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Metabolic Stability for Drug Discovery and Development

Pharmacokinetic and Biochemical Challenges

Collen M. Masimirembwa, *Ulf Bredberg* and *Tommy B. Andersson*

Department of Drug Metabolism and Pharmacokinetics & Bioanalytical Chemistry, AstraZeneca R &D Mölndal, Mölndal, Sweden

Abstract

Metabolic stability refers to the susceptibility of compounds to biotransformation in the context of selecting and/or designing drugs with favourable pharmacokinetic properties. Metabolic stability results are usually reported as measures of intrinsic clearance, from which secondary pharmacokinetic parameters such as bioavailability and half-life can be calculated when other data on volume of distribution and fraction absorbed are available. Since these parameters are very important in defining the pharmacological and toxicological profile of drugs as well as patient compliance, the pharmaceutical industry has a particular interest in optimising for metabolic stability during the drug discovery and development process. In the early phases of drug discovery, new chemical entities cannot be administered to humans; hence, predictions of these properties have to be made from *in vivo* animal, *in vitro* cellular/subcellular and computational systems. The utility of these systems to define the metabolic stability of compounds that is predictive of the human situation will be reviewed here. The timing of performing the studies in the discovery process and the impact of recent advances in research on drug absorption, distribution, metabolism and excretion (ADME) will be evaluated with respect to the scope and depth of metabolic stability issues.

Quantitative prediction of *in vivo* clearance from *in vitro* metabolism data has, for many compounds, been shown to be poor in retrospective studies. One explanation for this may be that there are components used in the equations for scaling that are missing or uncertain and should be an area of more research. For example, as a result of increased biochemical understanding of drug metabolism, old assumptions (e.g. that the liver is the principal site of first-pass metabolism) need revision and new knowledge (e.g. the relationship between transporters and drug metabolising enzymes) needs to be incorporated into *in vitro–in vivo* correlation models. With ADME parameters increasingly being determined on automated platforms, instead of using results from high throughput screening (HTS) campaigns as simple go/no-go filters, the time saved and the many compounds analysed using the robots should be invested in careful processing of the data. A logical step would be to investigate the potential to construct computational models to understand the factors governing metabolic stability. A rational approach to the use of HTS assays should aim to screen for many properties (e.g. physicochemical parameters, absorption, metabolism, protein binding, pharmaco-

kinetics in animals and pharmacology) in an integrated manner rather than screen against one property on many compounds, since it is likely that the final drug will represent a global average of these properties.

Metabolic stability studies represent some of the earliest *in vitro* studies used in the pharmaceutical industry in an effort to predict *in vivo* pharmacokinetics. Several excellent reviews have been published recently describing the *in vitro* methods[1,2] and their application in early screening.[3-7] In this brief overview, we will highlight the changing scope and depth of questions being asked about metabolic stability and raise critical issues about experimental designs, results interpretation and application.

The concept of *in vitro* metabolic stability derives its importance from being related to metabolic clearance *in vivo*. This metabolic clearance *in vivo* has a relationship with drug bioavailability and half-life, and hence gives information on how much and how frequently a drug could be given. Over the years, the quest has, therefore, been to derive *in vitro* models to predict *in vivo* clearance. Even though success with *in vitro* models has been limited, efforts have been initiated recently to derive computational models for the prediction of the parameters of drug absorption, distribution, metabolism and excretion (ADME) *in vitro* and/or *in vivo*. A number of factors are behind the recent resurgence in the importance of establishing the metabolic stability properties of compounds in the drug discovery process. These include increasing awareness of this property as being critical for the potential of a compound to make it to the market, and the potential of high throughput screening (HTS) predictive methodologies to reduce costs and length of time for optimising test compound pharmacokinetic properties. The need for volume and speed is being driven by the increased capacity for chemical synthesis, resulting from combinatorial chemistry, and the increased number of disease targets, resulting from advances in genomics, that the pharmaceutical industry can screen their libraries against.

1. The Pharmacokinetic Challenges: Are They the Same Old Questions?

The basis for the extensive use of *in vitro* liver systems for measuring metabolic stability is because they are believed to reflect *in vivo* hepatic plasma clearance (CL_H). Hepatic clearance is for many compounds a major determinant for the overall pharmacokinetic properties, i.e. the first-pass elimination of the drug (and hence the bioavailability), the systemic elimination and the half-life. The usefulness of *in vitro* predictions of hepatic metabolic clearance relies on the assumption that hepatic metabolic clearance is the major clearance mechanism, i.e. that biliary excretion as well as non-hepatic clearance mechanisms such as extra-hepatic metabolism and renal secretion are negligible. This is in many instances a relevant assumption, particularly for lipophilic compounds where protein binding can be expected to be high and renal clearance by glomerular filtration is expected to be low. However, at early stages of the drug discovery process, very little is known about non-metabolic or extra-hepatic clearance mechanisms for a new class of compounds, and the relative importance of hepatic metabolic clearance relative to other clearance mechanisms can, therefore, not be confirmed until *in vivo* pharmacokinetic data are available.

2. Liver Models for Predictions and Their Basic Assumptions

A number of liver models for relating *in vitro* metabolic clearance to *in vivo* clearance have been developed and applied over the years. The three most applied models are the 'well stirred', 'parallel tube' and 'dispersion' models.

In the 'well stirred' or 'venous equilibrium' model,[8,9] the liver is assumed to be a single 'well stirred' compartment and the unbound concentration in the venous blood leaving the organ is in equilibri-

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um with, and equal to, the intracellular unbound concentration in the hepatocytes.

The hepatic clearance, CL_H is expressed as:

$$CL_H = \frac{Q_H \bullet f_{ub} \bullet CL_{int}}{Q_H + f_{ub} \bullet CL_{int}}$$

where CL_{int} is the intrinsic clearance, reflecting the actual metabolic capacity of the enzyme system when there is free access to substrate, f_{ub} is the free fraction in whole blood and Q_H is the total liver blood flow.

In the 'parallel tube' model,[10] the liver is assumed to be composed of a number of parallel tubes, with enzymes uniformly distributed along the tubes. The unbound blood concentration at any point along the tube is assumed to be in equilibrium with the intracellular unbound concentration:

$$CL_H = Q_H\,(1 - e^{\frac{f_{ub} \bullet CL_{int}}{Q_H}})$$

The 'dispersion' model[11,12] is even more 'physiologically correct' than the other two models in that it also incorporates axial dispersion of blood caused by the branching and connections of the sinusoids. The degree of dispersion is expressed by the dispersion number, Dn. The model assumes that the diffusion of drug along the sinusoids is much more rapid than the blood transit through the liver. At its two extremes the dispersion model collapses to either the parallel tube model ($Dn \rightarrow 0$) or the well-stirred model ($Dn \rightarrow \infty$). The elimination capacity is expressed by the efficiency number, Rn:

$$Rn = \frac{f_{ub} \bullet CL_{int}}{Q_H}$$

The mathematical expression is more complex than for the other two models:

$$CL_H = Q_H \left[1 - \frac{4a}{(1+a)^2\; 2e^{(a-1)/2Dn} - (1-a)\;\; 2e^{(a-1)/2Dn}} \right]$$

where $a = (1 + 4\,Rn\,Dn)^{1/2}$.

The difference between these three liver models is in their description of the concentration profile across the liver. This has most effect for high clearance compounds, which results in the highest difference in concentration across the liver. A comparison of the predictive value of the three models was studied for 28 compounds,[13] and none of the models gave consistently better results than the other models. Since the 'well-stirred' model is the simplest in nature it has become the one most commonly used.

Apart from the difference mentioned here, all three models are based on the same three basic parameters: CL_{int}, f_{ub} and liver blood flow. They also share a number of assumptions, which are fundamental for their predictive value:

- Assumption 1: the unbound concentration in the hepatocytes is equal to the unbound concentration in blood and the equilibration between these two compartments is instant.

This assumption is absolutely fundamental for the predictive value of the models and also most difficult to experimentally check for and verify. This assumption relies in turn on two further assumptions, as follows:

- Assumption 2: only unbound drug is available for diffusion into the hepatocytes.
- Assumption 3: only passive diffusion is operating across the hepatocyte membrane.

The role of protein binding in the prediction is constantly under debate. Theory predicts that only free drug concentration in the blood is the driving force for drug transport into the hepatocyte. The degree of protein binding should therefore be corrected for in the prediction models. The uncertainty in how to deal with the protein binding parameter becomes a great problem when dealing with a completely new class of compounds where no *in vivo* pharmacokinetic information is available. This is particularly problematic for compounds with a high CL_{int} combined with a high degree of protein binding. Inclusion or not of f_{ub} in the calculation will determine whether the compound should be classified as a low or a high clearance compound (see section 3.1 for further discussion on protein binding).

In many instances, the fraction unbound in plasma is used instead of the fraction unbound in blood, f_{ub}, which is the correct parameter according to the

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liver models above. This approximation is reasonable except for cases where distribution into blood cells is extensive and the concentration in whole blood is several fold higher than the plasma concentration.

f_{ub} is seldom measured directly, but is normally derived from the free fraction in plasma, f_u, and the ratio of whole blood concentration, C_b, and plasma concentration, C_p. This is normally expressed as:

$$f_{ub} = f_u \bullet \frac{C_p}{C_b}$$

This transformation has one conceptual problem. One can easily see that for a compound with no protein binding ($f_u = 1$) and low or insignificant partitioning into blood cells ($C_p/C_b > 1$), f_{ub} becomes greater than unity, which is conceptually wrong.

An alternative way of transformation would be to also include the haematocrit (Hct):

$$f_{ub} = f_u \bullet \frac{C_p}{C_b} \bullet (1 - Hct)$$

This transformation takes care of the conceptual problem above but can be debated for other reasons, i.e. is the free concentration in plasma different to the free concentration in whole blood (a consequence of the alternative expression). This will not be further discussed here, but is worth noting.

However, even the use of the 'alternative' f_{ub} calculation would not account for the mispredictions often seen. The uncertainty in how to deal with the protein binding parameter in the predictions is most unsatisfactory both from a scientific and from a drug discovery point of view. There is still a great need for a better understanding of the role of different plasma proteins for transport across the hepatocyte membrane.

All liver models assume instant equilibrium between the blood compartment and the intracellular space. This assumption is probably reasonable in many instances, but diffusion rate across the membrane could be rate limiting for a compound with a high intrinsic clearance, particularly for compounds with an intermediate or low permeability. Active transport mechanisms may also be operating in either direction across the hepatocyte membrane, and this may severely violate the assumption of equal free concentration at both sides of the hepatocyte membrane. Active uptake will increase the free intracellular concentration relative to the free blood concentration, resulting in a higher rate of metabolism and thus a higher plasma clearance than predicted based on the blood concentrations. An active efflux, i.e. from the hepatocyte to the blood, will result in a lower clearance than predicted. The effect of transporters on *in vivo* clearance has been demonstrated by means of simulations.[14] By incorporating active transport into the liver models ('well stirred' and 'parallel tube'), these investigators demonstrated the great significance of the presence of uptake transporters on clearance. The impact of transport phenomena is however very difficult to capture with any *in vitro* system. The role of transporters is of course totally neglected when using liver microsomes. Even with hepatocytes, where transporters are present, the activity of these transporters may be lost or down-regulated during the preparation, or unknown cofactors may be missing.

- Assumption 4: *in vivo* intrinsic clearance can be estimated by scaling an *in vitro* derived measurement of intrinsic clearance.

The estimation of the *in vitro* intrinsic clearance may be obtained by measurement of the product(s) during steady-state conditions over a wide range of substrate concentrations. Maximum rate (V_{max}) and Michaelis constant (K_m) are estimated, and CL_{int} is calculated as the ratio (V_{max}/K_m). This method is not suitable in a drug discovery setting, since it requires knowledge about all the products formed. More recently, it has become popular to use the so-called depletion method, where the substrate is incubated at a low concentration, e.g. 1 μmol/L, and the concentration of the substrate is followed over time. CL_{int} is then estimated from the half-life and scaled to a whole liver by using standard scaling factors (discussed in section 3). The latter method is rapid but it is based on the assumptions that the substrate concentration is well below the K_m of all the important enzymes involved, that the system is stable during the incubation period (typically some 40–60 minutes) and that there is no significant product

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inhibition. Product inhibition is more likely to occur in simpler systems (liver microsomes or recombinant enzymes) where phase II metabolic enzymes are inactive or missing. The apparent affinity (i.e. K_m) can theoretically be lower in the *in vitro* systems than *in vivo*, due to the lack of endogenous ligands competing for the same enzyme in the *in vivo* situation. Disappearance of compound in the solution for other reasons than metabolism cannot be ruled out, e.g. adhesion to surfaces or non-metabolic degradation.

In all, the liver models discussed have a lot of merits but the predictions rely on some fundamental assumptions, which in some instances cannot be verified. The weakest point in the prediction of hepatic metabolic clearance is probably the assumptions related to intrahepatocyte concentration. There is a great need for more basic research in the area of transporters and the interplay between blood components (e.g. plasma proteins) and the hepatocyte surface and transporters. This knowledge gap has existed for many years and surprisingly little work has been conducted in this area in the context of drug clearance. Until this type of knowledge can be built into the prediction models discussed, the uncertainty in clearance predictions will remain relatively large. Meanwhile, the importance of early *in vivo* pharmacokinetic information in, for example, rats, in order to establish the correlation (or lack of) between the *in vitro* metabolic clearance and the actual *in vivo* clearance cannot be over-emphasised.

3. In Vitro Models: Applications and Challenges

Even though metabolic stability is considered critical for compound selection and optimisation, the basic *in vitro* models used in most screening programmes and the interpretation of results have not changed much during the last decades. The most noticeable change is the advancement of analytical methods and the speed at which *in vitro* incubations are done with robotised methods.

The prediction of *in vivo* clearance using existing models is in general poor. Even though this topic has been studied for several decades, the scientific understanding is still limited. The reasons for this could be several-fold: (i) important components governing metabolic clearance *in vivo* are missing or factors used are not right in the existing *in vitro–in vivo* scaling equations, and (ii) the *in vitro* systems used do not reflect the metabolic systems important for clearance *in vivo*.

3.1 In Vitro Systems

Most companies are using subcellular fractions such as liver microsomes, or possibly the fraction called S9 (the 9000*g* supernatant of a liver homogenate), to measure the disappearance of a compound over time, which is reported as half-life or intrinsic clearance. The experimental setup is simple and amenable to HTS, making this method widely utilised. When scaling the *in vitro* metabolic stability data, the amount of protein per g of liver is a key factor that directly affects the calculation of intrinsic clearance values for the whole organ. A frequently used number is 45mg of microsomal protein per g of liver in both rat and human studies.[15] The accuracy of this figure in many experimental setups is doubtful. Carlile et al.[15] showed that treatment of rats with phenobarbital and dexamethasone changed the amount of microsomal protein in the liver. The reported values in humans also show a large variation between laboratories, from 15–77mg per g of liver.[16] A part of the variation may be explained by interlaboratory differences in experimental procedures. However, there are probably interindividual variations in the amount of protein in the endoplasmic reticulum as a result of factors such as age, sex and medical treatment. To our knowledge, there are no studies that describe the variation in human liver. Such a study is needed, and it is also recommended that each laboratory carefully characterise the hepatic subfractionation method. The classical de Duve fractionation scheme[17] should be followed using specific enzyme markers to monitor carefully the yield and purity of the cell subfractions prepared.

Compounds incubated in human liver microsomes show a wide range of protein binding, dependent on their physicochemical properties.[18] The *in vitro* microsomal enzyme kinetics may be seriously

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distorted for drugs studied in the non-saturated binding range (mainly weak lipophilic bases) where both the substrate concentration and the protein concentration affect the fraction unbound. In theory, only the free fraction of the compound is available for metabolism and the concentration in the incubation should be corrected accordingly. Corresponding corrections should be done for the calculation of *in vivo* intrinsic clearance values where a value for the plasma protein binding is used (see section 2). However, it is not possible to measure the free fraction available at the enzyme site, which complicates studies that try to unravel which factors are important for calculating intrinsic clearance of compounds *in vivo*.

The issue of protein binding has been addressed in several studies.[1,2,19] Obach[2] suggested that protein binding can be disregarded for basic lipophilic drugs, since they are usually highly bound in both microsomes and human plasma. However for acidic compounds, which have a low protein binding in microsomes and high protein binding in plasma, taking protein binding in both systems into account gives the best predictions. However, no definitive rules can be made based solely on these simple physicochemical properties, and in most large screening programs on metabolic stability the free concentration of compounds is not measured. Estimates of intrinsic clearance and *in vivo* hepatic clearance predictions may thus be confounded, and comparison of compounds in large data sets may not be relevant.

Several *in vitro* studies on liver microsomes have shown that increasing protein concentration by adding albumin to the incubation yields lower K_m values when the kinetics are calculated using the free fraction.[16,20] These results indicate that 'protein binding' in microsomes and 'protein binding' when adding albumin affect the enzyme kinetics differently. Albumin added to the incubation may 'facilitate' the presentation of the substrate to the enzyme in the liver microsomes. In human liver slices, the K_m calculated from the free fraction of phenytoin was also lower when albumin was used in the media, which may indicate that protein not only may facilitate the presentation of the substance to the enzyme but also facilitate transport over the membrane.[20]

By incorporating microsomal binding into the calculation, the same K_m for *N*-demethylation of amitriptyline was obtained over a wide range of microsomal protein concentrations, which is a strong indicator that only the free substrate in liver microsomes is available for metabolism.[21] In line with this conclusion, the V_{max} for the reaction was not affected by correcting for microsomal binding.

A simpler model to screen for metabolic stability is overexpressed enzymes. This system offers the possibility to mix exact proportions of enzymes or incubate with single enzymes. This can be advantageous over liver microsomes, especially when studying human metabolism, because of the great variation in enzyme levels in human liver microsomes. However, the big disadvantage is that the studies are restricted to the enzymes available. There is also concern that we might not reproduce the coenzyme requirement for the different isoenzymes of cytochrome P450 (CYPs) and for different test compounds, as this has been shown to be important for some CYPs (e.g. CYP1A2 and 3A4) and for some compounds.[22] In drug discovery, studies using expressed enzymes are mainly used to identify enzymes responsible for the metabolism of drug candidates.

Using tissue slices and isolated cells could solve some of the problems encountered by using subcellular fractions of the liver or expressed enzymes. Compared with microsomes, cellular systems offer the complete range of enzymes operating in the cell, including conjugating enzymes, esterases and amidases in addition to the CYP enzymes. The cofactor supply should also resemble the *in vivo* situation instead of adding unphysiologically high amounts, which is the case in microsomal incubations. The substances also have to cross the membrane and inside the cell they are bound to intracellular proteins and presented to the metabolising enzymes as *in vivo*. Tissue slices also retain the intact cell architecture and all types of cells in the liver, which may be important for drug metabolism. The

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role of plasma protein binding, which is not dealt with when using subcellular systems, may also be solved by incubating liver cells in serum.[23]

Tissue slices have not been characterised to the same extent as microsomes and hepatocyte suspensions. Poor diffusion of substrate to the inner layer of the tissue slice has been discussed and associated with lower clearance values than for microsomes or hepatocyte suspensions.[24] Other research areas that need to be addressed are the stability of xenobiotic-metabolising enzymes and their cofactor supplies in the tissue used for cell and slice preparation and incubations. Furthermore, the impact of isolation and slicing procedures on plasma membrane integrity and associated transporter proteins need to be investigated.

Recently, several laboratories have described techniques to cryopreserve hepatocytes[25,26] and tissue slices.[27,28] Cryopreserved material considerably improves flexibility and access to the systems, especially as cryopreserved hepatocytes are now offered by several commercial companies. It is obvious that whole cell systems should offer a more reliable system for predicting *in vivo* metabolic clearance than subcellular systems. However, current procedures with cells or tissue slices are not easily applicable to HTS platforms because of practical constraints and access to tissue. Future research should concentrate on miniaturisation of methods and optimisation of incubation conditions.

3.2 Relevance for the In Vivo Situation

Many reports describe the success of predicting pharmacokinetic properties from *in vitro* data. However, published data probably overemphasise successful cases, whereas failed attempts are not reported. Further, for human predictions, only drugs on the market or compounds in development that have been approved for human use can be studied. Obach et al.[2] reported that the clearance of 23 out of 27 compounds was predicted within a 2-fold errors of actual *in vivo* clearance by using the half-life method in human liver microsomes (no plasma or microsome protein binding included). Several reports describe a tendency to under-predict *in vivo* clearance; e.g. Carlile et al.[16] reported for a series of CYP2C9 substrates that the predicted values from *in vitro* kinetics were in the range of 5–31% of actual *in vivo* clearance data. Houston[29] compared liver microsomes and isolated hepatocytes to predict *in vivo* clearance of 25 drugs. Both systems identified low clearance drugs, but microsomes under-predicted to a higher extent than hepatocytes for high clearance drugs. The reason for this would be interesting to study further. Shibata et al.[23] presented a good correlation between measured *in vivo* clearance values in rat and values predicted from incubation of 18 compounds in hepatocytes incubated in plasma, indicating the importance of protein in the incubation media.

Although many reports suggest successful predictions of *in vivo* clearance from *in vitro* metabolism data, anyone working with *in vitro* metabolic stability screening in the pharmaceutical industry is well aware that for a number of compounds no correlation exists between *in vitro* stability data and *in vivo* clearance of the drug. Figure 1 shows the results from 48 compounds in the same chemical series in a project within AstraZeneca; it is obvious that there is no correlation between *in vitro* half-life in rat liver S9 and measured blood clearance *in vivo*. Despite the poor correlation between *in vitro* metabolic clearance and *in vivo* clearance, metabolic instability was identified as a serious problem for the compound series. Major efforts were made to

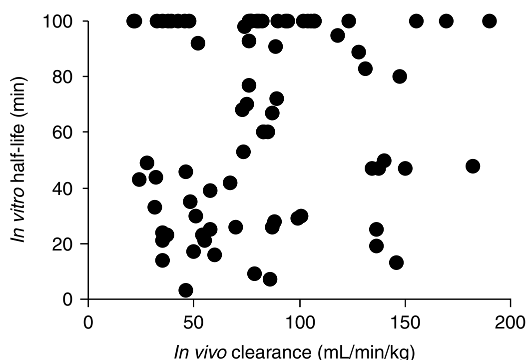

Fig. 1. Lack of correlation between *in vitro* half-life in rat liver 9000*g* supernatant (S9) and measured blood clearance *in vivo* for 48 compounds in the same chemical series in a project within AstraZeneca.

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understand the metabolic degradation pathways and how to stabilise the molecules. *In vivo* studies indicated other possible clearance routes, and Caco 2 cell assays showed that the compounds were substrates for transporter proteins. By using this integrative approach, the project could optimise the chemical lead, which initially exhibited quite impossible metabolic properties, without losing pharmacological effect.

Recently, Clarke and Jeffrey[30] presented results on hepatic microsomal metabolic stability in rat and *in vivo* clearance for over 1100 compounds from 49 chemistry programmes. When the compounds were categorised as low or high clearance *in vivo*, only 64% were predicted correctly from the *in vitro* data. About 24% were metabolically stable but had high clearance *in vivo*, which is not surprising since the liver microsomal *in vitro* method only tries to predict metabolic clearance involving CYP enzymes. What is more worrying is the large number of compounds that were metabolically unstable but were low clearance compounds *in vivo*. In programmes where metabolic stability is a key feature for selection, these compounds would be rejected despite good *in vivo* pharmacokinetics. In our screening programmes we have also encountered several examples where compounds that are extremely metabolically unstable *in vitro* in rat liver microsomes exhibit low clearance properties *in vivo*. The reason for this could be that liver microsomes create an artificial availability of the compound to the enzymes that, because of protein binding, permeability and drug transporters, does not occur *in vivo*. Clarke and Jeffrey[30] analysed their anomalous compounds and found that the plasma protein binding was significantly higher and that the volume of distribution was lower, which may indicate a restricted exposure of the metabolic enzymes to the compound. They also found that these compounds generally were more potent CYP inhibitors than the rest of the compounds.

The discussion points above illustrate the importance of evaluating if the *in vitro* metabolic stability data in a compound series correlate with actual measured clearance in the whole animal. Furthermore, chemical modifications to improve metabolic stability may in some cases give rise to problems with properties such as absorption or increased renal excretion, resulting in the overall pharmacokinetics not being improved. It is thus important that several relevant *in vitro* models are used to describe the disposition of the drug *in vivo*. An integrative way of working in early discovery is therefore vital, and *in vitro* results should always be contrasted with *in vivo* outcome in, for example, a rat model. An important task for drug metabolism and pharmacokinetics scientists in early drug discovery is to select representative compounds for whole animal studies by using relevant information from *in vitro* assays. In this process, computational models become more and more important.

A true, and urgently needed, evaluation is whether the predictions made from *in vitro* and *in vivo* preclinical studies are successful in human Phase 1 studies. Since such a naturally required appraisal is missing, it is tempting to suspect that these methods are not working as generally claimed because, if there is any success, it is at that junction it should be reported, not in retrospective studies of compounds on the market where the help of hindsight cannot be underestimated.

4. Chemical and Mechanistic Aspects of Metabolic Stability

The aim of preclinical metabolism studies during drug discovery and development is to nominate candidate drugs with predicted favourable pharmacokinetic and safety profiles for administration to humans. Figure 2 shows a general approach used in the pharmaceutical industry to achieve this. Because of the increased number of targets that companies are evaluating for drug development, and the increased number of compounds that they can screen for pharmacological activity, it is not always possible, strategic or economic to evaluate each compound *in vitro* or *in vivo*. At this level, general computational models that can predict metabolic properties such as sites of metabolism (hence metabolites), rates of metabolism and enzymes responsible could be useful as initial screens to assist in the

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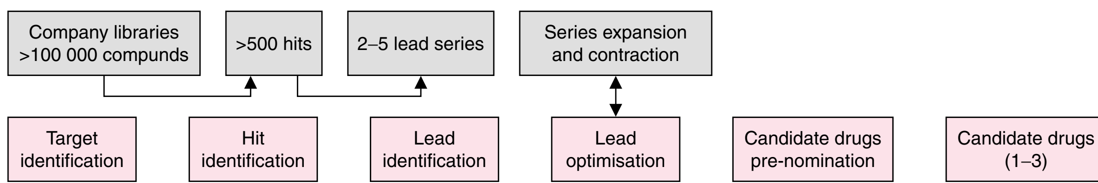

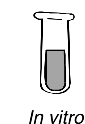

Fig. 2. General scheme of preclinical metabolism studies that are currently being performed to increase the chances of selecting candidate drugs that will be successful in the clinical phase of drug development. **CYP** = cytochrome P450.

design of *in vitro* and/or *in vivo* experiments that only analyse class representatives.

As the drug discovery process progresses to lead optimisation, general models and empirical data from *in vitro* and *in vivo* studies become insufficient to support efforts to optimise the chemistry of leads for desired metabolic properties. More specific computational models capable of accurate identification of enzyme-specific sites of metabolism, pharmacophore-based identification of enzyme-compound interactions and quantitative predictions of metabolic parameters become necessary (figure 2 and table I). Development and application of such computational models could facilitate fruitful communication between *in vitro–in vivo* metabolism scientists and medicinal chemists towards optimising compounds for metabolic stability.

There are currently many projects aimed at predicting the chemistry and biology associated with the metabolic stability of compounds. A number of companies specialise in computational prediction of drug metabolism and toxicology information, such as Molecular Design Limited (www.mdli.com), ArQule Inc. (www.arqule.com), MultiCASE Inc. (www.multicase.com), Accelrys Inc. (www.accelrys.com) and Amedis Pharmaceuticals (www.amedis.co.uk). Computer systems for the prediction of xenobiotic metabolism have been reviewed recently.[31] The increased understanding of the chemistry of drug biotransformation, drug metabolising systems and availability of better computational tools are beginning to yield promising models to predict metabolic stability (table I). Current models fall into the following general categories:

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Table I. Levels of biological complexity in predicting metabolism in which the chemistry of biotransformation is subject to the influence of biological factors, and levels of computational modelling complexity for both descriptive and quantitative predictions of drug metabolism. Factors are listed in increasing order of complexity

Level of complexity	Factors
Metabolic complexity	
Functional group chemistry	Oxidation (cytochrome P450, flavin mono-oxygenase)
	Hydrolysis (epoxide hydrolase, carboxyesterase)
	Conjugation (glucuronidation, acetylation, sulfation)
Proximal group effects	Activating or deactivating adjacent groups (e.g. radical stabilising systems)
	Steric hindrance (e.g. halogen atoms)
Global factors	Ionisation, hydrophobicity
	Pharmacophore
Biological factors	Biological system (tissue and cellular distribution)
	Interindividual factors (species, sex, genetics)
	Intra-individual factors (age, health, induction, inhibition)
Predictive modelling complexity	
General categorisations	Based on two-dimensional descriptors (e.g. molecular weight, volume, shape, hydrophobicity, hydrogen bond acceptors/donors) to predict the likely enzyme with which a compound will interact
Qualitative models	Based on two- and three-dimensional descriptors, quantum chemistry, molecular fragmentation and database searches (e.g. pharmacophore, active-site docking and electron density calculations) to predict likely substrates, inhibitors and site of metabolism
Quantitative models	As for qualitative models, plus molecular and/or neural network algorithm to relate descriptors to biological data and derive quantitative predictive models for reaction constants or intrinsic clearance

1. qualitative structural selectivity models
2. quantitative structural activity relationship
3. categorisation methods
4. non-linear methods (genetic algorithms and neural networks)
5. knowledge/rule based methods (relational databases/recursive partitioning).

The modelling approaches generally seek to understand the chemical descriptors in two or three-dimensional space that define substrates, inhibitors or inducers of drug metabolising enzymes. Other approaches generally seek to understand the enzyme active site geometry and chemistry that could give an insight of why specific enzymes interact with particular compounds. Promising approaches of combining compound molecular descriptors and homology models of enzyme active sites are increasing being used to develop more predictive qualitative and quantitative models.

Because of both a biased knowledge base and the fact that CYP is the major enzyme system involved in the metabolism of pharmaceuticals, work on this enzyme is most abundant. For this important family of enzymes, a number of reviews on active site structure and the general features of their substrates, inhibitors and inducers that summarise pre-2000 research have been published.[32-37] Work on the other major drug metabolising enzymes, flavin mono-oxygenase, carboxyesterase, epoxide hydrolase, UDP-glucuronosyltransferase (UGT), *N*-acetyltransferase and sulfotransferase, significantly lags behind.

4.1 Models to Predict Specific Enzyme-Substrate Binding Affinity

Qualitative structure activity relationship (SAR) and quantitative structure activity relationship (QSAR) models have been derived in two (2D) and three (3D) dimensions for CYP substrates and/or inhibitors. The computational methods used have been reviewed by Ekins et al.,[38] and include CoMFA, CATALYST, VolSurf/GRID and ALMOND.

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Lewis et al.[39] have made enormous contributions in using 2D molecular descriptors and active-site homology models of CYPs to define general features of isoform-specific substrates and deriving models that predict their binding affinity. Lewis et al.[32] have also combined protein homology modelling, substrate docking and mutagenesis data with 2D descriptors to better understand CYP-substrate interactions. Recent excellent reviews on pharmacophoric models based on 3D molecular descriptors have been published for CYP1A1, 1A2, 2A6, 2B6, 2C9, 2D6, 2E1 and 3A4 substrates and inhibitors.[38,40] With respect to the induction of CYPs, QSARs for CYP1A and CYP2B have been developed.[41] Using CATALYST, Ekins derived a 3D pharmacophore model for inducers of CYP3A4 that bind to the pregnane X receptor (PXR).[42]

In deriving SAR relationships for either substrates or inhibitors, the daunting challenge is to find alignment rules and active conformers for the usually diverse and flexible compounds capable of interacting with each drug metabolising enzyme isoform. In understanding the active site chemistry of enzymes, the limiting factors have been lack of crystal structures of human CYPs and a poor understanding of ligand-induced protein dynamics on substrate entry, catalysis and metabolite exit. Most of the enzyme-substrate interaction models derived are qualitative, with either little work or success in deriving predictive quantitative models for measures of interaction such as binding constant (K_s), K_m or inhibition constant (K_i). General trends of increasing hydrophobicity (log D) being associated with lower K_i, K_s and K_m and increased CL_{int} have been observed for some CYP-mediated metabolism.[43,44] Most enzyme-ligand interactions involving CYPs and other drug metabolising enzymes are, however, driven by more complex descriptors other than log D. These have to do with the chemistry and architecture of the active sites of the enzymes and the physicochemical properties (e.g. steric, hydrogen bond donor and acceptor properties, flexibility and stereochemistry) of the substrates, as demonstrated in a number of studies.[35,38,45] Recent success in crystallising membrane-bound rabbit CYP2C5[46] facilitated the derivation of a 3D-QSAR model for CYP2C9 inhibitors by docking ligands into the CYP2C9 homology to define alignment rules and select likely active conformers.[45] Astex™ Technology has recently announced solution of the 3D crystal structures of human CYP2C9 and CYP3A4.[47] This represents a major breakthrough in CYP research, as crystallisation of CYP has eluded scientists for decades, and is likely to provide a methodological framework to determine the structures of other human CYPs. In the meantime, it will improve homology modelling of other human CYPs, and co-crystallisation with substrates and inhibitors will improve our understanding of CYP-ligand interactions.

4.2 Models to Predict Position of Metabolic Attack in Molecules

Usually a molecule has many potential sites at which it could be metabolised. For CYP, the general belief is that the enzyme active site hypervalent iron-oxene species is a very powerful oxidising agent, which will attack most sites presented to it. This has led to the belief that if one can predict how the substrates will dock into the active site (the substrate-enzyme 3D pharmacophore), then one could reduce the possible sites for metabolism thereafter as being dictated by electronic and/or optimal distance and/or angle to the iron-oxene. Evidence is also increasing that for some CYPs and substrates, enzyme-substrate fit is important, whereas for others only the metabolic lability (electronic factors) of sites on the molecule are important. The latter case has been observed for CYP3A4 substrates, where there is evidence for molecules tumbling in the presumed big active site of the enzyme, leading to metabolism at distantly located multiple sites.[48] Reasonable success in prediction of site of metabolism has been achieved for CYP2C9,[49] CYP2D6,[50] and CYP3A4.[48] Knowledge of the CYP2D6 substrate pharmacophore has assisted in the design of a substrate for this enzyme for use in *in vitro* metabolism studies.[51]

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4.3 Models to Predict Rate of Metabolism

Predicting that a substrate interacts with a particular enzyme (qualitatively) and with what affinity (K_s or K_m) and predicting the site of metabolism does not tell you how fast that substrate will be metabolised. This is a fundamental issue, as most substrates are capable of interacting with many drug metabolising enzymes and being metabolised at similar or different sites by these enzymes. The quantitative measure of a route of metabolism becomes crucial for knowledge of the relative contribution of a particular enzyme to drug stability and the generation of a specific metabolite. The difficult of deriving such QSAR is demonstrated by the fact that although enzyme-ligand and site of metabolism models for CYP2D6 have been around for some years now, no quantitative model has been developed to assist scientists in judging the relative importance of this infamously polymorphic enzyme in the metabolism of test compounds. Reasonable success in this area has been achieved for CYP2E1 substrates,[52-54] carboxylesterases[55] and UGTs.[56] Evidence of allosteric auto- and hetero-metabolic activation associated with some CYPs (CYP2B6, 2C9 and 3A4 so far) adds further complexity to modelling drug metabolism.

4.4 General Models to Predict Complex Parameters

Non-mechanism-based models have also been developed to predict measures such as bioavailability that are themselves the sum of complex mechanisms, for instance absorption and metabolism. These models are either based on some sophisticated 'black-box' algorithm (neural network or artificial intelligence) or on some defining pharmacophoric features that are qualitatively and/or quantitatively associated with some drug disposition parameter. Modelling for metabolic stability of compounds in human liver microsomes (which possess many drug metabolising enzymes) by Ekins and Obach[57] and compound bioavailability (across many biological barriers) by Yoshida and Topliss[58] using pharmacophoric approaches represent such approaches. Completely or partially 'black-box' approaches to predict drug bioavailability and clearance have also been reported.[59,60] These general models have been proposed to be useful in preliminary screens of large compound collections and not so much in lead optimisation.

Understanding of the chemical basis of metabolic stability will strongly complement *in vitro* and *in vivo* data in the selection and design of compounds with desired properties. Preliminary success with *in silico* models has already encouraged others to use such models very early in the drug discovery process, either to characterise compound libraries for favourable metabolic stability or to rank virtual library compounds during lead optimisation such that only those with predicted preferred properties are synthesised (figure 2). When looking at the complexity of biotransformation, as indicated in table I, and the issues of data quality and quantity, the use of these computational models in the drug discovery process should be implemented with caution.

5. Conclusion

HTS platforms are allowing us to generate a large amount of information on metabolic stability and enzyme identification. However, the information should not primarily be used to select compounds with the belief that it can predict acceptable *in vivo* clearance. Instead, the data can give insight into the chemical basis of metabolic stability by applying computational techniques. To obtain reliable predictions, it is first necessary to run *in vivo* pharmacokinetic studies on a number of representative compounds to establish a real correlation with *in vitro* results.

Computational methods are expected to enable evaluation of metabolic issues very early in the drug discovery process and to facilitate fruitful interactions between metabolism/pharmacokinetics scientists and medicinal chemists in the optimisation of lead compounds. Indeed, development and application of computational methods for predicting and modifying human ADME is currently a major focus of attention in the pharmaceutical industry. Over-optimistic projections of the impact these methods will have on the drug discovery process are being

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made, sometimes displaying the same naivety as was seen with the unrealistic expectations for HTS platforms. As with *in vitro* systems, the science behind the biological parameters to which this sophisticated software is being applied is still not well understood.

In this review, we have tried to encourage scientists to exercise caution in their interpretation of results and to avoid the temptation to report only successes, as this gives the pharmaceutical industry false optimism and leads to inappropriate investment strategies. Nevertheless, predictions of *in vitro* properties from computational models seem to be getting better, whereas the bridge from *in vitro* and/or animal studies to humans remains formidable. The issues around the non-physiological nature of *in vitro* systems, interspecies differences and the complexity of pharmacokinetics *in vivo* still limit our quantitative and qualitative predictions of metabolic stability in humans. These old issues and new challenges will probably haunt us for a while as we seek to integrate our understanding of metabolic stability into the drug discovery process.

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Correspondence and offprints: Dr *Collen M. Masimirembwa*, Department DMPK & Bioanalytical Chemistry, AstraZeneca Mölndal, S-431 83 Mölndal, Sweden.
E-mail: collen.masimirembwa@astrazeneca.com

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