicated to highly polar materials that are relatively efficiently excreted in the bile and urine[30]. For example, glucuronides are Phase II conjugates that are very water-soluble and generally less toxic metabolites of the parent drug, and are rapidly excreted in the urine.

In some cases, conjugates can undergo hydrolysis and reconjugation in a process known as enterohepatic recirculation. Because Phase II conjugates are anions, entry to and exit from the hepatocyte is dependent on the properties of the conjugate toward the organic anion transporter.

Not all conjugates are benign, however, and there is some evidence that Phase II conjugates of metabolites of toxic chemicals can be carriers of carcinogenic activity[31]. It is possible that certain carcinogens are transported from sites of high monooxygenase activity after conjugation and hydrolysis to sites of low monooxygenase activity that become selective targets for cellular toxicity[31,32]. Among the many Phase II enzymes involved in the conjugation of drugs or xenobiotics are the multiple forms of microsomal or cytosolic catalysts[4]:

- Glutathione *S*-transferases (microsomal and cytosolic),
- UDP-glucuronosyltransferases (microsomal),
- Sulfotransferases (cytosolic),
- Methyltransferases (cytosolic), and
- *N*-Acetyltransferases (cytosolic).

The conjugating enzymes are generally highly dependent on cofactors and/or cosubstrates. For that reason, microsomal incubations that are supplemented with only NADPH to assess monooxygenase activity often do not contain the necessary ingredients for conjugase activity. Another limitation is that many conjugase enzyme forms are present in the cytosol but not in the microsome fraction. In order to include Phase I and Phase II enzyme reactions, the S9 fraction or hepatocytes or tissue slices could be used advantageously to examine drug metabolism of both types of enzyme.

A bank of adult human liver specimens has been characterized with regard to seven different Phase II enzyme reactions[4]. It is notable that there was significant interindividual variability in the activity of the Phase II enzymes examined[4,15]. Historically, Phase II conjugation enzymes (i.e. *N*-acetyltransferases) were among the first examples of human drug-metabolizing polymorphism discovered. In addition, it is known that multiple forms of Phase II enzymes possessing distinct substrate specificity exist, and these are, undoubtedly, differently expressed in various adult human liver preparations. This probably accounts for the fact that, in the study cited[4], acetaminophen glucuronidation did not correlate with that of 7α-ethynylestradiol sulfation. While the substrates listed in Table 3 are not necessarily selective for a specific form of conjugating enzyme, the level of enzyme activity nevertheless provides an indication of general conjugation ability.

To obviate the need for extensive parallel drug metabolism studies in the presence of human liver microsomes and, separately, human liver cytosol to characterize Phase I and Phase II reactions, an integrated approach using human hepatocytes or liver slices can be undertaken.

Adult human liver hepatocytes and liver slices

The ability to mimic the *in vivo* condition in an *in vitro* experiment with a full complement of adult human Phase I and Phase II drug metabolizing enzymes is an attractive feature of using human hepatocytes in the drug discovery regimen. For maximal cell viability, in-house procurement of human hepatocyte preparations is probably optimal, although commercially available hepatocytes are also available. Hepatocyte isolation involves obtaining hepatic tissue from whole liver, or by surgical operation or biopsy, followed by collagenase digestion. Optimal recovery of Phase I and II enzyme activity has been observed with cell cultures in suspension, although monolayer cell cultures can be employed, albeit with some significant loss of Phase I enzyme activity. The enzyme activity of the hepatocytes is usually highly dependent upon the quality of the tissue and the proficiency of the cell culture technique. An advantage of the use of hepatocytes is their reliability as an *in vivo* model of human drug metabolism, although cell viability can be quite variable; for example, cryopreserved cells often lose 50% of their viability upon thawing[33].

Human liver slices are generally more resistant to loss of Phase I and Phase II enzyme activity than are hepatocytes and can have remarkable stability if properly prepared[34]. Because disruption of the integrity of the tissue is considerably less in human liver slice preparations than in the course of procurement and culture of hepatocytes, a relatively accurate representation of human liver *in vivo* drug metabolism can be achieved using liver slices. With further advances in storage and cryopreservation, human liver slices may provide an important supplemental method to validate *in vitro* observations of drug metabolism that accurately mimic the *in vivo* situation.

Regardless of whether the profile of the metabolites of a drug development candidate is obtained from human liver microsomes, hepatocytes or liver slices, the aim is to identify and verify the specific monooxygenases involved. With the recent application of molecular biological approaches to produce commercially available drug metabolizing enzymes, rapid progress in the identification of the specific monooxygenases responsible for the drug candidate transformation can be established.

Table 3. General functional conjugase substrate activities[a]

Substrate	Activity[b]	Range	Fold
Acetaminophen	Glucuronidation	22–210	10×
Acetaminophen	Sulfation	7–86	13×
17α-Ethynylestradiol	Glucuronidation	35–233	8×
17α-Ethynylestradiol	Sulfation	4–31	8×
Isoniazid	Acetylation	10–190	18×
Mercaptopurine	Methylation	26–42	2×
3,4-Dichloronitrobenzene	GSH conjugation[c]	0.8–1.7	2×

[a] Adapted from Ref. 4 with permission from Marcel Dekker Inc.
[b] Activities were measured with liver cytosol except glucuronidation, and are expressed as pmol min^{-1} mg^{-1} protein, except 3,4-dichloronitrobenzene–GSH conjugation, which is expressed as nmol min^{-1} mg^{-1} protein
[c] GSH, glutathione

Substantially pure human liver drug metabolizing enzymes

It is important to identify the specific Phase I or Phase II drug metabolizing enzyme because of the possibility that the drug candidate (or a metabolite) causes drug–drug interactions, is involved in polymorphic metabolism or is metabolized by an enzyme whose expression is regulated. Drug metabolism polymorphisms may lead to adverse drug reactions if a normally efficiently metabolized drug is not metabolically cleared. In addition, it is important to recognize when one agent is metabolized by the same enzyme that metabolizes another drug. Because the availability of highly purified enzymes from tissue sources is limited, the use of cDNA-expressed enzymes from prokaryotes or stably transfected cell lines potentially affords an unlimited supply of drug metabolizing enzymes. Substantially pure preparations of cDNA-expressed drug metabolizing enzymes or fusion proteins are useful in the verification that a particular enzyme can perform the reaction observed in other *in vitro* or *in vivo* systems. However, there are limitations as to the usefulness of the recombinant proteins, just as there are limitations to the utility of other drug metabolizing systems. To compare the relative contribution of each enzyme to the metabolism of the drug candidate, it is important to determine that:

- The amount of enzyme present (i.e. nanomoles of protein with a selective functional activity) is comparable and at an appropriate level.
- The amount of cofactors or coenzymes present is saturating and not in any way limiting the transformation of interest. For example, under physiological conditions, in the hepatocyte, cytochrome P-450 reductase is present in large excess

compared with the CYP monooxygenase; consequently, in the recombinant system, reductase may need to be added.

- The amount of substrate present approaches physiological concentration, and the kinetic parameters determined are relevant to the pharmacokinetic picture (see the discussion of pharmacokinetics, below).
- The response to inhibitors or alternative competitive substrates is likewise in agreement with that observed in other systems.

If the above criteria are met, knowledge of the particular Phase I or Phase II enzyme involved in the metabolism of the drug candidate can be useful to predict the influence of drug metabolism on clearance and other pharmacokinetic parameters. Recently, the use of *in vitro–in vivo* correlates of human drug metabolizing activity has been shown to be extremely useful in drug development. As described below, the ability to predict pharmacokinetic parameters from *in vitro* metabolic data is likely to lead to improved efficiency and cost-effectiveness in drug development.

In vitro–in vivo correlations

In principle, good approximations of pharmacokinetic parameters can be obtained from *in vitro* studies[35,36]. Once the values are available, the next key step is to extrapolate the parameters to humans. Interspecies allometric scaling and physiological modeling are two common approaches and are especially reliable for compounds that are largely eliminated by biliary, renal or pulmonary excretion[37].

Interspecies scaling can be used to interpolate and extrapolate between mammals based on their fundamental physiological and anatomical similarities; for example, pharmacokinetic parameters are related across species according to body weight raised to a certain power[38]. Allometric scaling is useful for estimating pharmacokinetic parameters, profiles and toxic endpoints. It may lead to a reduction in the number of animals and human subjects needed in a study, as well as predict the most appropriate large animal and correct dose to use in toxicity testing studies of drug candidates[38].

For *in vitro–in vivo* correlations, the hepatic clearance is the most important pharmacokinetic parameter for predicting *in vivo* clearance from *in vitro* data. Hepatic clearance (CL_h) is influenced by hepatic blood flow (Q_h), the fraction of unbound drug (f_u) and intrinsic clearance (CL_i; i.e. the intrinsic ability of the organ to clear the drug candidate that is not bound to blood cells or plasma proteins). Assuming the instantaneous and complete mixing of the drug candidate in the blood of the organism[39], hepatic clearance can be expressed as:

$$CL_h = [Q_h \times f_u \times CL_i] / [Q_h + (f_u \times CL_i)] \quad (1)$$

Because hepatic clearance (CL_h) is the product of hepatic blood flow (Q_h) and the hepatic extraction ratio (ER_h) (i.e. the ratio of the rate of drug candidate elimination to the rate of presentation to the liver), the hepatic extraction ratio can be written as:

$$ER_h = [f_u \times CL_i] / [Q_h + (f_u \times CL_i)] \quad (2)$$

For drug candidates with a high extraction ratio, clearance often approaches hepatic blood flow (Q_h). For drug candidates with a low extraction ratio, clearance is approximated by $f_u \times CL_i$. Because the intrinsic ability of an organ to metabolize a chemical is related to the activity of the metabolic enzymes, Equation 2 can be related to the Michaelis–Menten equation:

$$\text{Rate of metabolism} = (V_{max} \times C) / (K_m + C) \quad (3)$$

A relationship between CL, Q, ER and rate of metabolism becomes apparent. If it is assumed that V_{max} is the maximum rate at which drug metabolism occurs and K_m is the Michaelis–Menten constant, dividing both sides of Equation 3 by the systemic concentration of a chemical (C) gives:

$$\text{Rate of metabolism} / C = CL_m = V_{max} / (K_m + C) \quad (4)$$

Thus, Equation 4 shows the relationship between classical enzyme kinetics and pharmacokinetics which can be rewritten as:

$$f_u \times CL_i = V_{max} / (K_m + C) \quad (5)$$

As discussed above, for drug candidates with a low extraction ratio (i.e. $CL_m = f_u \times CL_i$), V_{max} and K_m values (which can be readily obtained from *in vitro* experiments) can be used to predict *in vivo* clearance parameters[35]. This is a powerful method to obtain relatively costly human pharmacokinetic data from inexpensive *in vitro* studies. Of course, hepatic clearance may not be the only process leading to drug candidate elimination (renal, pulmonary, etc., elimination may also be important), but it is often the prominent route of elimination.

Another approach is to relate the clearance values obtained from *in vitro* metabolic data to *in vivo* pharmacokinetic data from animals. *In vivo* clearance values from each animal species can be normalized by the ratio of *in vitro* metabolic clearance and related to human values. Some examples are available in the literature[40–42]. Correlations of various animal data can be useful to validate human clearance parameters[43]; see, for example, the following equation (a, animal; n, normalized; h, human; m, microsomes):

$$CL_{a,n} = CL_a \times CL_{h,m} / (CL_{a,m}) \quad (6)$$

Procurement of *in vitro* kinetic parameters is not restricted to microsomes. As described above, meaningful metabolic clearance data can also be obtained from hepatocytes and liver slices. If a drug candidate is largely metabolized via one prominent pathway, it is also possible to relate *in vitro* data from recombinant enzyme studies and extrapolate to *in vivo* clearance. Of course, kinetic studies with substantially pure enzymes must be validated with other *in vitro* hepatic preparations. Finally, by taking into account the intrinsic clearance values and the relative abundance of Phase I or Phase II drug metabolizing enzymes, the identification of the principal enzyme responsible for metabolism of a drug candidate can be obtained.

In conclusion, the strategy outlined above to obtain *in vitro* drug metabolism data to estimate *in vivo* parameters involves the following steps:

- Obtain the K_m and V_{max} values for the drug candidate in question,
- Use appropriate scaling parameters, relate *in vitro* clearance to intrinsic *in vivo* values,
- Employ a liver clearance model that accounts for the intrinsic clearance through the particular metabolic pathway of interest, and
- The strategy must include a consideration of other elimination routes for the drug, both metabolic and otherwise.

While the preference here has been to stress the use of data derived from cDNA-expressed enzymes and microsomes, the use of results from hepatocytes and tissue slices may provide even better predictors of CL_i. The fact that the Michaelis–Menten model accurately describes the complexities of kinetic relationships of metabolism of drug candidates in microsomes, hepatocytes and other hepatic preparations suggests that, at least for the moment, more complicated analyses are not required. The recent report that *in vitro–in vivo* data for rat liver CYP catalyzed drug metabolism could be correlated over four orders of magnitude[44] supports the idea that, in the near future, correlations just as powerful will be obtained for human liver tissue. This will provide valuable tools to the medicinal chemist, the metabolic chemist, the molecular toxicologist and the clinician in the drug development industry.

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