Screening for human ADME/Tox drug properties in drug discovery

Albert P. Li

There is no doubt that ADME/Tox drug properties, absorption, distribution, metabolism, elimination and toxicity, are properties crucial to the final clinical success of a drug candidate. It has been estimated that nearly 50% of drugs fail because of unacceptable efficacy, which includes poor bioavailability as a result of ineffective intestinal absorption and undesirable metabolic stability[1]. It has also been estimated that up to 40% of drug candidates have failed in the past because of safety issues[2]. In this review, the methodologies that are available for use in drug development as *in vitro* human-based screens for ADME/Tox drug properties are discussed.

***Albert P. Li**
Chief Scientific Officer
In Vitro Technologies
1450 S. Rolling Rd
Baltimore
MD 21227, USA
*tel: +1 410 455 1242
fax: +1 410 455 1245
e-mail: lialbert@invitrotech.com

▼ In addition to the relatively large number of drug candidates that fail during clinical trials, some drugs have been taken off the market after successful clinical trials. One reason for this is the incidence of drug–drug interactions. A recent example was in June 1998, when the anti-hypertensive and anti-anginal medication mibefradil was voluntarily withdrawn from the market by its manufacturer because of concerns over drug–drug interactions (V. Suga, 8 June 1998, News Release: Roche announces voluntary withdrawal of Posicor. Hoffmann-La Roche, Basel, Switzerland). Other cases of voluntary market withdrawal because of drug–drug interactions include the drugs soruvidine and terfenadine. The antiviral drug, soruvidine, was withdrawn from the Japanese market by its manufacturer within the year of its introduction because of its fatal interactions with the antitumor 5-fluorouracil (5-FU) prodrugs[3], and the non-sedating antihistamine terfenadine was withdrawn from the worldwide market by its manufacturer after co-administration with the antifungal ketoconazole resulted in cardiotoxicity[4].

Unexpected drug toxicity is another factor that might lead to the withdrawal of a drug from the market. Recently, in November 2000, a public health advisory was issued by the Food and Drug Administration (Rockville, MD, USA) concerning the risk of hemorrhagic stroke, or bleeding into the brain, associated with phenylpropanolamine hydrochloride, a common ingredient used both in cough and cold medicines as a decongestant, and in over-the-counter weight loss products. In this advisory, the FDA requested that drug companies discontinue marketing products that contain phenylpropanolamine (FDA, 6 November 2000, Safety of phenylpropanolamine, FDA/Center for Drug Evaluation and Research). In March 2000, troglitazone, a thiazolidinedione compound introduced in 1997 for the treatment of type 2 (non-insulin-dependent) diabetes, was withdrawn from the market upon the request of the FDA after several cases of severe hepatotoxicity were reported (FDA, 21 March 2000, Rezulin to be withdrawn from the market. FDA News: HHS News, P00-8).

It is, therefore, becoming apparent that in addition to pharmacological properties, ADME/Tox properties are crucial determinants of the ultimate clinical success of a drug. This realization has led to the early introduction of ADME/Tox screening during the drug discovery process, in an effort to select against drugs with problematic ADME/Tox profiles.

In this review, the scientific concept and practice of human-based, *in vitro* ADME/Tox screening approaches are described. Approaches with laboratory animals, which are also important tools in preclinical drug development, will not be discussed.

Intestinal absorption

Oral delivery is the most desirable route of drug administration. Therefore, it is important to develop drugs that can be absorbed

1359-6446/01/$ – see front matter PII: S1359-6446(01)01712-3

effectively through the intestinal epithelium (mucosa). There are several known mechanisms of intestinal drug absorption[5].

The major mechanism for drug uptake through the intestinal epithelium is passive diffusion that is driven by a concentration gradient. Passive diffusion can occur between cell junctions (paracellular transport) or through the cytoplasm (transcellular transport). Lipophilic compounds, such as testosterone, can cross the plasma membrane easily and, therefore, are mainly transported transcellularly. Hydrophilic compounds, such as mannitol, are relatively impermeable across the cell membrane and will, therefore, be transported predominantly via the paracellular route.

In addition to passive diffusion, some substances, such as amino acids and glucose, are actively transported by specific transporters, a process that requires energy expenditure. An important feature of the intestinal epithelium is that some substances that enter the cytoplasm of mucosal cells can be transported back to the intestinal lumen (efflux). This efflux process is mainly a function of a transporter in the plasma membrane called P-glycoprotein (P-gp). P-gp substrates, such as vinblastine, enter the intestinal mucosal cells via passive diffusion, and a portion of the substrate is transported out of the cell and into the intestinal lumen by P-gp. Inhibition of P-gp with an inhibitor, such as verapamil, would, therefore, increase the intestinal absorption of P-gp substrates. These different mechanisms of drug transport are depicted in Fig. 1.

The existence of multiple drug transport pathways is one of the reasons that intestinal drug permeability cannot be accurately estimated based solely on physicochemical factors (e.g. pK_a, lipophilicity and solubility). For the screening of many compounds, an *in vitro* model of the intestinal mucosa is needed. To date, the most widely accepted human cell-based model for intestinal permeability is the Caco-2 cell system[6].

The Caco-2 screening assay for intestinal absorption

A wealth of data has been collected on the application of Caco-2 cells as a model to evaluate the intestinal absorption of drugs[7]. These cells, although derived from a human colon adenocarcinoma, possess many of the functional and morphological characteristics of normal, differentiated enterocytes when cultured as confluent cells. Caco-2 cells are routinely cultured in a cell-culture plate (transwell), which is specifically designed for drug uptake studies (Fig. 2). A transwell plate comprises an inner well, which is placed in a larger outer well. The bottom of the inner well consists of a semi-permeable membrane, upon which Caco-2 cells are grown to confluence.

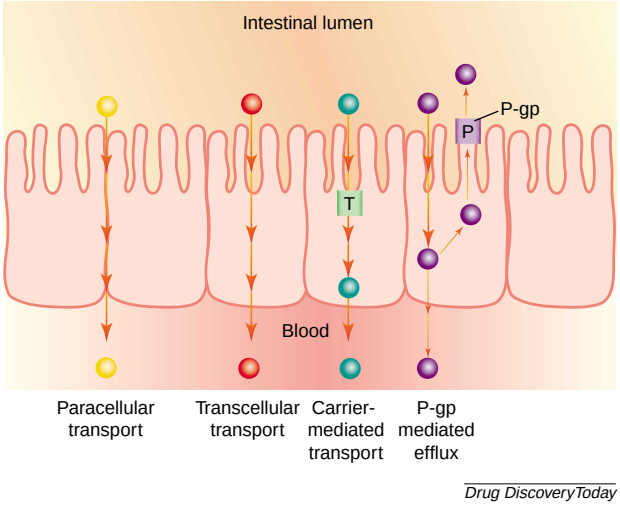

Figure 1. The multiple mechanisms of transport through the intestinal epithelium. After oral administration, a drug can be absorbed into the systemic circulation via passive diffusion through the cells (transcellular transport), between cells via cell–cell junctions (paracellular transport) or via a transporter (active transport). P-glycoprotein (P-gp) is a transporter found associated with the plasma membrane of intestinal mucosal epithelium that actively pumps some drugs back into the intestinal lumen after they are absorbed into the cells. Reproduced, with permission, from Clark Design, Baltimore, MD, USA

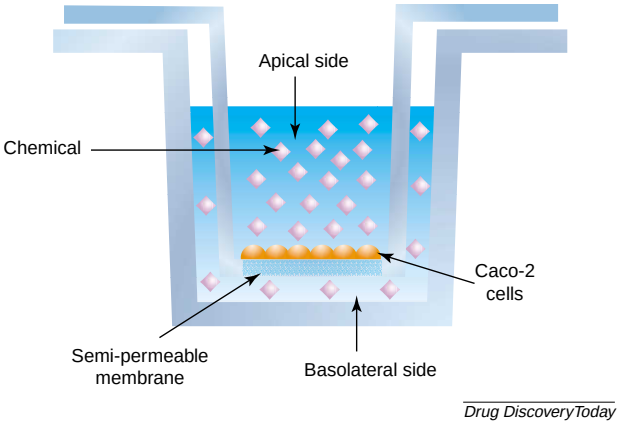

Figure 2. The Caco-2 permeability assay. Caco-2 cells are cultured as confluent monolayers on the porous membrane of an inner well situated within an outer well. Transport studies are performed by placing the compounds to be studied in the inner well (apical side) and monitoring the amount of the test compound in the outer well (basolateral side). For this assay to be useful, the Caco-2 cell barrier needs to have tight cell–cell junctions. This is usually achieved by culturing the cells for >21 days. However, it has since been demonstrated that tight cell–cell junctions and the expression of P-glycoprotein can be achieved after only three days in culture. Reproduced, with permission, from Clark Design, Baltimore, MD, USA

To evaluate transport, a drug is added to the medium above the Caco-2 cells (lumenal or apical compartment). Uptake of the drug is then monitored by quantifying the amount of the drug in the medium on the opposite side of the membrane (basolateral compartment). Data are expressed as apparent permeability coefficients (P*app*, cm sec^{-1}), which are calculated by using the following equation (Eqn 1):

$$P_{app}\ (\text{cm sec}^{-1}) = \frac{\text{amount transported}}{\text{area} \times \text{initial concentration} \times \text{time}} \quad [1]$$

The Caco-2 system can be used to evaluate the uptake of drugs across the cytoplasm (transcellular uptake), between cells (paracellular uptake), and by transporter-mediated active uptake. The role of P-gp in the uptake of a particular drug can also be evaluated by using a specific P-gp inhibitor, such as verapamil. The P*app* of a P-gp substrate would be enhanced by the inhibition of its efflux from the Caco-2 cells by such an inhibitor.

Several procedures have been established to evaluate whether particular Caco-2 cell cultures have the appropriate properties to study active transport, including the tightness of the cell junctions and the expression of P-gp. The tightness of the cell junction is determined by measuring transepithelial electrical resistance (TEER), and by measuring P*app* for a chemical with low permeability, such as mannitol. The expression of P-gp is evaluated by measuring the uptake of a P-gp substrate, usually vinblastine, in the presence or absence of a P-gp inhibitor, usually verapamil.

A major application of the Caco-2 permeability assay is the screening of chemical libraries to identify structures that can be readily absorbed via the intestinal epithelium. This application requires many Caco-2 cell plates with appropriate properties. This routinely involves culturing the Caco-2 cells for at least three weeks (21-day culture) for the cells to acquire the differentiated properties of intestinal mucosal cells. The application of Caco-2 cells in HTS has been greatly enhanced by the use of the three-day Caco-2 culture system[8]. Using a proprietary procedure, it is possible to culture Caco-2 cells for three days and still acquire the desirable properties of intestinal epithelium, which are almost identical to those of conventional 21-day cultures. Using this procedure, the Caco-2 cell cultures routinely display acceptable TEER values, mannitol P*app* values and P-gp activity[8].

Screening for Caco-2 permeability is performed using Caco-2 cells cultured in 24-well transwells. This study measures the uptake of compounds from the lumen (inside the transwell) to the blood (outside the transwell). A typical protocol involves the addition of 50 μM of the test compound to the medium in the inner well with an incubation period of 1 h to allow transport, followed by harvesting of the cells and quantification of total uptake, usually by LC–MS.

Drug metabolism

A drug that is absorbed orally is transported via the portal circulation to the liver, where it is subjected to hepatic metabolism followed by elimination as bile or via the kidneys. A typical drug metabolism pathway is the oxidation of the parent drug (phase I oxidation), followed by conjugation of the oxidized moiety with highly polar molecules, such as glucose, sulfate, methionine, cysteine or glutathione (phase II conjugation). The key enzymes for phase I oxidation are the isoforms of the cytochrome P450 (CYP) family. The major human CYP isoforms involved in drug metabolism are CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4[9] and, of these, CYP3A4 is responsible for the majority of xenobiotic metabolism: almost 50% of known pharmaceuticals. The key phase II enzymes include UDP-dependent glucuronosyl transferase (UGT), phenol sulfotranferase (PST), estrogen sulfotransferase (EST), and glutathione-*S*-transferase (GST). Like CYP, the phase II enzymes also exist as multiple isoforms.

Drug metabolism is a key determinant of several important drug properties:

- Metabolic stability: a drug that is rapidly metabolized, that is, a drug with low metabolic stability, will require multiple daily dosing or continuous infusion to maintain an adequate therapeutic plasma level. Likewise, a highly stable drug, that is, a drug that is not readily metabolized and eliminated, could have a prolonged half-life, which might influence its safety.
- Drug–drug interactions: a major cause of drug–drug interactions is the interference of the metabolism of one drug by a co-administered drug.
- Drug toxicity: a drug might be rendered non-toxic (i.e. detoxification) or more toxic (i.e. metabolic activation) by metabolism.

It is widely believed that our inability to accurately predict the properties of a drug in humans using data obtained from laboratory animals is, at least in part, a result of species differences in drug metabolism.

Liver microsomes and hepatocytes as experimental models

Because the liver is the major organ for drug metabolism, increased throughput screening assays have been developed to determine the metabolic stability of drugs. Two experimental systems – liver microsomes and intact hepatocytes – are currently widely used (Fig. 3).

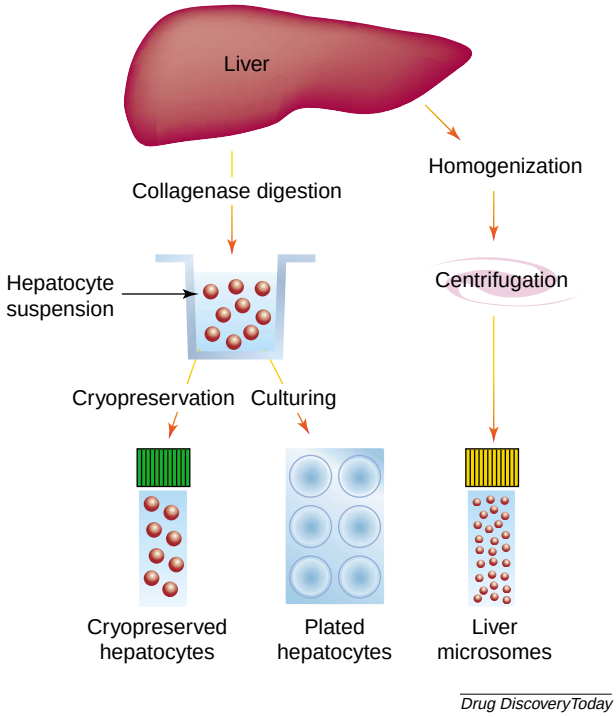

Figure 3. Preparation of liver microsomes and hepatocytes. Liver microsomes can be prepared from previously frozen tissues, whereas hepatocytes can only be prepared from a freshly isolated liver. Hepatocytes can either be plated onto collagen-coated plates as primary cultures or used in suspension for studies of metabolism, cytotoxicity and drug–drug interaction. However, cultured hepatocytes are required for use in enzyme induction studies. Hepatocytes can be cryopreserved and stored in liquid nitrogen for later use, which greatly enhances the utility of this experimental system, particularly where human hepatocytes are concerned.
Reproduced, with permission, from Clark Design, Baltimore, MD, USA

Liver microsomes are prepared by the homogenization of a liver, followed by centrifugation of the homogenate at 9,000–10,000 × g to yield a supernatant fraction (known as S9 or S10). The 'microsomal pellet' is prepared via centrifugation of the S9 or S10 fraction at 100,000 × g and contains the smooth endoplasmic reticulum where the enzymes responsible for phase I oxidation, including the cytochrome P450 monooxygenases, reside. With the exception of UGT, most phase II enzymes are cytosolic and are, therefore, absent from the liver microsomes. In general, liver microsomes are used to evaluate phase I oxidation by supplementing the microsomes with the cofactor NADPH (usually as an NADPH-regenerating system consisting of glucose-6-phosphate, $NADP^+$ and glucose-6-phosphate dehydrogenase). UGT can be specifically studied by supplementing liver microsomes with the cofactor for glucuronidation, uridine diphosphate glucuronic acid (UDPGA).

Intact hepatocytes are isolated from livers by a procedure called two-step collagenase digestion. A freshly isolated liver is perfused: first with an isotonic buffer solution containing a calcium chelating agent to clear the blood and to loosen cell–cell junctions (as a result of calcium removal), followed by a collagenase solution to dissociate the hepatocytes from the liver parenchyma[10]. This procedure has been successful in the preparation of hepatocytes from multiple species, including man. One rat liver typically yields approximately 1×10^9 hepatocytes, and one human liver, depending on the size of the fragment used for isolation, yields as many as 50×10^9 hepatocytes. The isolated hepatocytes can be used for metabolism and cytotoxicity studies, either as a cell suspension or as primary cell cultures. For convenient use of the isolated hepatocytes, cryopreservation procedures have been developed[11]. Cryopreserved hepatocytes retain both phase I and phase II drug metabolizing enzyme (DME) activities[12] and, human hepatocytes in particular, represent an experimental system that can be used routinely for HTS of human metabolic stability[12].

Screening for metabolic stability

Metabolic stability screening using microsomes or hepatocytes (Fig. 4) involves the incubation of the drug candidate in a 96-well plate, followed by quantification by LC–MS of the amount of the parent compound that remains after metabolism. A typical study would evaluate a series of structurally related compounds, with the most stable compound being that which is least metabolized.

The metabolic stability assay is routinely performed using liver microsomes and NADPH, as mentioned previously, and can be automated[13]. The use of microsomes allows evaluation of the readiness of drugs to undergo oxidative metabolism. However, it is now known that for some drugs, phase II metabolism or metabolism involving non-microsomal enzymes is more important than phase I oxidation. The use of microsomes, therefore, could create a bias towards phase I oxidation, which might not be the key pathway *in vivo*, and could lead to erroneous conclusions on metabolic stability. A further complication is the recent observation that some drugs can bind to the non-enzymatic proportion of microsomal proteins under *in vitro* experimental conditions. This non-specific 'ubiquitous' binding can lead to an inverse relationship between metabolic rate and microsomal protein concentration[14] and thus, for these drugs, it would be difficult to obtain physiologically relevant data using microsomes.

Cryopreserved hepatocytes represent the ideal system for the screening of metabolic stability[12]. Intact hepatocytes contain all hepatic DMEs, both microsomal and

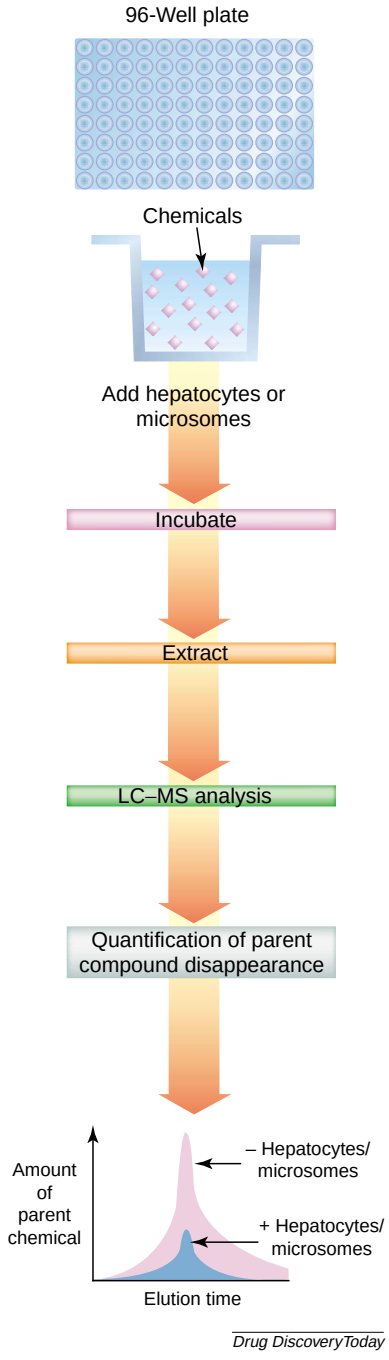

Figure 4. Metabolic stability screening assay. This assay can be performed using 96-well plates with a porous membrane at the bottom. Test compounds are mixed with either hepatocytes or liver microsomes and incubated for a period of time. After incubation, an organic solvent, such as acetonitrile, is added to stop the reaction and to extract any non-covalently bound compounds from macromolecules. The plates are then centrifuged so that the liquid fraction can be filtered into a second 96-well recipient plate, and the concentration of test compound is determined using, for example, LC–MS. Reproduced, with permission, from Clark Design, Baltimore, MD, USA

cytosolic, as well as all cofactors, including those required for phase I oxidation and phase II conjugation. Further, these components are present in isolated intact hepatocytes at levels comparable with those under physiological conditions. Correspondingly, the determination of metabolic stability using intact hepatocytes would be physiologically relevant because in hepatocytes, the drugs are subjected to metabolism by all relevant pathways that exist in the liver *in vivo*. When screening novel compounds with unknown biotransformation pathways, the use of intact hepatocytes should, therefore, provide physiologically relevant data on metabolic stability.

Screening of metabolic stability with liver microsomes or human hepatocytes can be performed in 96-well plates to enhance throughput[12]. Typically, the wells are preloaded with test compounds in an isotonic buffer (e.g. Krebs-Henseleit buffer). Liver microsomes and enzyme cofactors or intact hepatocytes are added to the wells to initiate the study. After the incubation period (0.5–1 h for microsomes and 2–4 h for hepatocytes), an equal volume of an organic solvent (e.g. acetonitrile) is added to stop the reaction and extract the test compounds from the assay. The plates are then centrifuged to filter the reaction mixture through the porous membrane into a new 96-well recipient plate, where the samples are analyzed by LC–MS (Fig. 4).

The results of metabolic stability are generally expressed as percentage of parent compound disappearance, represented by Eqn 2:

$$\%\ \text{parent compound disappearance} = \left\{1 - \left[\frac{\text{parent compound concentration (after incubation)}}{\text{parent compound concentration (before incubation)}}\right]\right\} \times 100 \quad [2]$$

Cassette-dosing (dosing of one well with several compounds) is an approach used to enhance the throughput of metabolic screening, because LC–MS enables the analysis of the disappearance of individual compounds in a mixture. However, cassette-dosing can lead to erroneous results because of chemical–chemical interactions. Single-compound dosing followed by the pooling of samples for cassette analysis is, therefore, a preferred approach.

Drug–drug interactions

It is now well known that a drug can affect the metabolic stability of another drug, a concept known as 'pharmacokinetic drug–drug interactions'[15]. Cases of this are plentiful, for example, the antifungal ketoconazole, a potent inhibitor of CYP3A4, causes drug–drug interactions with drugs that are substrates of CYP3A4. One high profile case of this is the interaction between ketoconazole and

terfenadine, as mentioned previously. Co-administration of ketoconazole and terfenadine leads to increased plasma levels of terfenadine, resulting in cardiotoxicity. The interaction between terfenadine and ketoconazole is known as an inhibitory drug–drug interaction, where one drug (ketoconazole) inhibits the metabolism of another drug (terfenadine), leading to a higher than intended plasma level. The major clinical consequence of inhibitory drug–drug interactions is undesired drug toxicity[16].

Another mechanism of drug interaction is 'inductive' drug–drug interactions. In this case, a drug affects the metabolic stability of another drug via the induction of DME pathways. An example of this is the macrolide antibiotic, rifampicin, which is one of the most potent inducers of DMEs in humans. It has been mainly characterized as an inducer of CYP3A4, although it is also known to induce CYP2A6, CYP2C9, and most recently, the phase II sulfation pathway. An inducing drug would lead to more rapid metabolism of co-administered drugs that are substrates of the induced DMEs. The major clinical consequence of this induction is therapeutic failure, because of the unintentional lowering of the plasma level of the affected drug to non-therapeutic levels[15]. Rifampicin co-administration, for instance, has been reported to lower plasma levels of cyclosporin, erythromycin and oral contraceptives to non-therapeutic levels. Because the mechanisms of pharmacokinetic drug–drug interactions are relatively well-defined, the following screening assays have been developed to help identify structures that might have drug–drug interaction potential.

Screening for inhibitory drug–drug interactions

HTS for P450 inhibition

Using genetically engineered microsomes (cDNA-expressed microsomes) that contain only one specific human P450 isoform, an extremely high-throughput screening assay has been developed[16]. Test compounds are incubated with microsomes in the presence of P450 substrates that enable metabolism to be quantified using fluorescence.

Liver microsome P450 inhibition

Although genetically engineered microsomes enable HTS for P450 inhibition, the generated data might need confirmation using human liver microsomes that contain all of the P450 isoforms in a human liver. A drug could be metabolized by one isoform to metabolites that might be more or less effective at inhibiting another P450 isoform. In this assay, test compounds are incubated with liver microsomes in the presence of various isoform-specific substrates[17]. The substrates commonly used are: phenacetin (CYP1A2), coumarin (CYP2A6), tolbutamide (CYP2C9), *S*-mephenytoin (CYP2C19), dextromethorphan (CYP2D6), chlorzoxazone (CYP2E1) and testosterone (CYP3A4). The formation of metabolites representing the activity of specific isoforms is then quantified using HPLC or LC–MS.

Human hepatocyte P450 inhibition

Cryopreserved human hepatocytes pooled from multiple donors are used in this assay. The use of intact hepatocytes allows the compounds to be evaluated under the most physiologically relevant conditions: in the presence of all hepatic DMEs and cofactors[12]. Another advantage of the use of intact hepatocytes is that the assay allows the partitioning of test compounds between the extracellular matrix and the hepatocytes, so that the inhibitory potential of a drug based on the expected plasma drug concentration can be predicted. The assay is typically performed using 96-well plates that are first preloaded with test compounds, followed by the addition of hepatocytes and P450 substrates (Fig. 5a).

The data for inhibitory drug–drug interactions are usually expressed as IC_{50} values; however, the inhibitory coefficient K_i can also be derived by performing experiments with multiple substrate and inhibitor concentrations. In general, for screening purposes, one concentration (e.g. 10 μM) of the test compound is used and results are expressed as percentage inhibition (Eqn 3):

$$\%\ \text{inhibition} = \left\{1 - \left[\frac{\text{activity (inhibitor)}}{\text{activity (solvent control)}}\right]\right\} \times 100 \quad [3]$$

A common approach to the screening for potential inhibitory drug–drug interactions during drug discovery is to use cDNA-expressed microsomes for high-throughput initial screening and liver microsomes or intact hepatocytes for confirmation. Intact hepatocytes are the most appropriate model for the prediction of drug–drug interaction potential based on drug concentrations in plasma and, therefore, should be used for final drug candidate selection or for the definitive evaluation of the drug–drug interaction potential of drug candidates.

Screening for inductive drug–drug interactions

HTS for CYP3A4 induction

Recently, the mechanism of CYP3A4 induction has been defined[18]: compounds that induce CYP3A4 activate the pregnane-X-receptor (PXR), which binds to a response element in the *CYP3A4* gene called the pregnane-X-receptor response element (PXRE)[18]. A high-throughput induction assay for CYP3A4 has been developed using a genetically engineered cell line that expresses a PXRE–luciferase reporter gene[19]. Induction of CYP3A4, by the xenobiotic-mediated binding of PXR to PXRE, leads to the activation

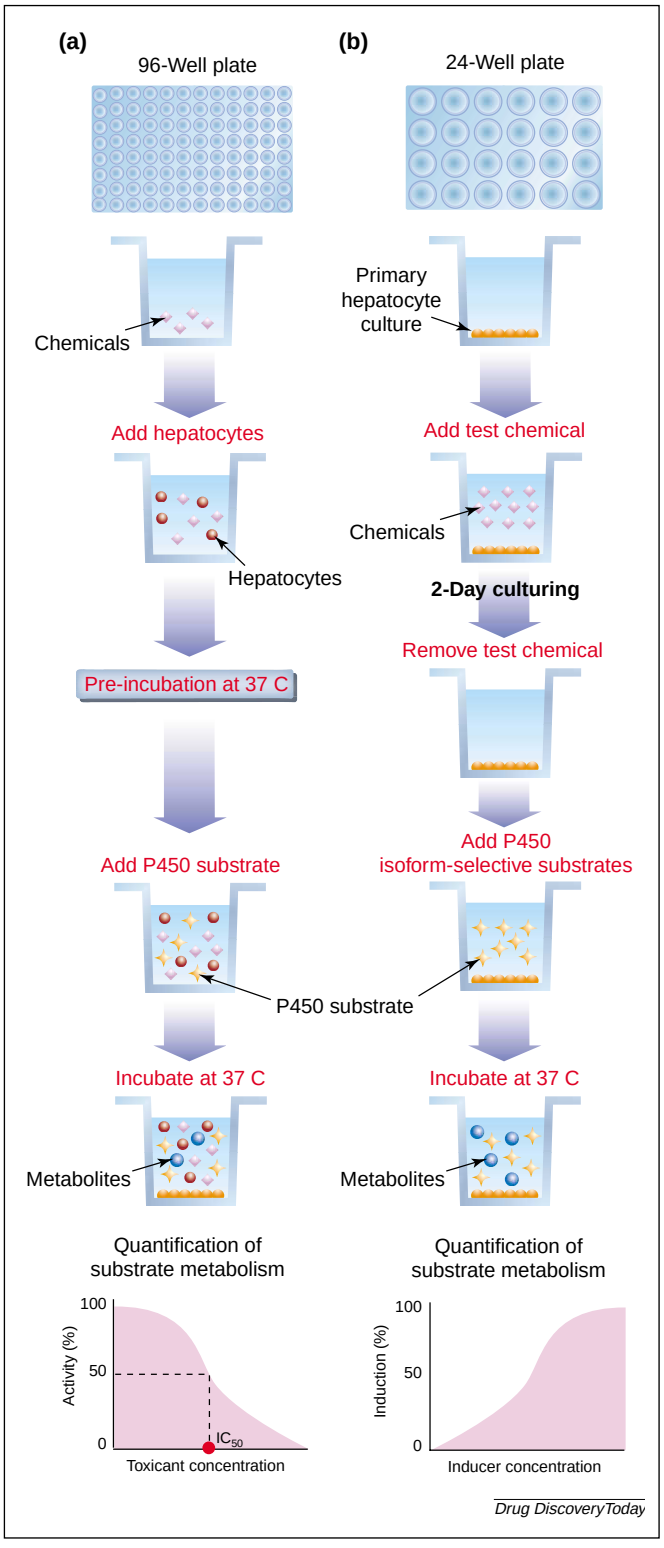

Figure 5. (a) P450 inhibition assay. Test compounds are preincubated with either liver microsomes or hepatocytes followed by the addition of P450 substrates. After further incubation, the rate of metabolism is determined by quantification of specific metabolites. P450 inhibition is indicated by a dose-dependent decrease in activity. For screening assays, a single concentration of the test compound is used, but for more extensive studies of inhibitory potential, multiple concentrations of test compounds can be used for the determination of IC_{50} or K_i values. **(b)** P450 induction assay. This assay uses cultured hepatocytes that are treated with the test compounds for >2 days. Isoform-specific P450 substrates are then added to the cells to quantify the activities of specific P450 isoforms. Induction is indicated by an increase in activity in treated cells compared with untreated control cells. This is a robust assay: to date, all known inducers of human P450 isoforms induce the relevant enzymes in cultured human hepatocytes.
Reproduced, with permission, from Clark Design, Baltimore, MD, USA

of luciferase synthesis, which can be quantified using a chemiluminescent substrate, luciferin.

Human hepatocyte P450 induction

The inductive effect of a xenobiotic on all P450 isoforms can be evaluated by incubation of the compound with primary human hepatocytes, followed by quantification of different P450 isoform activities by incubating the treated hepatocytes with isoform-specific substrates (Fig. 5b)[20]. In some laboratories, P450 activities are determined in microsomes prepared from the treated hepatocytes. Western blotting of P450-isoform proteins, as well as northern blotting for *CYP*-isoform mRNA, are also used for the evaluation of P450 induction.

Results from such assays are typically expressed as percentage induction (Eqn 4):

$$\%\ \text{induction} = \left[\frac{\text{activity (treatment)} - \text{activity (solvent control)}}{\text{activity (solvent control)}}\right] \times 100 \quad [4]$$

Although the current emphasis on inductive drug–drug interactions is on P450 induction, it is now known that phase II conjugation can also be induced. The most notable example is the loss of efficacy of oral contraceptives in patients co-administered with rifampicin, which has been found to involve not only CYP3A4 induction, but possibly the induction of phase II sulfation enzymes[21]. The use of intact human hepatocytes allows the evaluation of all possible DMEs.

Toxicology

Drug toxicity is a crucially important drug property. A desirable drug would have a high therapeutic index, that is, the plasma level required to exert a toxic effect would be significantly higher than that required for therapeutic efficacy. The potential for toxicity remains the most unpredictable property of a drug. Human toxicity continues to occur in clinical trials of drug candidates that are apparently found to be safe during preclinical trials, leading to restricted use and, in some cases, market withdrawal.

Toxicity is arguably the most difficult drug property to adequately screen, because it could be species-specific,

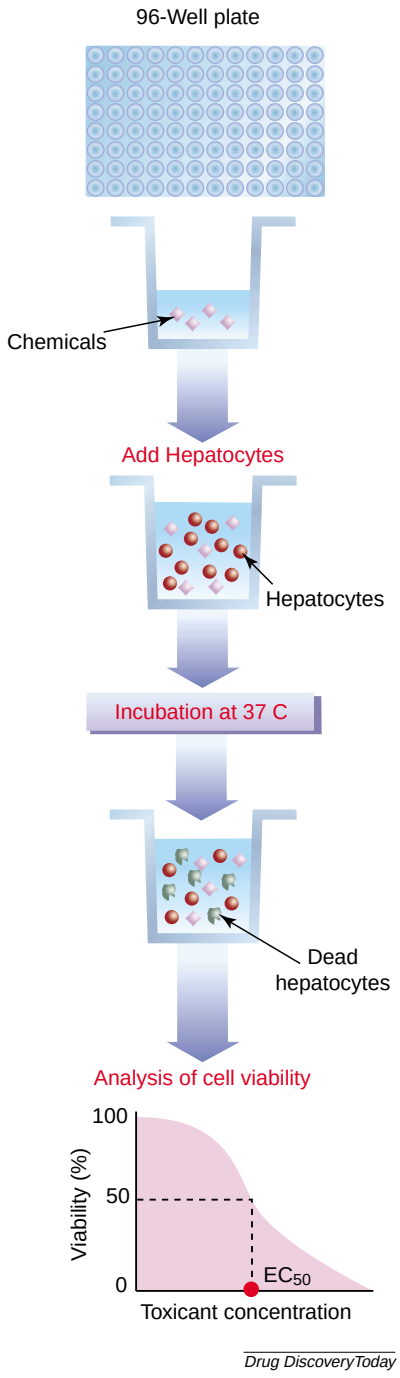

Figure 6. Screening assay for hepatotoxicity. Hepatocytes are treated with the test compounds and cell viability is determined using various endpoints. The endpoints commonly used include: quantification of ATP content, release of cytoplasmic enzymes, mitochondrial functions, dye uptake, macromolecular synthesis and cellular glutathione content. For screening assays, a single concentration of test compound is usually used; however, cytotoxicity can be further studied using multiple concentrations, enabling the determination of dose–response curves and EC_{50} values.
Reproduced, with permission, from Clark Design, Baltimore, MD, USA

organ-specific, and could involve multiple host factors and chronic dosing regimens, all of which cannot be adequately modeled experimentally. However, as hepatotoxicity is a major manifestation of drug toxicity, and it is known that toxicity can be influenced by drug metabolism, screening for toxicity using intact hepatocytes (Fig. 6)[22] is an approach being adopted in many drug discovery and development laboratories.

Commonly used toxicity screening assays

ATP measurement

ATP measurement can be an extremely high-throughput methodology. In general, this assay involves treating viable cells in 96-well or 384-well plates with the test compound at various concentrations (for dose–response analysis) or at single concentrations (e.g. 50 μM) for screening. After the treatment period, the cells are lysed and the ATP content per well is quantified based on chemiluminescence using a luciferin-luciferase assay. This study can be performed with all cell types, including cryopreserved human hepatocytes[23,24].

MTT assay

This assay measures the metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a yellow tetrazolium salt, which, when reduced by the mitochondria of metabolically active cells, forms a blue formazan dye precipitate that can be extracted using organic solvents. The blue extracted fraction can then be colorimetrically quantified at 550 nm. This assay can be applied to all cell types[24], including cryopreserved human hepatocytes[12], using 96-well or larger culture plates. However, the assay measures cell viability based on mitochondrial activity, and so testing of compounds that selectively affect mitochondria might result in an overestimation of toxicity.

Enzyme release

Release of cytoplasmic enzymes[22,24], such as lactate dehydrogenase (LDH), and the liver-specific cytosolic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are commonly used endpoints for the evaluation of cytotoxicity. Reagent kits are available for the colorimetric quantification of enzyme activities. Cells are treated with the test compounds and then the enzyme activities in the medium or in the remaining cells are evaluated. This assay, therefore, measures plasma membrane integrity, and can be used with all cell types. In general, enzyme release assays have a relatively high level of background activity (particularly when using cryopreserved hepatocytes) and are less sensitive than MTT and ATP assays.

Neutral red uptake

Neutral red is a dye that is preferentially absorbed into the lysosomes of viable cells[24]. In this assay, after compound treatment, the cells are incubated with neutral red to allow uptake. The cells are then destained and the red color quantified at 540 nm using a microplate reader. This assay measures viability as lysosomal activity, and so results might be inaccurate if the chemicals tested have differential effects on lysosomes. This assay can also be used with any cell type.

Macromolecular synthesis

In this assay, the incorporation of DNA, RNA and protein precursors into their respective macromolecules is quantified[22,24]. This typically involves the incorporation of radiolabeled precursors, for example, ^{3}H- or ^{14}C-labeled thymidine, uridine or leucine as DNA, RNA, and protein precursors, respectively. The radioactivity of the macromolecules, usually determined as trichloroacetic-acid-precipitable materials, is then quantified. This assay can be used for all dividing cells. For non-dividing cells such as hepatocytes, RNA and protein synthesis can be used as endpoints. Macromolecular synthesis is a sensitive technique for the measurement of cell viability; however, a major drawback is the need for the use of radiolabeled isotopes.

Glutathione measurement

Glutathione (GSH) scavenges electrophilic moieties, and most toxic metabolites are highly reactive electrophilic molecules that readily react with, and thereby deplete, cellular GSH content. GSH quantification can be readily performed via fluorescence spectrophotometry using *O*-phthaldehyde (OPT) as a reagent. GSH reacts with OPT, yielding a fluorescent product that can be activated at 350 nm with an emission peak at 420 nm[25]. The measurement of GSH depletion is usually performed with primary hepatocytes[24], and is considered a 'specialized' cytotoxicity assay that is useful for determining the formation of reactive metabolites.

Cytotoxicity data are generally expressed as percentage relative viability (Eqn 5):

$$\%\ \text{relative viability} = \frac{\text{viability (treatment)}}{\text{viability (vehicle control)}} \times 100 \qquad [5]$$

Application of toxicity data

Cytotoxicity assays are used routinely by toxicologists for the evaluation of toxic mechanisms, and have recently gained acceptance by discovery scientists to aid the selection and design of chemical structures for further development. In general, cytotoxicity data already exist when pharmacological screening using intact cells is performed. However, most pharmacological assays are performed using tumor cell lines, which contain little drug-metabolizing capacity. Data obtained using these cells reflect mainly the 'intrinsic toxicity' of the parent compounds, but yield little information about either the attenuation of parent toxicity by metabolic detoxification, or the metabolic activation of the parent compound as a result of the formation of toxic metabolites. Toxicity screening with intact human hepatocytes, however, allows the evaluation of these phenomena and, therefore, should yield data that would reflect human hepatotoxicity *in vivo* more accurately.

Conclusions

ADME/Tox properties are important parameters for the selection of drug candidates for development. Drug candidate selection involving both pharmacological properties and ADME/Tox screening should lead to an enhanced probability of clinical success. Screening tools for intestinal absorption, metabolic stability, drug–drug interactions and toxicity have been developed using human-based *in vitro* systems. Such tools can be applied to screen compounds during drug discovery, to aid medicinal chemists in structural design for drug optimization, and to mechanistically define drug–drug interaction potential and toxicity.

Future directions should include high content assays (assays yielding extensive information, such as expression genomics and proteomics) as well as the development of extensive databases correlating chemical structure and ADME/Tox drug properties. The high content assays could lead to the discovery of endpoints useful for the accurate prediction of drug toxicity. This is particularly important for toxicity that is difficult to detect, such as idiosyncratic drug toxicity that occurs at such low frequency that it escapes detection during clinical trials.

The highest-throughput screening methodologies will be virtual screening: prediction of drug properties based on chemical structure. This will only be possible with access to highly reproducible data obtained under well-defined experimental conditions. One goal of ADME/Tox screening should be the development of a database correlating chemical structures and biological endpoints.

Although present approaches will continue to be refined and new directions will be developed, experimental systems already exist for extensively defining the human drug properties of drug candidates, allowing the selection of drug candidates with acceptable pharmacological properties, intestinal permeability, metabolic stability, drug–drug interaction potential and toxicological potential. This will inevitably save time and costs in drug discovery and development by enhancing the probability of the success of drug candidates in clinical trials.

References

1 Kennedy, T. (1997) Managing the discovery/development interface. *Drug Discov. Today* 2, 436–444
2 DiMasi, J.A. (1995) Success rates for new drugs entering clinical testing in the United States. *Clin. Pharmacol. Ther.* 58, 1–14
3 Watabe, T. (1996) Strategic proposals for predicting drug–drug interactions during new drug development: based on sixteen deaths caused by interactions of the new antiviral sorivudine with 5-fluorouracil prodrugs. *J. Toxicol. Sci.* 21, 299–300
4 FDA (1992) Warnings based on non-sedating antihistamines terfenadine and astemizole. *J. Am. Med. Assoc.* 268, 705
5 Borchardt, R.T. *et al.* (1991) Pharmaceutical applications of cell culture: an overview. In *Pharmaceutical Applications of Cell and Tissue Culture to Drug Transport* (Wilson, G. *et al.*, eds), pp. 1–14, Plenum Press
6 Hildago, I.J. *et al.* (1989) Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* 96, 736–749
7 Yamashita, S. *et al.* (2000) Optimized conditions for prediction of intestinal drug permeability using Caco-2 cells. *Eur. J. Pharm. Sci.* 10, 195–204
8 Li, A.P.S. *et al.* (2001) Caco-2 Cell Screening Assay for Intestinal Absorption. In *High Throughput Screening: Supplement to Biomedical Products*. (January), pp. 6–9, Cahners
9 Wrighton, S.A. and Stevens, J.C. (1992) The human hepatic cytochromes P450 involved in drug metabolism. *Crit. Rev. Toxicol.* 22, 1–21
10 Li, A.P. *et al.* (1992) Isolation and culturing of hepatocytes from human liver. *J. Tissue Cult. Methods* 14, 139–146
11 Loretz, L.J. *et al.* (1989) Optimization of cryopreservation procedures for rat and human hepatocytes. *Xenobiotica* 19, 489–498
12 Li, A.P. *et al.* (1999) Cryopreserved human hepatocytes: characterization of DME activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug–drug interaction potential. *Chem. Biol. Interact.* 121, 17–35
13 Linget, J.M. and du Vignaud, P. (1999) Automation of metabolic stability studies in microsomes, cytosol and plasma using a 215 Gilson liquid handler. *J. Pharm. Biomed. Anal.* 19, 893–901
14 Obach, R.S. (1999) Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: an examination of *in vitro* half-life approach and non-specific binding to microsomes. *Drug Metab. Dispos.* 27, 1350–1359
15 Li, A.P., ed. (1997) *Advances in Pharmacology: Pharmacokinetic Drug–Drug Interactions* (Vol. 43), Academic Press
16 Miller, V.P. *et al.* (2000) Fluorometric high-throughput screening for inhibitors of cytochrome P450. *Ann. New York Acad. Sci.* 919, 26–32
17 Rodrigues, A.D. and Wong, S.L. (1997) Application of human liver microsomes in metabolism-based drug–drug interactions: *in vitro–in vivo* correlations and the Abbott Laboratories experience. In *Advances in Pharmacology: Pharmacokinetic Drug–Drug Interactions* (Vol. 43) (Li, A.P., ed.), pp. 65–101, Academic Press
18 Lehmann, J.M. *et al.* (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J. Clin. Invest.* 102, 1016–1023
19 Moore, J.T. and Kliewer, S.A. (2000) Use of the nuclear receptor PXR to predict drug interactions. *Toxicology* 153, 1–10
20 Li, A.P. (1997) Primary hepatocyte cultures as an *in vitro* experimental model for the evaluation of pharmacokinetic drug–drug interactions. In *Advances in Pharmacology: Pharmacokinetic Drug–Drug Interactions* (Vol. 43) (Li, A.P., ed.), pp.103–130, Academic Press
21 Li, A.P. *et al.* (1999) Effects of cytochrome P450 inducers on 17-alpha-ethinylestradiol (EE2) conjugation by primary human heptocytes. *Br. J. Clin. Pharmacol.* 48, 733–742
22 Li, A.P. (1994) Primary hepatocytes as an *in vitro* toxicological system. In In Vitro *Toxicology* (Gad, S., ed.), pp. 195–220, Raven Press
23 Lu, C. *et al.* (2000) A high-throughput screening assay for hepatotoxicity using cryopreserved animal and human hepatocytes. *The Toxicologist* 54, 206
24 Waterfield, C.J. *et al.* (1998) Ethionine toxicity *in vitro*: the correlation of data from rat hepatocyte suspensions and monolayers with *in vivo* observations. *Arch. Toxicol.* 72, 588–596
25 Hissin, P.J. and Hill, R. (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* 74, 214–226

Contributions to *Drug Discovery Today*

Drug Discovery Today publishes topical information on all aspects of drug discovery – molecular targets, lead identification, lead optimization and associated technologies, drug delivery, gene therapy, vaccine development and clinical trials – together with overviews of the current status of compound classes and approaches in specific therapeutic areas or disease states. Areas of pharmaceutical development that relate to the potential and viability of drug candidates are also included, as are those relating to the strategic, organizational and logistic issues underlying pharmaceutical R&D.

Authors should aim for topicality rather than comprehensive coverage. Ultimately, articles should improve the reader's understanding of the field addressed and should therefore assist in the increasingly important decision-making processes for which drug discovery and development scientists are responsible.

Please note that publication of Review articles is subject to satisfactory expert peer and editorial review. The publication of Update and Editorial articles is subject to satisfactory editorial review. In addition, personal perspectives published in *Drug Discovery Today* do not represent the view of the journal or its editorial staff.

If you would like to contribute to the Reviews, Monitor or Editorial sections of *Drug Discovery Today* in the future, please submit your proposals to: Dr Debbie Tranter, Editor (e-mail: deborah.tranter@current-trends.com). If you would like to contribute to the Update section, please submit your proposals to: Dr Rebecca Lawrence, News & Features Editor (e-mail: rebecca.lawrence@current-trends.com).