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Review

An update on in vitro test methods in human hepatic drug biotransformation research: pros and cons

Esther F.A. Brandon,[a,*] Christiaan D. Raap,[a] Irma Meijerman,[a] Jos H. Beijnen,[a,b] and Jan H.M. Schellens[a,b]

[a] *Division of Drug Toxicology, Department of Biomedical Analysis, Faculty of Pharmaceutical Sciences, Utrecht University, The Netherlands*
[b] *The Netherlands Cancer Institute, Amsterdam, The Netherlands*

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Abstract

The liver is the predominant organ in which biotransformation of foreign compounds takes place, although other organs may also be involved in drug biotransformation. Ideally, an in vitro model for drug biotransformation should accurately resemble biotransformation in vivo in the liver. Several in vitro human liver models have been developed in the past few decades, including supersomes, microsomes, cytosol, S9 fraction, cell lines, transgenic cell lines, primary hepatocytes, liver slices, and perfused liver. A general advantage of these models is a reduced complexity of the study system. On the other hand, there are several more or less serious specific drawbacks for each model, which prevents their widespread use and acceptance by the regulatory authorities as an alternative for in vivo screening. This review describes the practical aspects of selected in vitro human liver models with comparisons between the methods.

Keywords: Microsomes; Supersomes; Human liver fractions; Cell lines; Liver slices; Biotransformation; In vitro techniques

Introduction

The development of a new therapeutic agent always involves a preclinical screening stage. During this stage the main pharmacokinetic, pharmacodynamic, and toxicological properties of the candidate drug are investigated. The preclinical investigation is based on both in vitro models and in vivo experiments in various animal species (Koster et al., 1997).

Drug biotransformation is one of the most important factors that can affect the overall therapeutic and toxic profile of a drug. It can lead to detoxification and excretion of the drug, but also to bioactivation. For this reason, drug biotransformation is a pivotal factor in the early developmental stage of new drugs. Biotransformation occurs in many tissues, with the liver as the most important organ, but also the kidneys, skin, lungs, and intestine can be involved (Lu, 1996). The liver is the largest internal organ of the human body and is strategically located between the digestive tract and the other parts of the body (Moffett et al., 1993).

Drug biotransformation is divided into two types of reactions, namely phase I (hydrolysis, oxidation, and reduction) and phase II reactions (conjugation). The biotransformation pathway of a drug is mediated by phase I, phase II, or a combination of both. The cytochrome P450 (CYP)[1] enzyme superfamily plays a dominating role in the phase I biotransformation and is mainly present in the liver (Derelanko and Hollinger, 1995; Rang et al., 1996). Many dif-

* Corresponding author. Division of Drug Toxicology, Department of Biomedical Analysis, Faculty of Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands. Fax: +31-0-30-2535180.

E-mail address: e.f.a.brandon@pharm.uu.nl (E.F.A. Brandon).

[1] Abbreviations used: Acetyl CoA, acetyl coenzyme A; CYP, cytochrome P450; DOC, dynamic organ culture; GT, glutathione; HLM, human liver microsomes; NAT, *N*-acetyl transferase; PAPS, adenosine-3′- phosphate-5′-phosphosulphate; ST, sulfotransferase; UDPGA, uridine diphosphoglucuronic acid; UGT, uridine diphosphoglucuronosyl transferase; UW, University of Wisconsin Solution.

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Table 1A
An overview of the main human CYP isoforms (CYP1-4) involved in drug biotransformation and their occurrence[a] and the model substrates used to quantify the CYP isozyme activity

Isoform	Occurrence	Model substrates	Recommendation[b]
CYP1A1	Mainly extrahepatic	7-ethoxyresorufin *O*-deethylation	
CYP1A2	Liver	Phenacetin *O*-deethylation	Preferred
		Caffeine N3-demethylation	Acceptable
CYP2A6	Liver	Coumarin C7-hydroxylation	Preferred
CYP2B1/2		Pentoxyresorufin O-dealkylation	
CYP2B6	Liver	(S)-mephenytoin N-desmethylation	Preferred
		Bupropion hydroxylation	Acceptable
CYP2C8	Liver	Paclitaxel C6-α-hydroxylation	Preferred
	Intestine		
CYP2C9	Liver	(S)-warfarin C6-, C7 hydroxylation	Preferred
	Intestine	Diclofenac 4′-hydroxylation	Acceptable
		Tolbutamide para CH_3-hydroxylation	Acceptable
CYP2C18/2C19	Liver	(S)-mephenytoin C4′-hydroxylation	Preferred
CYP2D6	Liver	Bufuralol C1′-hydroxylation	Preferred
	Intestine	Dextromorphan O-demethylation	Preferred
	Kidney	Codeine O-demethylation	Acceptable
CYP2E1	Liver	Chlorzoxazone C6-hydroxylation	Preferred
	Intestine	Lauric acid C (ω1)-hydroxylation	Acceptable
	Leukocytes		
CYP3A4	Liver	Midazolam C1′-hydroxylation	Preferred
	GI tract	Testosterone C6-β-hydroxylation	Preferred
CYP4A11	Liver	Lauric acid ω-hydroxylation	
	Kidney		

[a] Birkett et al., 1993; Schenkman and Greim, 1993; Crommentuyn et al., 1998.
[b] Recommendation according to the FDA, American Association of Pharmaceutical Sciences (AAPS), and the European Federation of Pharmaceutical Sciences (Basel conference 2000) (Tucker et al., 2001).

ferent CYP isoforms have been characterized, which are categorized in families based on their sequence. Table 1A gives an overview of the human CYP-families 1 to 4, which are the main CYPs involved in drug biotransformation (Birkett et al., 1993; Crommentuyn et al., 1998; Schenkman and Greim, 1993) and lists the various model substrates that are used to quantify specific CYP activity. Table 1B lists the various inducers and inhibitors of the different isozymes (Tucker et al., 2001).

Phase II enzymes (e.g., uridine diphosphoglucuronosyl transferase (UGT), *N*-acetyl transferase (NAT), glutathione *S*-transferase (GST), and sulfotransferase (ST)) also have an important role in the detoxification and/or excretion rate of xenobiotics (Lu, 1996). The elucidation of the entire human biotransformation pathway, both phase I and phase II biotransformation, is essential in the early development of a candidate drug.

It is becoming increasingly apparent that drug transporters (phase III) influence not only the therapeutic efficacy but also the absorption, distribution, and elimination of a drug (Zhang et al., 2003). The drug transporters are located in epithelial and endothelial cells of the liver, gastrointestinal tract, kidney, blood–brain barrier, and other organs. They are responsible for the transport of most of the commonly prescribed drugs across cellular barriers and thus for the concentration at the target or biotransformation site. Multidrug resistance proteins (MRP; *p*-glycoprotein and others) have been shown to be important in explaining the pharma-

Table 1B
An overview of commonly known inhibitors and inducers of the CYP isozyme activity[a]

Isoform	Inhibitor	Inducer
CYP1A1	α-Naphtoflavone	Polycyclic hydrocarbons
CYP1A2	Furafylline	Smoking
		3-Methylcholanthrene
		Char-grilled meat
		Rifampicine
CYP2A6	Sulfaphenazole	Pyrazole
		Barbiturates
CYP2B1/2		
CYP2B6	Sertraline	
CYP2C8	Glitazones	Rifampicine
		Barbiturates
CYP2C9	Sulfaphenazole	Rifampicine
		Phenobarbital
CYP2C18/2C19	Ticlopidine	Rifampicine
	Ketoconazole	Carbamazepine
CYP2D6	Quinidine	
	Haloperidol	
CYP2E1	Diethyl-dithiocarbamate	Ethanol
CYP3A4	Ketoconazole	Rifampicine
	Grapefruit juice	Barbiturates
CYP4A11	17-Octadecynoic acid	

[a] Birkett et al., 1993; Schenkman and Greim, 1993; Crommentuyn et al., 1998; Tucker et al., 2001.

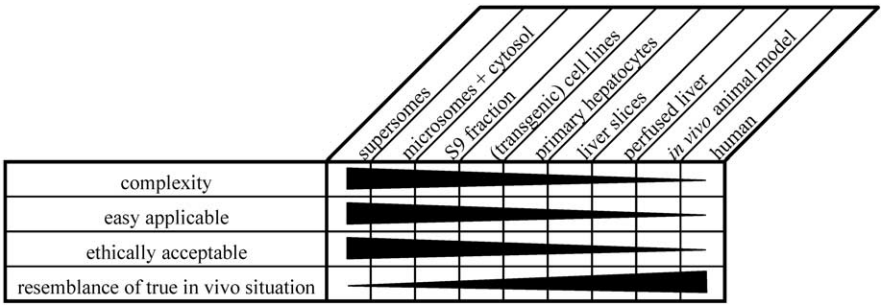

Fig. 1. In vitro and in vivo models used in the development of new drugs, ranging from human to isolated enzymes, in order of in vivo resemblance.

cokinetics of a drug in man (Fricker and Miller, 2002). Thus, the elucidation of the influence of drug transporters on the adsorption disposition metabolism elimination of a drug is essential in the early developmental stage of new drugs.

A key question in human drug biotransformation research is how to make reliable extrapolations from the in vitro or in vivo model to clinical practice. Thus, the objective is to establish a useful model system with a strong predictive power for human biotransformation. Several models have been developed in the past, ranging from (recombinant) isolated enzymes to the intact perfused liver (see Fig. 1). They are used to obtain early information about biotransformation pathways and to predict drug–drug interactions at the metabolic level (Ekins et al., 2000). The quality of the human liver used in the preparations of the different in vitro methods described is a dominant factor in the outcome of the in vitro studies, especially in precision-cut liver slices and isolated hepatocytes (Fisher et al., 2001b). Livers that are not suitable for transplantation or liver sections from biopsies are used and, in order to ensure a viable cell yield as high as possible or, in the case of cell fractions, the highest enzyme activity, the liver or liver section needs to be processed as soon as possible after the resection. The optimal model system in a given situation depends on a number of factors, such as in vivo resemblance, expense, availability of the model, and ethical considerations. In vitro data from human and animal models can be used to choose the best in vivo model (e.g., mouse, rat dog) for further testing. In conclusion, it can be stated that an in vitro model is always a compromise between convenience and relevance.

Current guidelines for human drug development allow in vitro systems to be used in supportive studies, and therefore in vitro data should be used mainly qualitatively (http://www.eudra.org/emea.html and http://www.fda.gov). For example, when in vitro data show a lack of drug–drug interaction, no in vivo experiments have to be performed, but when a drug–drug interaction is demonstrated, then in vivo experiments have to follow (http://www.fda.gov). A complete replacement of animal experiments by in vitro models in the near future seems to be an unrealistic scenario, because a lack of validation prevents acceptance by regulatory authorities (Anderson and Russell, 1995; Clark, 1994).

In this review, an overview of different in vitro models (supersomes, microsomes, cytosol, S9 fraction, cell lines, transgenic cell lines, primary hepatocytes, liver slices, and perfused liver) for human biotransformation, with their advantages and disadvantages, is given. The phase III in vitro models (e.g., confluent cell monolayers) are beyond the scope of this review and are already extensively described, with their advantages and disadvantages, by Zhang et al. (2003).

Human CYP and UGT supersomes (baculovirus-insect-cell-expressed)

Insect cells lack endogenous cytochrome P450 and uridine diphosphoglucuronosyl transferase activity and, therefore, microsomes, which consist of vesicles of the hepatocyte endoplasmic reticulum, of human CYP- or UGT-transfected insect cells can be a useful tool in human biotransformation studies. Since the expression is baculovirus mediated, microsomes of these cells are sometimes referred to as baculosomes (Chen et al., 1997), but more often as supersomes (Gentest; Becton Dickinson Company, Woburn, MA offers them under this trade name).

The availability of specifically expressed human CYPs and UGTs in supersomes allows the investigation of the contribution of a single metabolic enzyme to the biotransformation pathway of the compound under investigation. At present all common human CYPs, coexpressed with NADPH-cytochrome P450 reductase and optionally cytochrome b_5, and UGTs are offered in supersomes. A control experiment, an incubation with nontransfected supersomes, must always be conducted (Araya and Wikwall, 1999). A NADPH-regenerating system (which consists of β-NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase) or NADPH is required to supply the energy demand of the CYPs (Taavitsainen et al., 2000). For the UGT activity, uridine diphosphoglucuronic acid (UDPGA) has to be added as a cofactor.

The specific CYP and UGT activity can be measured with various model substrates, e.g., midazolam C1′-hy-

droxylation for CYP3A4 and estradiol 3-glucuronidation for UGT1A (Birkett et al., 1993; Crommentuyn et al., 1998; Schenkman and Greim, 1993; and http://www.gentest.com). The CYP and UGT activity is usually provided by the supplier of the supersomes. Supersomes are commercially available from different companies.

Advantages and disadvantages

A major advantage of supersomes is that they can be used to study not only isozyme-specific drug biotransformation, but also drug–drug interactions (e.g., fluvoxamine–theophylline interaction at CYP1A2; Yoa et al., 2001). In the past few years the development of new CYP and UGT supersomes has increased considerably. The different genotypes of the CYP isozymes (e.g., CYP2C9*1, CYP2C9*2, and CYP2C9*3) are now also commercially available (http://www.gentest.com). Hence, the influence of different polymorphisms on the drug biotransformation pattern also can be studied.

A disadvantage is that, in UGT supersomes, the UGT active site is shielded behind a hydrophobic barrier, resulting in latency of glucuronidations. However, this disadvantage can be overcome by using a pore-forming agent, e.g., alamethicin (Fisher et al., 2001a).

Future

Supersomes are a valuable supplement to human liver microsomes (Huang et al., 2000; Rendic et al., 1999) and therefore it is likely that their application will increase in the future (Gasser et al., 1999). More detailed information will become available in the future about the specific advantages and disadvantages of this model compared to the other in vitro models.

Human liver microsomes

Human liver microsomes (HLM) still account for the most popular in vitro model, providing an affordable way to give a good indication of the CYP and UGT metabolic profile. Also, the influence of specific isozymes can be studied in the presence of specific inhibitors (Birkett et al., 1993). The large availability of human microsomes and their simplicity in use contribute to the popularity of this in vitro model.

Liver microsomes consist of vesicles of the hepatocyte endoplasmic reticulum and are prepared by differential centrifugation (Pelkonen et al., 1974) and thus contain almost only CYP and UGT enzymes. Liver preparations, other than from fresh human liver, can also be used (e.g., liver slices, liver cell lines, and primary hepatocytes) for preparation of microsomes (Olsen et al., 1997; Skaanild and Friis, 2000). The CYP and UGT enzyme activity can be measured by various model substrates. In commercially available HLM, the CYP activity is already characterized by the supplier (Bradford, 1976; Lowry et al., 1951; Peterson, 1977). As in supersomes, NADPH regenerating system or NADPH is required to supply the energy demand of the CYPs and UDPGA and alamethicin for UGT activity.

Table 2
Advantages and disadvantages of human liver microsomes, the most popular model in human drug biotransformation research

Advantages	Disadvantages
Easily applicable	Unsuitable for quantitative measurements
Affordable	Incomplete representation of in vivo situation
Well established	Only CYP and UGT enzymes
Interindividual variation can be studied	

The activity of HLMs can vary substantially between individuals. This problem, however, can be successfully solved by the application of pooled microsomes, which results in a representative enzyme activity (Araya and Wikwall, 1999). These pools can be purchased from different companies. Individual human liver microsomes can also be used to screen for the interindividual variability in the biotransformation of a drug. It is also possible to identify the critical CYP involved in the biotransformation of the drug using individual HLMs by correlating the enzyme activity of a particular CYP, using a bank of human donors, to the metabolism of the drug. The influence of gender on drug biotransformation can be investigated with gender-specific HLM pools. Different animal liver microsomes (e.g., mouse, rat, and monkey) can also be purchased from different companies. The results obtained can be used to screen for the best in vivo model for human drug biotransformation.

Advantages and disadvantages

The major advantages of microsomes are low costs, simplicity in use, and they are one of the best-characterized in vitro systems for drug biotransformation research. However, some major drawbacks exist (see also Table 2). First, it should be noted that results obtained with microsomes cannot be used for quantitative estimations of in vivo human biotransformation, because CYPs and UGTs are enriched in the microsomal fraction and there is no competition with other enzymes. This results in higher biotransformation rates in microsomes compared to the human in vivo situation, but also compared to primary hepatocytes and liver slices (Sidelmann et al., 1996) . Additionally, the absence of other enzymes (e.g., NAT, GST, and ST) and cytosolic cofactors can leave metabolites formed in intact liver cells unnoticed (Crommentuyn et al., 1998).

Future

The limitations make microsomes useful only for qualitative routine CYP and UGT screening in the early phase of

drug development and not for quantitative prediction of human biotransformation. However, some equations exist to extrapolate the in vitro HLM data to in vivo pharmacokinetics values for humans, but there is disagreement about the best equations to be used.

Human liver cytosol fractions

The liver cytosolic fraction contains the soluble phase II enzymes, e.g., *N*-acetyl transferase, glutathione *S*-transferase, and sulfotransferase (http://www.gentest.com). It is obtained by differential centrifugation of whole-liver homogenate, like microsomes. For the catalytic activity of the phase II enzymes, addition of exogenous cofactors, e.g., acetyl coenzyme A (acetyl CoA), dithiothreitol (DTT), and acetyl CoA regenerating system for NAT, adenosine-3′-phosphate-5′-phosphosulfate (PAPS) for ST, and glutathione (GT) for GST, is necessary (http://www.gentest.com). Human liver cytosol is commercially available from different companies, such as Gentest Corporation (Becton Dickinson Company, Woburn, MA) and XenoTech (Kansas City, KS).

The specific NAT isozymes are also commercially available in cytosol without the other soluble phase II enzymes. These enzymes are prepared from the cytosolic (soluble) fraction of insect cells infected with recombinant baculovirus. The influence of both NAT1 and NAT2 isozymes on the biotransformation pathway can be studied with this system (http://www.gentest.com).

Advantages and disadvantages

The main advantage is the presence of only three enzymes in the cytosolic fraction at higher concentrations compared to human liver S9 fraction. The biotransformation capacity of NAT, ST, and GST can be studied separately or in combination depending on the cofactors added. A disadvantage is that only the soluble phase II enzymes are present in the liver cytosol fraction and that therefore the UGT, which is located on the endoplasmic reticulum, metabolic pathways cannot be investigated with this model.

Future

So far, cytosol has not been used very often in drug biotransformation research (Favetta et al., 2000; Frandsen and Alexander, 2000; Long et al., 2001). It will probably play a more important role in the future, because researchers are more and more aware that not only knowledge of biotransformation by CYP but knowledge of the entire biotransformation pathway is of importance.

Human liver S9 fractions

The liver S9 fraction contains both microsomal and cytosolic fractions. Similar to supersomes and microsomes, a NADPH-regenerating system or NADPH solution is required to supply the energy demand of the CYP enzymes (http://www.gentest.com). For the catalytic activity of phase II enzymes, addition of exogenous cofactors is necessary: UDPGA and alamethicin for UGT; acetyl CoA, DTT, and acetyl CoA regenerating system for NAT; PAPS for ST; and GT for GST.

Human liver S9 fraction is mainly used in combination with the Ames test, which is a simple and rapid in vitro method for detecting the mutagenicity of chemicals (Maron and Ames, 1983). The test plays a critical role in development of new drugs and is used for predicting possible mutagenicity of a compound. However, many procarcinogens remain inactive until enzymatic transformation and thus a metabolic activation system, e.g., human liver S9 fraction, is necessary for testing not only the genotoxicity of a drug, but also its metabolites in humans (Hakura et al., 1999).

Advantages and disadvantages

Compared with microsomes and cytosol, S9 fractions offer a more complete representation of the metabolic profile, as they contain both phase I and phase II activity. In some cases with S9 fractions, metabolites are formed that are not produced by either the cytosolic fraction or the microsomal fraction alone, due to a phase I reaction followed by phase II biotransformation. However, a disadvantage is the overall lower enzyme activity in the S9 fraction compared to microsomes or cytosol, which may leave some metabolites unnoticed.

Future

Liver S9 fractions have been used since the 1970s, but not as extensively as microsomes (Mae et al., 2000; Mandagere et al., 2002; Sumida et al., 2001). It is a useful tool to study the human biotransformation, but the best option is to use it in addition to microsomes and cytosol. It is likely that the S9 fraction is going to be more widely used in the future, because of the importance to elucidate the entire biotransformation pathway, not only metabolites produced by phase I or phase II, but also by a combination of both.

Liver cell lines

Liver cell lines as an in vitro model are less popular compared to other described models. This is mainly due to their dedifferentiated cellular characteristics and incomplete expression of all families of metabolic enzymes. Human liver cell lines can be isolated from primary tumors of the liver parenchyma, seen after chronic hepatitis or cirrhosis (Crommelin and Sindelar, 1997).

An important requirement of cell lines as a model is that

Table 3
An overview of the known human hepatoma cell lines[a]

Designation	Origin	Enzymes active (constitutive)
Hep G2	Hepatocellular carcinoma	CYP 1A, CYP 3A, and UGT
BC2	Hepatoma	CYP 1A1/2, 2A6, 2B6, 2C9, 2E1, 3A4, and GST and UGT
Hep 3B	Hepatocellular carcinoma	CYP 1A1
C3A	Hepatoblastoma	CYP 3A
PLC/PRF/5	Hepatoma	GST
SNU-398	Hepatocellular carcinoma	
SNU-449	Hepatocellular carcinoma	
SNU-182	Hepatocellular carcinoma	
SNU-475	Hepatocellular carcinoma	
SK-Hep-1		

Note. The Hep G2 cell line is the most frequently applied cell line in human drug biotransformation studies (Almar and Dierickx, 1990; Doostdar et al., 1988; Fardel et al., 1992; Galijatovic et al., 1999; Gomez-Lechon et al., 2001; Ricci et al., 1999; Urani et al., 1998; Vickers et al., 1992; Walle et al., 2000).

[a] http://www.atcc.org.

they must resemble the normal physiology of human hepatocytes in vivo. The usefulness of hepatoma cell lines as in vitro model therefore falls or stands with their ability to express human phase I and phase II enzymes. Currently available human liver cell lines are presented in Table 3 (http://www.atcc.org). Only a few are used in biotransformation studies. There are also different animal hepatoma cell lines (http://www.atcc.org), but they are unpopular in human drug biotransformation research in which human systems are preferred. Established cell lines can be readily obtained from specialized companies such as ATCC (Manassas, VA).

Hep G2 cell line

The most frequently used and best-characterized human hepatoma cell line is the Hep G2 cell line (Doostdar et al., 1988; Fardel et al., 1992; Galijatovic et al., 1999; Urani et al., 1998; Vickers et al., 1992; Walle et al., 2000). This cell line, established in 1979, still has a variety of liver-specific metabolic functions. Under standard culturing conditions, the cell line shows nearly undetectable levels of functional CYP. However, various isoforms can be induced by pretreatment with inducing agents. Exposure to 3-methylcholanthrene and rifampicin results in higher levels of CYP 1A and 3A, respectively, compared to untreated cells. Compared to freshly isolated human hepatocytes, however, the overall CYP activity remains low (Fardel et al., 1992). Also, the composition of the culture medium has a significant effect on the metabolic enzyme activity in Hep G2 cells. Earle's medium gives a strong increase of the activity of CYP 1A and 2B compared to Dulbecco's medium and Williams' E medium (Doostdar et al., 1988).

In a direct comparison between Hep G2 cells, human liver slices, and human liver microsomes, the Hep G2 cells were shown to be an unsuitable model for biotransformation of cyclosporin A, which is mainly metabolized by CYP 3A. In contrast to human liver slices and microsomes, the cells generated only one of three primary metabolites of cyclosporin A. This underlines again that outcomes of experiments with human cell lines should be interpreted with caution (Vickers et al., 1992).

BC2 cell line

The cell line BC2 has been established more recently (in 1998) (Gomez-Lechon et al., 2001) and has been shown to express many drug metabolizing enzymes, especially the most important drug-metabolizing CYPs (1A1/2, 2A6, 2B6, 2C9, 2E1, and 3A4) and the phase II enzymes GST and UGT. It should be noted, however, that basal activities of these enzymes are very low and remain low after induction compared to freshly isolated human hepatocytes. It is the first cell line that combines the convenience of stable phenotypic expression in culture of a large set of drug-metabolizing enzymes (Gomez-Lechon et al., 2001); however, it has not been used by other research groups other than the group that isolated the cell line.

Other hepatoma cell lines

Other human hepatoma cell lines that have been used for cytotoxicity studies are the PLC/PRF/5 (Almar and Dierickx, 1990) and the Hep 3B (Ricci et al., 1999) cell lines. These cell lines have rarely been used for drug biotransformation studies, because their enzyme expression levels are very low and are difficult to induce.

Advantages and disadvantages

Compared to primary hepatocytes, cell lines are generally easier to culture and have relatively stable enzyme concentrations. However, an important disadvantage is the absence or low expression level of most important phase I and phase II drug- metabolizing enzymes, which limits its application. It is difficult to detect metabolites in cell lines and it is also difficult to investigate the individual CYPs or other enzymes due to their low expression levels.

Future

It is likely that the cell lines will only be used in the enzyme-induced state for drug biotransformation studies and most likely in combination with cytotoxicity studies of the drug and its metabolites.

Transgenic cell lines

Another approach to obtain a cell line expressing phase I and/or phase II enzymes is the recombinant expression of the human enzyme in a cell line. At present all known human CYPs involved in drug biotransformation have successfully been over-expressed in cells and these cell lines are available for research (Gasser et al., 1999). Also human UGTs have been successfully transfected into the V79 cell line (Wooster et al., 1993).

Cell lines may be transfected at high efficiency using protoplast fusion, centrifugation of lysozyme-treated bacteria bearing the desired vector with the parent cells in the presence of polyethylene glycol. Stable expression of human CYPs in a human cell line was first achieved in 1993 by Crespi et al. The V79 Chinese hamster cell line (Doehmer, 1993; Philip et al. 1999; Wooster et al., 1993) and the Hep G2 cell line (Caro and Cederbaum, 2001; Dai and Cederbaum, 1995; Delescluse et al., 2001; Feierman et al., 2002; Jover et al., 1998; Ono et al., 1995) have since then been engineered for stable expression of one or more CYPs and UGTs, but also other cell lines have been used to study human biotransformation (Cavin et al., 2001; Crespi et al., 1993; Hu et al., 1999; Kawahara et al., 2000; Lewis et al., 1999; Van Vleet et al., 2002). Gentest developed the MLC-5 human lymphoblast cell line, which stably expresses CYP1A2, CYP2A6, CYP2E1, and CYP3A4 and microsomal epoxide hydrolase (http://www.gentest.com).

Advantages and disadvantages

Transfected cell lines are often as easy to culture as nontransfected cell lines and their main advantage over nontransfected cells is the higher expression of CYP and UGT isozymes. The expression levels are high enough to perform biotransformation experiments. The transgenic cell lines can be readily obtained from specialized companies, such as ATCC and Gentest. Unfortunately, transgenic cell lines can be very expensive compared to the other in vitro models.

Transgenic cell lines, like supersomes, allow the study of single enzyme reactions. It is possible to elucidate the influence of one isoenzyme or a combination of a number of isoenzymes on the biotransformation and to screen for differences in cytotoxicity of the metabolites. Transfected cell lines can also be used to generate metabolites for structure elucidation and pharmacological characterization and to assess potential drug–drug interactions at the metabolic level. A limitation is that only one or a few isozymes are expressed, which is not a complete reflection of the in vivo situation.

Future

In the future, it is likely that more transgenic cell lines will be used to study human biotransformation, because the enzymatic levels are high and stable enough to study the biotransformation. Hopefully, more and more cell lines that express several (iso)enzymes will become available at moderate cost.

Hepatocytes

Primary hepatocytes

Primary hepatocytes are a popular in vitro system for drug biotransformation research due to their strong resemblance of in vivo human liver. Detailed reviews dealing with this model have been published previously (Cross and Bayliss, 2000; Hengstler et al., 2000; Puviani et al., 1998) and, therefore, primary hepatocytes will be discussed here only briefly.

Hepatocytes of various animal species can be isolated from the liver by the traditional collagenase perfusion operation developed by Howard and Pesch (Howard et al., 1967). This method requires the whole liver, which is not available in the case of human liver. Human liver is mainly obtained from patients that undergo partial liver resection, e.g., because of liver metastasis. Therefore, a method for human liver parts has been developed (Puviani et al., 1998) and this is a modification of the traditional collagenase perfusion (Berry et al., 1991). Preferably, the perfusion takes place immediately after resection. When this is not possible, the tissue can be stored at 4°C for up to 48 h in University of Wisconsin (UW) solution, without relevant loss of viability (Guyomard et al., 1990).

Cultured hepatocytes

Once isolated, hepatocytes can be held in suspension, in which case they remain viable for only a few hours, or they can be maintained in monolayer culture for a maximum of 4 weeks. Both cultured hepatocytes (Chenery et al., 1987; Le Bigot et al., 1987) and suspensions of primary hepatocytes (Bayliss et al., 1999; Berry et al., 1992; Cross et al., 1995) have repeatedly proven to be powerful tools to analyze the specific metabolic profile of a variety of drugs with good in vitro–in vivo correlations (Bayliss et al., 1999; Berry et al., 1992; Chenery et al., 1987; Cross et al., 1995; Le Bigot et al., 1987).

However, it has been widely recognized that cultured hepatocytes are subject to a gradual loss of liver-specific functions, with special reference to a decreased CYP expression. This loss is different for the specific CYP isoforms; for some isoforms it becomes evident after a few days of culture (CYP 2E1 and CYP 3A4), while others remain nearly unaffected by the isolation and culturing processes (CYP 1A2 and CYP 2C9) (George et al., 1997). Various culturing methods have been explored in an effort to maintain the liver-specific characteristics of hepatocytes during prolonged culture. These include the application of

culturing matrices (e.g., double-layer collagen gel sandwich; this culture method can be used to study not only biotransformation, but also transporter-mediated biliary excretion) (Ammann and Maier, 1997; De Smet et al., 2000; Jauregui et al., 1994; Koebe et al., 2000); the addition of specific nutrients, hormones, and inducers to the culture medium (Block et al., 1996; 't Hoen et al., 2000; Sewer and Morgan, 1997; Vernia et al., 2001); and also the coculturing of hepatocytes with other cell types (e.g., the hepatic Kupffer cells) (Milosevic et al., 1999).

Advantages and disadvantages

An advantage of isolated hepatocytes compared to liver slices and perfused liver is the possibility of cryopreservation. Cryopreserved hepatocytes have been shown to retain the activity of most phase I and phase II enzymes (Annaert et al., 2001; Silva et al., 1999). Due to successful cryopreservation techniques, human hepatocytes are now commercially available (Hengstler et al., 2000). Due to their widespread use in drug biotransformation research, isolated hepatocytes have become a well-established and well-characterized in vitro model and, with special techniques, isolated hepatocytes can be made viable for up to 4 weeks. However, it should be noted that prolonged culture conditions result in a more complex data interpretation, since outcomes partly depend on culture system factors.

A disadvantage is the lack of liver nonhepatocyte cells. Although hepatocytes account for the vast majority of the liver volume (about 80%), other cells such as Kupffer cells may be necessary for cofactor supply. Another problem encountered with human hepatocytes, as with human liver microsomes, is the considerable interindividual variation. This problem can be overcome by using mixtures of hepatocytes from multiple donors to mimic an average enzyme content. Also, animal primary hepatocytes are used in human biotransformation studies and they can be used, like HLM, to choose the best animal in vivo model, the in vivo model that has the most resemblance with the human biotransformation pathway.

Future

Despite the disadvantages (see Table 4), isolated hepatocytes are a useful tool to predict human biotransformation and are therefore often used in human drug biotransformation studies and will continue to be used in the future.

Table 4
Primary hepatocytes and their advantages and disadvantages in human biotransformation studies

Advantages	Disadvantages
Well established	Isolation can be complicated and time consuming
Well characterized	Only preselected cells can be studied
Viability for up to 4 weeks	Cell damage during isolation
Study of mediators and enzyme inducers possible	Cellular interactions more difficult to study
Viable cell enrichment possible	
Cryopreservation possible	
Drug transporters still present and operational	

Liver slices

Cultures of tissue slices were developed in the 1920s by Otto Heinrich Wartburg. His technique was adopted by later workers, including Hans Adolf Krebs who used razor blade-cut tissue slices to study the biotransformation of amino acids in a variety of organs of different species (Krebs, 1933). Today, the incubation of liver slices in nutrient-enriched media offers a powerful tool to study biotransformation in vitro.

Initially, it proved to be difficult to produce uniform slices, which led to irreproducible results. Even after the development of more precise slicing devices (e.g., the McIlwain tissue chopper) in the early 1970s, the technique remained unpopular. This was partially due to good results obtained with primary hepatocytes, which became the model of choice for drug biotransformation studies. Liver slices as a model for human drug biotransformation studies fell further into disuse (Ekins, 1996; Fell, 1976).

The development of high-precision tissue slicers, however, set the stage for the "renaissance" of liver slices in in vitro biotransformation studies. The Krumdieck tissue slicer, for example, allows the rapid production of equally sized slices of less than 250 μm thickness (Krumdieck et al., 1980). The Brendel-Vitron is essentially a simpler slicing device but gives access to slices of equal quality (Bach et al., 1996; Price et al., 1998). The thin slices obtained with the Krumdieck and the Brendel-Vitron slicers realistically and reliably represent the in vivo situation and have been used to study the biotransformation of many compounds (Ekins, 1996).

The resected tissue can be stored at 4°C in UW solution up to 48 h without loss of phase I and phase II enzyme activity (Olinga et al., 1997b). However, the long-term storage of liver slices in liquid nitrogen has been shown to be complicated and there is no optimal cryopreservation protocol. No commercially human liver slices are yet available. The duration of the CYP activity is short and this is probably due to impaired diffusion of nutrients and oxygen in the tissue slice. A recent study with rat liver slices showed that the amount of many CYPs drops below half of the initial value within 24 h (Hashemi et al., 2000). However, CYP induction has been reported, e.g., rifampicin has been reported to induce human CYP3A (Lake et al. 1997) and Aroclor 1254, omeprazole, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3,3′-diindolylmethane induce human CYP1A (Lake et al., 1996, 1998).

Dynamic organ culturing (DOC) was developed to prolong the limited viability period of liver slices. In this

technique, the slice is continuously exposed to both culture medium and gas atmosphere (Smith et al., 1985). Some variants of DOC were developed later (Brendel et al., 1987; Olinga et al., 1997a).

In terms of qualitative and quantitative biotransformation properties, slices have been shown to be comparable to perfused liver, a more complicated model that is discussed below.

Advantages and disadvantages

The advantages and disadvantages of using liver slices in human drug biotransformation studies are summarized in Table 5. One of the main advantages is the nonrequirement for digestive enzymes and thus the intact cellular tissue architecture, allowing for observing biotransformation in nonhepatocytes. Also, the possibility to study the induction of CYP isoforms by new drugs is a main advantage. The most prominent disadvantages include inadequate penetration of the medium into the inner part of the slice, damaged cells on the slice outer edges with impaired biotransformation, and the short viability time period of 5 days. Also, the optimal incubation method is highly dependent on the applications of the liver slices.

Future

Tissue slice cultures are a powerful tool to study biotransformation in vitro, but the drawbacks mentioned still prevent its large-scale application.

Isolated perfused liver

Although an isolated perfused liver is considered to be the best representation of the in vivo situation, it has never been used with human liver and only on a small scale with animal livers. Many specific drawbacks make the animal perfused liver less attractive as a model for biotransformation studies.

Table 5
Comparison of advantages and disadvantages of precision cut liver slices used in human drug research

Advantages	Disadvantages
Nonrequirement of harmful proteases	Inadequate penetration of the medium
Intact cellular interactions	Damaged cells on the outer edges
Normal spatial arrangement	Limited viable period
Morphological studies possible	Technology still being developed and optimized
	Viable cell enrichment not possible
	Noninducible by CYP inducers
	Cryopreservation needs further optimization
	Expensive equipment necessary

Table 6
Advantages and disadvantages of the isolated perfused liver model in human biotransformation research

Advantages	Disadvantages
Best representation of in vivo situation	Delicate model, difficult to handle
Bile can be collected and analyzed	Limited experimental viable period
Three-dimensional architecture	Poor reproducibility
All cell types, so also biotransformation by nonhepatocytes	No human liver available

The procedure for perfusion of intact rat liver was described in detail in 1959 by Brauer et al. The perfusion is carried out with Krebs-Henseleit buffer as perfusate, while other media such as diluted blood solutions have also been reported (Alexander et al., 1998). The test compound under investigation should be dissolved in the medium and the viability period of the liver is only 3 h (Wu et al., 1999).

Advantages and disadvantages

There are several reasons why the perfused liver has not been widely used in human biotransformation studies. There are no human livers available for such studies and animal livers are not always the correct model for human drug biotransformation. Also the perfused liver method is labor intensive, has poor reproducibility, and the functional integrity is limited to 3 h. An overview of the advantages and disadvantages is given in Table 6. Since an intact perfused liver has only slight advantages over precision-cut liver slices, the latter will be preferentially used for practical reasons. In view of animal welfare, it is an inappropriate model as well, as the ratio of animal to experiment is 1:1.

Future

The perfused animal liver is a useful model only in cases in which bile secretion is of importance or when validation of other in vitro methods is required.

Conclusion

Although the isolated perfused liver gives an excellent representation of the in vivo situation, practical inconveniences, such as unavailability of human liver, poor reproducibility, and test limitation of 3 h prevents the method from being used on a large scale. Liver slices only have slight disadvantages over the isolated perfused liver and liver slices are thus preferred over the perfused liver model. Therefore, perfused animal liver is the model of choice only in biotransformation studies when bile excretion is necessary.

Liver slices and primary hepatocyte suspensions also

Table 7
An overview of different in vitro models and their advantages and disadvantages

In vitro technique	Advantages	Disadvantages
Human CYP and UGT supersomes	One isozyme present Different genotypes High enzyme activities	Difficult extrapolation to HLM and in vivo
Human liver microsomes	Affordable Study of individual, gender-, and species-specific biotransformation	Unsuitable for quantitative measurements Only CYP and UGT enzymes
Human liver cytosol	NAT, ST, and GST activity depends on cofactors present High enzyme activities Study of individual, gender-, and species-specific biotransformation	Only NAT, ST, and GST
Human liver S9 fraction	Both phase I and II Study of individual, gender-, and species-specific biotransformation	Lower enzyme activity than in microsomes and cytosol
Human liver cell lines	Easy to culture Relatively stable enzyme expression levels CYPs inducible	Low expression levels
Transgenic cell lines	Easy to culture Higher expression levels Study of one isozyme or a combination of CYPs	Incomplete representation of in vivo situation Only a few isozymes are expressed
Primary hepatocytes	Well established and characterized Study of mediators and enzyme inducers possible Drug transporters still present and operational	Isolation can be complicated and time consuming Only preselected cells can be studied Cell damage during isolation
Liver slices	Intact cellular interactions Morphological studies possible Interindividual variation can be studied	Inadequate penetration Damaged cells on the edges Limited viable period Expensive equipment
Isolates perfused liver	Bile formation Three-dimensional architecture	Delicate model Limited viable period Poor reproducibility No human liver available

give a good picture of the in vivo metabolic profile and offer a more efficient use of tissue. A disadvantage of these models, however, is a rapid decline of viability and metabolic capacity within hours after isolation. Cultures of primary hepatocytes have a longer viability period, but the decline of some enzymes is still rapid. Methods to prolong the viability period with maintenance of hepatospecific functions have been developed for liver slices and cultured primary hepatocytes, but can complicate data interpretation. A disadvantage of the primary hepatocytes compared to the liver slices is that the normal liver integrity is not maintained. However, an advantage of the cultured primary hepatocytes is that the decline of the enzymes can be reduced by adding inducers of these enzymes to the culture medium, which is not possible in liver slices.

Established cell lines have a relatively stable phenotype in culture compared to primary hepatocytes, liver slices, and perfused liver, but they usually lack or overexpress many essential enzymes specific for the liver, which limits their use. Transgenic cell lines, with an established CYP expression, are a better option, but there is no transgenic cell line at this moment that represents the true in vivo human hepatocyte. Established and transgenic cell lines offer a model to study a combination of biotransformation and cytotoxicity of the drug and its metabolites.

Subcellular liver fractions are widely used to characterize the metabolic profile of novel compounds. Microsomes can be used to obtain information on CYP- and UGT-mediated biotransformation (phase I), while cytosol can be used to study phase II biotransformation of the soluble phase II enzymes (NAT, GST, and GST). CYP and UGT supersomes and NAT cytosol provide information about CYP, UGT, or NAT isozymes. S9 fractions can be used to study both phase I and phase II biotransformation at the same time. Supersomes and other sources of artificially expressed human CYPs are valuable to identify new metabolites and to elucidate the contribution of individual CYPs, UGTs, and NATs to the biotransformation of the compound under investigation. However, a disadvantage of the subcellular fractions is that the drug biotransformation is not influenced by drug transporters, which is normally the case in intact cells and organs.

Biotransformation research of a new drug can start with a simple model while the model can become more complex at later stages. The best sequence is to start with microsomes and cytosol, then CYP and UGT supersomes and NAT cytosol, the S9 fraction, followed by (transfected) cell lines and primary hepatocytes, and finally liver slices. Also drug–drug interactions, the influence of polymorphisms can be studied using different in vitro techniques. Table 7 summarizes the different in vitro techniques with their main advantages and disadvantages. An overview of the different models of choice and their preference in use is shown in Table 8. The perfused liver should only be used in cases of bile excretion study and is not a good model for biotransformation research.

Table 8
An overview of different in vitro models to be used at different stages in drug biotransformation research

Drug research	In vitro model	Arguments
Drug biotransformation	(1) Pooled microsomes and cytosol (gender-specific fractions can be used)	(1) Combination of enzymes in one model, CYPs and UGTs in microsomes and soluble phase II enzymes in cytosol
	(2) Supersomes and NAT cytosol	(2) One specific isozyme present
	(3) Human liver S9 fraction	(3) Combination of phase I and II enzymes
	(4) (Transgenic) cell lines and primary hepatocytes	(4) Intact cell to study
	(5) Liver slices	(5) Intact liver structure
	(6) Perfused animal liver	(6) Only for bile excretion
Isolation of metabolites	(1) CYP or UGT supersomes or NAT cytosol	(1) Metabolite of one specific isozyme (higher yield)
	(2) Microsomes or cytosol	(2) Only when isoenzyme is not present as isolated enzyme
	(3) S9 fraction	(3) Only when metabolite is combination of phase I and II biotransformation
Drug–drug interaction	(1) Microsomes or cytosol	(1) Combination of enzymes in one model
	(2) CYP or UGT supersomes or NAT cytosol	(2) Interaction at one specific isozyme
	(3) Primary hepatocytes	(3) Intact cell
Influence of polymorphisms	(1) Supersomes	(1) CYP polymorphisms in supersomes available
	(2) Microsomes or cytosol	(2) From one patient that shows that polymorphism
	(3) Primary hepatocytes	(3) From one patient that shows that polymorphism
Toxicity of drug and its metabolites	(1) Primary hepatocytes	(1) Intact cell and cytotoxicity is measurable
	(2) (Transgenic) cell lines	(2) Influence of one or combination of enzymes on cytotoxicity can be studied
	(3) Liver slices	(3) Intact liver structure
Choose animal as in vivo model	(1) Microsomes and/or cytosol	(1) Different animal models are available
	(2) Supersomes	(2) Rat CYP supersomes are available
	(3) S9 fraction	(3) Different animal models are available
	(4) Primary hepatocytes	(4) Intact cell and different animal models are available

Although, at present, in vitro models are unable to replace in vivo screening completely, they offer promising features. They can reduce the number of animals needed and offer a less complex way to elucidate the human biotransformation pathway of a new drug. It is likely that the different in vitro techniques used to study human biotransformation, except perfused liver, will become increasingly important in the early development stage of a new drug before starting in vivo experiments, so that the most promising drugs are selected and in vivo testing can be performed as efficiently as possible.

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