Section 1

Models for Hepatotoxicity Testing

1

Current *in vitro* Models to Study Drug-Induced Liver Injury

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1.1 Introduction

In a published report on the root causes of failed drugs over a 10-year period, it was concluded that the pharmaceutical industry as a whole is still facing the same challenge of selecting drug candidates with high efficacy and low toxicity (Schuster *et al.*, 2005). Among all human organ toxicities, hepatotoxicity and cardiovascular toxicity were the two most prominent causes, accounting for two out of three market withdrawals in the last decade (Schuster *et al.*, 2005). In clinical drug development phases, about half of attrition were due to insufficient efficacy and one third were due to toxicity (Schuster *et al.*, 2005). While regulatory animal toxicity testing was able to identify more than 70 % of human toxicities in a retrospective analysis, hepatotoxicity in humans had the poorest correlation with regulatory animal toxicity tests (Olson *et al.*, 2000). In only half of new pharmaceuticals that produced hepatotoxicity in the clinical stage was there any concordant signals in animal toxicity studies (Olson *et al.*, 2000). Obviously, identifying better models to predict human hepatotoxicity is a critical need for the pharmaceutical industry.

Drug-induced liver injury (DILI) can be broadly classified into two categories, based on incidence, animal model predictability and dose-dependency. DILI type 1 (DILI-1) is characterized by relatively higher incidence, reproducible in at least one animal species and dose-dependent increase in incidence and severity of the observed injury. Acetaminophen is a classic case of DILI-1 and accounts for nearly half of acute liver failure in the United States (Larson *et al.*, 2005). It can be modeled in more than one strain of rodents (Mehendale, 2005)

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and has a clear dose-dependency in both animals and humans. In humans, the reported ratio of median dose leading to acute liver failure to maximally recommended therapeutic dose is 2 for unintentional overdose to 6 for intentional overdose (Larson *et al.*, 2005). DILI-2, on the other hand, has a relatively lower incidence, occurring at therapeutic dosesfrom 1 in every 1000 patients to 1 in every 100 000 patients (Lee, 2003). It is typically not predicted by the classic animal models of rodents, dogs and monkeys. Because of its rare incidence and large patient-to-patient variability, there is no clear indication of dose-dependency, although within the same susceptible patient, an underlying concentration–response may be present.

DILI-2 is also referred to as *idiosyncratic hepatotoxicity*. The term 'idiosyncratic' derives from the Greek meaning 'mixture of characteristics' and in the context of drug toxicity refers to the combination of genetic and non-genetic factors that make a patient susceptible to drug injury. Numerous epidemiological studies have identified increasing age (Andrade *et al.*, 2005), females (Ostapowicz *et al.*, 2002) and certain disease-associations (Boelsterli, 2003) as the top demographic factors associated with higher incidence of DILI. While the reasons for such drug idiosyncracy are not entirely clear, most likely there are multiple factors that encompass both toxicokinetic, and toxicodynamic or adaptive reasons. In terms of toxicokinetic reasons, there have been numerous cases where factors such as age, gender, disease states, enzyme induction and inhibition, genetic polymorphism, food and gut micro-organisms have been implicated in causing variability in the pharmacokinetics of drugs. These factors can easily cause several-fold variations in drug concentrations among individuals given the drug at the same dose (Tam, 1993). In addition, the practice of *polypharmacy* (patients taking multiple drugs) tends to increase with age, which may surpass the predictability of well-controlled clinical trials on drug–drug interaction studies that are typically conducted on a limited number of drug pairs (FDA, 1999). In addition, the presence of disease states, including inflammation and infection, can affect both drug metabolism (Renton, 2004) and drug transport (Fernandez *et al.*, 2004; Hartmann *et al.*, 2002). In terms of adaptive reasons, the human liver has a remarkable ability to regenerate. Indeed, livers that underwent partial hepatectomy can regenerate and patients can go on to live normal lives. In addition, most of idiosyncratic hepatotoxicity was evident only after weeks of drug therapy, not upon a single dose of drug administration. The liver's adaptive response after the initial drug 'insult' is probably crucial to the final manifestation of the full-blown liver injury. These adaptive responses almost certainly involve tissue repair (or lack thereof) (Mehendale, 2005), and the innate and adaptive immune systems (Holt and Ju, 2006). Numerous factors, including genetic polymorphism in tissue repair and immune responses, systemic and/or tissue inflammation, disease states, and continued challenge by polypharmacy, can affect a particular patient's response to drug-induced liver injury. The mechanisms of such adaptive responses, including signal transduction pathways (Jaeschke and Bajt, 2006; Schwabe and Brenner, 2006), interactions between hepatocytes and cells of the host immune systems (Minagawa *et al.*, 2004) and cellular decision-making (Malhi *et al.*, 2006), will likely be an important and fruitful area of research in the post-genomic era.

In vitro cellular models of drug toxicity have unique and important roles to play in order to understand both the mechanisms of initial drug injury and the many signal transduction pathways involved in tissue repair. This present chapter will focus primarily on the use of *in vitro* models of drug-induced liver injury. The better known mechanisms of drug-induced liver injury have been reviewed in previous publications (Begriche *et al.*,

Table 1.1 *Major mechanisms of drug-induced liver injury*

Mechanism	Prototypical drug	Reference
Altered lipid metabolism causing fatty liver	Amineptine, amiodarone, doxycycline, tetracycline, tianeptine, pirprofen	Letteron *et al.*, 2003, and references therein
Decreased bile salt clearance causing cholestasis	Cyclosporine A, estradiol-17 beta-D-glucuronide, taurolithocholate, ethinyl estradiol	Crocenzi *et al.*, 2003a,b; Micheline *et al.*, 2002; Roman *et al.*, 2003
Formation of protein adduct causing immune reaction	Flucloxacillin, diclofenac, tienilic acid, sulfamethoxazole, halothane	Aithal *et al.*, 2004; Carey and van Pelt, 2005; Robin *et al.*, 1996; Sanderson *et al.*, 2006
Increased oxidative stress leading to cellular injury	Sodium diethyldithiocarbamate, diclofenac, ketoconazole, acetaminophen and drugs that cause steatohepatitis	Amin and Hamza, 2005; Reid *et al.*, 2005
Decreased mitochondrial function leading to apoptosis or necrosis	Nimesulide, amiodarone, tamoxifen, stavudine, zidovudine and drugs that cause steatohepatitis	Begriche *et al.*, 2006; Tay *et al.*, 2005
Cytotoxic T cell-mediated cell killing	Sulfamethoxazole, lidocaine, carbamazepine, lamotrigine, phenindione	Sanderson *et al.*, 2006, and references therein
Incomplete or dysregulated tissue repair	Acetaminophen, carbon tetrachloride, chloroform, thioacetamide, trichloroethylene, allyl alcohol	Mehendale, 2005, and references therein

2006; Jaeschke *et al.*, 2002a; Kaplowitz, 2002; Lee, 2003). These are summarized in Table 1.1. A single small-molecule drug may invoke a multitude of such mechanisms (e.g. amiodarone, perhexiline, diclofenac and acetaminophen). This has led some researchers to postulate the 'multi-hit' hypothesis of drug hepatotoxicity (Begriche *et al.*, 2006; Letteron *et al.*, 2003; Pirmohamed *et al.*, 2002). Cellular models based on human liver tissues have unique capabilities for studying such DILI mechanisms, as illustrated by the examples shown in Table 1.2. However, before describing the applications of *in vitro* models, we will first explore the various types of *in vitro* models that are available to researchers today.

1.2 *In Vitro* Models to Study DILI

1.2.1 Primary Hepatocytes

Currently, the primary hepatocytes system is the *in vitro* model of choice for studying drug metabolism and hepatotoxicity of new drugs (Castell *et al.*, 2006; Davila *et al.*, 1998;

Table 1.2 *Representative applications of cell-based assays to study drug-induced liver injury*

Applications	Methodology	Representative reference
Multiparameter cytotoxicity	Combine several different readouts including multi-spectral cytometric analysis	O'Brien *et al.*, 2006
Steatosis	Neutral lipid stain (e.g. Oil Red O)	Amacher and Martin, 1997
Cholestasis	Uptake and efflux of taurocholate	Kostrubsky *et al.*, 2006
Phospholipidosis	Phospholipid accumulation in cytoplasm or lysosomal stain	Gum *et al.*, 2001; O'Brien *et al.*, 2006
Reactive metabolite	GSH adduct formation; GSH depletion (e.g. monochlorobimane)	Lilius *et al.*, 1996; Thompson *et al.*, 1998)
Oxidative stress	Redox-sensitive dyes	Lautraite *et al.*, 2003; LeBel *et al.*, 1992; Wang and Joseph, 1999
Mitochondria damage	Mitochondria membrane potential dyes (e.g. TMRM)	Haskins *et al.*, 2001
Identify targets of toxicological importance	RNAi technology and/or specific inhibitors	Lee and Sinko, 2006; Pichler *et al.*, 2005; Tan *et al.*, 2005; Xu *et al.*, 2005

Gebhardt *et al.*, 2003; Gomez-Lechon *et al.*, 2004; Guillouzo *et al.*, 1977; LeCluyse, 2001; Sinz and Kim, 2006). Primary hepatocytes represent a unique system since they are able to retain, under a refined cultured condition, Phase I and II enzyme activities, as well as their inducibility by xenobiotics. However, primary hepatocytes that fail to preserve and/or re-establish cell polarity and the expression of liver-specific genes are less responsive to drugs and therefore do not accurately reflect the metabolic potential of the liver tissue (Berthiaume *et al.*, 1996; Kocarek *et al.*, 1992; LeCluyse *et al.*, 1996; Tuschl and Mueller, 2006; Waring *et al.*, 2003)

It is well established that primary hepatocytes cultured on plastic surfaces and allowed to form an epithelial monolayer (2-dimensional configuration) lose not only up to 75 % of total CYP450 during the first 24-h period, but also liver-specific functions and differentiation processes (Davila and Morris, 1999; Farkas and Tannembaum, 2005; Gomez-Lechon *et al.*, 2004; LeCluyse, 2001); therefore, it is critical that cell culture conditions be refined for studying liver metabolism and toxicity of drugs in humans. Several optimized culture conditions have been reported to maintain liver-specific functions and gene responsiveness (co-factors expression) to levels comparable with those *in vivo*; these include the use of extracellular matrix or complex substrata (e.g. collagen and/or Matrigel®, sandwich configuration) (Farkas and Tannembaum, 2005; LeCluyse, 2001; Schuetz *et al.*, 1988; Sidhu *et al.*, 1994), chemically defined culture conditions (e.g. the use of low concentrations of insulin and dexamethasone in a serum-free amino acid-rich culture medium) (Enat *et al.*,

1984; LeCluyse, 2001; Sidhu *et al.*, 1994) and co-culture hepatocytes with other cell types (e.g. sinusoidal cells, kidney epithelial cells, kupffer cells) (Begue *et al.*, 1984; Donato *et al.*, 1994).

Primary rat hepatocytes cultured and overlaid with Matrigel® in an optimized media enables the hepatocytes to retain and maintain longer-term viability and expression of more stable differentiated liver function, such as Phase I and II metabolizing enzymes, and responsiveness to drugs and chemicals (Davila and Morris, 1999; Davila *et al.*, 1998). Matrigel® is basically a solubilized basement membrane preparation extracted from the Engelbreth–Holm–Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix (ECM) proteins. This complex matrix is mainly composed of laminin, collagen IV, heparan sulfate proteoglycans and entactin, which polymerizes at room temperature to produce biologically active matrix material resembling the mammalian cellular basement membrane. We have previously reported on the development of a modified sandwich configuration model initially described by Dunn (Dunn *et al.*, 1991) and others (Kocarek *et al.*, 1992; LeCluyse, 2001; Schuetz *et al.*, 1988; Sidhu *et al.*, 1994), where freshly isolated hepatocytes are resuspended, plated and cultured in a Matrigel®–Matrigel® system with serum-free media containing insulin and hydrocortisone (10^{-7} M) (Davila and Morris, 1999). One advantage of using this model is that the concentration of Matrigel® can be easily monitored before and after the initial plating. The concentration of ECM used for culturing is known to affect the morphology and functionality of the hepatocytes. In preliminary studies, we found that rat hepatocytes, cultured and overlaid with Matrigel® at 0.35 mg/ml, acquire a 3-dimensional (3D) configuration and are reorganized as acinar structures; cells become more cuboidal with a distinctive canalicular network and the polarized phenotype and function of normal hepatocytes are well preserved (Davila and Morris, 1999) . Under these culture conditions, the basal levels of a variety of liver genes are maintained and can be induced/stabilized by xenobiotics to levels that are comparable to those achieved *in vivo.* The mRNA levels of most of the rat CYP450 enzymes (e.g. CYP1A, CYP2B, CYP3A and CYP4A) can be induced 2–4 h after the initial plating. This is an improvement from the traditional methods using primary hepatocytes where cells have been reported to be unresponsive to inducers during the first 24–48 h (Kocarek *et al.*, 1992; Richert *et al.*, 2002; Silva *et al.*, 1998). Freshly isolated rat hepatocytes cultured under this refined culture condition are proven to be a valuable and important *in vitro* toxicological approach to assess the chemical-induced changes in expression of rat liver CYP450 and Phase II conjugating enzymes (Davila and Morris, 1999; Davila *et al.*, 1998). However, the ultimate species of interest in predicting liver metabolism and toxicity of drugs is human.

1.2.1.1 Refinement of a Primary Human Hepatocyte System for Drug Metabolism and Toxicity

Primary human hepatocytes are a useful model system to potentially predict the metabolism and toxicity of compounds and allow a better understanding of the mechanism of actions of drugs in the human population. Caveats in using primary human hepatocytes are the limited supply of human liver tissues and the ability of such cultures to loose their phenotype after culturing. Despite their limitations, primary human hepatocytes are currently the system of choice to predict human risk before new chemical entities (NCEs) are first tested in Phase I clinical trials. In this section, we describe the refinement of a feasible, reproducible and simple cell culture system that could be used for routine toxicological applications early during drug discovery and development. This model has been specifically refined for assessing the

metabolism and toxicity of drugs. Validation studies supporting the use of this optimized human hepatocyte model for metabolism-mediated toxicity studies include: (1) microscopy analysis to identify hepatocytes in a 3D configuration with well define intracellular structures characteristic of normal and functional hepatocytes; (2) the measurement of several constitutive and inducible expressions of human Phase I (CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2D6, CYP2E1, CYP3A4, CYP4A11 and fatty acyl CoA oxidase, FACO) and Phase II (uridine diphosphate-glucuronosyltransferase, UDPGT2B7; sulfotransferase, SULT2A1; glutathione-S-transferase, GSTYa) biotransformation enzymes at the mRNA and protein and activity level; (3) *in vivo* and *in vitro* comparison of relative Phase I enzyme mRNA levels; (4) the differential expression of CYP450 enzymes in human and rat hepatocytes following treatment with various inducing agents.

1.2.1.2 Procurement, Isolation and Culturing of Human Hepatocytes

Human donor livers used in these studies were received from the National Disease Research Interchange (NDRI, PA) within 16 h of patient procurement. Livers were screened at the NDRI for evidence of infectious agents and preserved with ViaSpan (Dupont/Pharma, Wilmington, DE) upon removal from donors. Livers were only accepted from donors with minimal fat content $< 10\,\%$, normal liver enzyme levels, low visible fibrosis, medications which would not be considered as potent liver enzyme inducers (particularly, CYP450s) and patients ranging from 14 to 60 years old. Freshly isolated human hepatocytes were obtained from The University of Pittsburgh, Department of Pathology (Stephen Strom's laboratory) within 24 h after cell isolation and from our laboratory as indicated below.

The isolation of human hepatocytes from the whole liver was performed by a three-step perfusion technique using liver sections *in situ*, as described by Strom (Strom *et al.*, 1996). The culturing of freshly isolated human hepatocytes was then processed as indicated for rat hepatocytes (Davila and Morris, 1999) with some modification. Briefly, after isolation, liver cells were filtered through a sterile nylon mesh (100 μm) and collected in several 50-ml conical test tubes. Cells were centrifuged twice at $30 \times$ g for 2 min and then the final pellet was resuspended with 30 ml Williams E solution containing hydrocortisone and insulin (10^{-7} M) and glutamine (4 mM). Ten ml of diluted Percoll (30 vol %) were placed in the bottom of each tube and centrifuged at $1100 \times$ g for 5 min. The pellets were washed with Williams E at $30 \times$ g for 2 min and then resuspended in 20 ml Williams E solution. Viability of the isolated hepatocytes used in these studies was typically greater than 90 %. The final cell suspension was resuspended in diluted Matrigel® solution (0.35 mg/ml) to a final cell density of 4×10^5 cells/ml and plated in 12 (1 ml) or 6 (2 ml) well plates, as previously indicated for rat hepatocytes (Davila and Morris, 1999).

1.2.1.3 Cell Culture Treatment

Hepatocytes were treated for two consecutive days at 24 h and 48 h after initial plating with optimal concentrations of phenobarbital, PB (100 μM), rifampicin, RIF (10 μM), 3-methylcholanthrene, 3MC (1 μM), clofibrate, CLO (50 μM), tamoxifen (TAM, 10 μM), omeprazole (OMZ, 20 μM), pregnenolone-16α-carbonitrile (PCN, 1 μM) and hydrocortisone (HC, 20 μM). Compounds were dissolved in dimethyl sulfoxide (DMSO) and then diluted with Williams E prior to administration to the cultures (final DMSO concentration was 0.1 vol %). The concentrations of the inducers used in these studies were selected

based on preliminary experiments to determine the concentration of inducer producing the highest levels of enzyme expression in the absence of toxicity (data not shown). When the enzyme was not inducible, a maximum non-toxic concentration was used (50–100 μM). The concentrations of inducers used in this study were not toxic to the hepatocytes as indicated by morphological examination, MTT reduction assay and recovery of total RNA (data not shown).

1.2.1.4 PCR and Fluorogenic cDNA Probes

Messenger RNA was determined using real-time PCR. Primer sets specific for human cyclophylin (CYC, house-keeping gene), CYP450 1A1, 1A2, 2B6, 2C9, 2D6, 2E1, 3A4, 4A11, FACO, UDPGT2B7, GSTYa and SULT2A1, and TaqMan fluorogenic probes specific for each primer set are described in Tables 1.3 and 1.4, respectively. The TaqMan fluorogenic probes were designed in accordance to the guidelines issued by Perkin-Elmer. The cDNA samples were analyzed using an ABI Prism 7700 (ABI) sequence detection system (Perkin Elmer Biosynthesis, Foster City, CA). The results generated by the ABI system are expressed as cycle threshold (Ct), representing the PCR cycle at which an increase in TaqMan probes fluorescence is detected (specific signal) above the baseline signal for each amplicon (Table 1.5, A–F). The target gene Ct (Table 1.5, C) for a sample is then normalized by subtracting the target gene Ct from its specific cyclophilin Ct (Table 1.5, B), therefore adjusting all individual samples to the expression of a common housekeeping gene (ΔCt, Table 1.5, D). The resulting value for each sample is then further expressed as the difference in ΔCt for the sample compared to the difference in ΔCt for the vehicle control (ΔΔCt, Table 1.5, E) with the lowest ΔCt value across experiments. ΔΔCt provides a value that is equal to how many cycles each sample is from the experimental control. The final calculation yields a relative quatitation of mRNA expression for each sample by expressing the results as fold induction above the experimental level control. All studies were repeated a minimum of five times, unless otherwise indicated, and representative data are presented.

1.2.1.5 Statistical Analysis

Data are presented as ± SE of the mean and statistically analyzed, using Dunnett's T-test ($P < 0.05$). Each determination was performed in triplicate ($n = 3$ wells) and the data presented are from one of five separate experiments, unless otherwise indicated, giving qualitatively similar results.

1.2.1.6 Results and Discussion

Microscopy analysis. Phase contrast and electron micrographs of three-day old primary human hepatocytes cultured with Matrigel® in a well defined serum-free culture medium are presented in Figure 1.1. Primary human hepatocytes cultured with Matrigel® are organized as acinar structures and acquire a 3-dimensional (3D) configuration, cells retain cell polarity and phenotype (Figure 1.1(a)) and viability for several weeks. This technique, where freshly isolated hepatocytes are suspended and plated in a culture media containing diluted Matrigel® (0.35 mg/ml) and then overlaid with a second layer of Matrigel, allows the development of a 3D configuration. Cells cultured in this fashion reorganize into structures that are characteristic of the tissue of origin, adopting an *in vivo*-like morphology (Berthiaume *et al.*, 1996; Davila and Morris, 1999; Farkas and Tannembaum, 2005;

Table 1.3 *Oligonucleotides for PCR primers for human CYC, CYP450s, FACO, UDPGT, GST and ST*

Target gene, accession #	5′ Sense primer (5′ to 3′)	3′ Anti-sense primer (5′ to 3′)	Fragment size (bp)
Cyclophilin (CYC), #Y00052	CTTgTCCATggCAAATgCTg	gTgATCTTCTTgCTggTCTTgC	179
CYP1A1, #K03191	gATgAgAACgCCAATgTCCAg	CTgCCAATCACTgTgTCTAgC	175
CYP1A2, #NM_000761	ACAACgCTgAATggCTTC	gCTgAACTCCAgTTgCTgT	266
CYP2B6, #X13494	CACTCATCAgCTCTgTATTCg	gTAgACTCTCTCTgCAACATgAg	355
CYP3A4, #D11131, #M18907	TgCTATgAgACTTgAgAgg	gCAAACCTCATgCCAATgCAg	236
CYP4A11, #NM_000778	TATggCCTTCACCACAACC	TCAACACAAgTCgTgCAATgg	243
CYP2E1, #J02625	ATgAAgCAACCCgAgACACCA	AACAACTCCATgCgAgCCAg	234
CYP2C9, #M61855	AATggACATgAACAACCCTCA	CTCAgggTTgTgCTTgTCgT	158
CYP2D6, #M33388	CgTgACATCgAAgTACAgg	gAgAAgCTgAAgTgCTgCAgC	261
Fatty acyl-coA oxidase (FACO), #U07866	gTTCgAgCAAgTgAggCAC	CAAgCACAgAgCCAAgTgTC	294
UDP-glucuronosyltransferase (UDPGT-PB), #J05428	gCATCTACgAggCAATCTACCA	CACATCCAAAgAgTggTACTg	343
Glutathione-*S*-transferase (GST)-Ya, # M14777	gAgATTgATgggATgAAgC	AggTAgTCTTgTCCATggCTC	268
Hydroxysteroid sulfotransferase (ST), #X84816	CTgAgTTATgAggAgCTg	CTCAgAAgTTgTgCTTTgTCC	196

Table 1.4 *Fluorogenic probe sequences for human CYC, CYP450s, FACO, UDPGT, GST and ST. TaqMan probes were designed in accordance to the guidelines issued by Perkin-Elmer*

Target gene	Fluorogenic probe (TaqMan)
Cyclophilin (CYC)	CCACAATATTCATgCCTTCTTTCAC
CYP1A1	CTTggATCTTTCTCTgTACCCTgg
CYP1A2	CAgCATCATCTTCTCACTCAAgg
CYP2B6	CCAAggACCTCATCgACACCTACCT
CYP3A4	CCAAgCTATgCTCTTCACCgTgAC
CYP4A11	TCTgCTCAACACAgCCACgCTTTC
CYP2E1	ACAgTCgTAgTgCCAACTCTggACTC
CYP2C9	ACAAgTCAACTgCAgTgTTTTCCAAgCT
CYP2D6	CACTCATCACCAACCTgTCATCggT
Fatty acyl-coA oxidase (FACO)	CAACCAAAgCAACAgCATCTgAgC
UDP-glucuronosyltransferase (UDPGT-PB)	CAATCCAgAAgACTgCTCgATCCA
Glutathione-*S*-transferase (GST)-Ya	CAgACCAgAgCCATTCTCAACTAC
Hydroxysteroid sulfotransferase (ST)	CTCAAgAACAgCTCCTTTCAgAgC

LeCluyse *et al.*, 2005) As indicated by electron microscopy (Figure 1.1(b)), these cells exhibit distinctive structural characteristics of *in vivo* hepatocytes, including numerous mitochondria, bile canaliculi, nuclei, glycogen, gap junctions and a rich smooth and rough endoplasmic reticulum. This suggests the presence in this cell system of the machinery needed for the cells to express a wide array of liver-specific functions

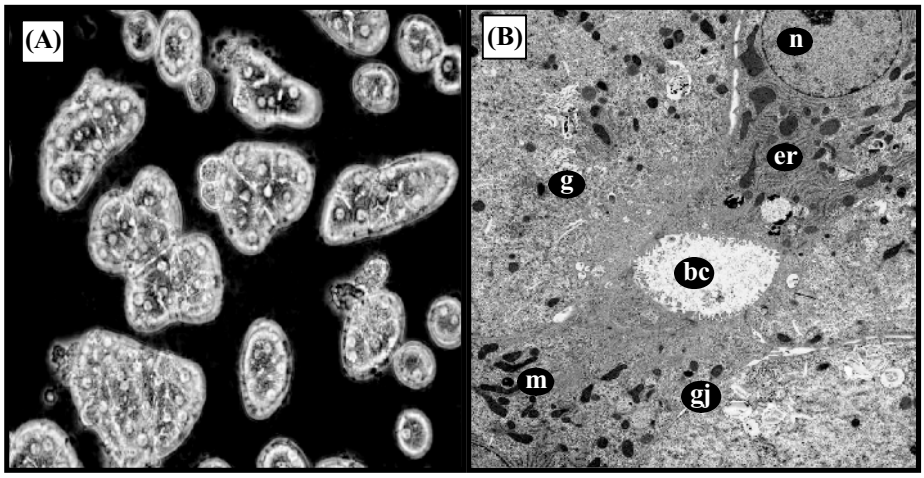

Figure 1.1 *Phase contrast (A, ×320) and electron micrographs (B, × 1200) of human hepatocytes plated and overlaid with Matrigel® for three days. Freshly isolated human hepatocytes suspended, plated and cultured with Matrigel at (0.35 mg/ml) are reorganized as acinar strucutures and acquired a 3D configuration. The electron micrograph shows a well-defined nucleus (n), endoplasmic reticulum (er), mitochondria (m), bile canaliculi (bc), glycogen droplets (g) and gap junctions (gj)*

Table 1.5 *Example of the results generated by the ABI 7700 sequence detector, showing TaqMan calculations to yield relative quantitation (mRNA expression)*

A Treatment	B Cyclophilin-Ct	C Target gene-Ct	D ΔCt	E $\Delta\Delta$Ct	F Relative quantitation
Control	18.2	32.45	(Control)C $-$ B $=$ 14.25	ΔCt Control $-$ ΔCt Control $=$ 0.00	$2^{-\Delta\Delta Ct}$(Control) $=$ 1.00
Sample A	17.2	28.45	(Sample A)C $-$ B $=$ 11.25	ΔCt Sample A $-$ ΔCt Control $=$ $-$3.00	$2^{-\Delta\Delta Ct}$(Sample A) $=$ 8.00
Sample B	17.5	26.83	(Sample B)C $-$ B $=$ 9.33	ΔCt Sample B $-$ ΔCt Control $=$ $-$4.92	$2^{-\Delta\Delta Ct}$(Sample B) $=$ 30.27

Measurement of several Phase I and II biotransformation enzyme expressions in primary human hepatocytes. In this study, we found that both the constitutive and inducible expression of the major families and subfamilies of human mRNA CYP450 (CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2D6, CYP3A4, CYP2E1, CYP4A11) and Phase II (UDPGT2B7, SULT2A1, GSTYa) enzymes, as well as the rate of protein synthesis, are maintained in culture for several days. The rank ordering for the average basal mRNA expression levels of human CYP450 and Phase II isoforms expressed in this primary cell culture system from the lowest to the highest was as follows: CYP4A11 < CYP2B6 < CYP1A1 < SULT2A1 < CYP3A4 \ < CYP2D6 < CYP1A2 < CYP2E1 < GSTYa < CYP2C9 < UDPGT2B7 < FACO. The expression of Phase I and II enzymes at the mRNA, protein and activity levels (e.g. CYP1A and CYP3A) in three-day old cell cultures are reported in Figures 1.2, 1.3 and 1.4, respectively.

In agreement with other investigators (Bowen *et al.*, 2000; LeCluyse, 2001; Strom *et al.*, 2001), we found that CYP1A1, CYP1A2, CYP2B6 and CYP3A4 are inducible by 3MC, and PB and RIF, respectively, in human hepatocytes (Figures 1.2 and 1.3). Interestingly, the basal levels of CYP3A4 and CYP2B6 in human hepatocytes cultured by a Matrigel®–Matrigel® system did not disappear within the 48 h in culture as previously reported by others (Kocarek *et al.*, 1992; Richert *et al.*, 2006). One explanation would be the difference of the substrata and the culture media components used in these studies. However, in agreement with others (Donato *et al.*, 1995; Silva *et al.*, 1998), some variability in the induction of CYP3A and CYP2B among the donors was observed. Seven out of ten liver donors responded to RIF at 10 μM, while two out of ten cell cultures responded to PB at 100 μM. Furthermore, we have also found that RIF induces CYP1A1 mRNA in all of the human hepatocytes tested. CYP2C9, CYP2D6 and CYP2E1 were found to be unaffected by the inducing agents used in this study. This lack of responsiveness of these enzymes has been previously documented and may be explained by genetic polymorphism, differences in substrate specificity or the high constitutive levels of the enzyme (Elkins and Wrighton, 1999; Guengerich, 1997; Mimura *et al.*, 1993; Waxman *et al.*, 1991). In addition, the disruption of circulating levels of growth hormone patterns *in vitro* may have altered the constitutive levels and inducibility of some enzymes, e.g. of CYP2D6 and CYP2C9 (Gonzalez, 1996; Neuman *et al.*, 1993; Prough *et al.*, 1996; Waxman *et al.*, 1991). These secondary contributors to enzyme induction were not studied as part of this investigation. Moreover, we found that CLO was not a good inducer of CYP4A11 and FACO; however, we have found that WY14,643 and cyprofibrate at 20 μM are good inducers of CYP4A11, 20- and 7-fold higher than controls, respectively (data not shown).

To further characterize this *in vitro* human hepatocyte system, we studied the effects of liver enzyme inducers on the expression of several Phase II enzymes, UDPGT2B7, GSTYa, and SULT2A1 at the mRNA level (Figure 1.2(g–l)). In contrast to the expression of CYP450 enzymes, Phase II enzyme expression and activity have been scarcely studied in human liver samples. One reason is the lack of specific substrate probes for the various isoenzymes and the lack of sensitive analytical methodology (Burchell and Coughtrie, 1989; Eaton and Bammler, 1999). However, RT–PCR technology allows us to assess the ability of a drug to modulate hepatic Phase II gene expression, thus offering a specific and rapid alternative to quantification of gene induction by immunodetection or substrate metabolism. UDPGT2B7, GSTYa and SULT2A1 were found to be expressed at a high basal

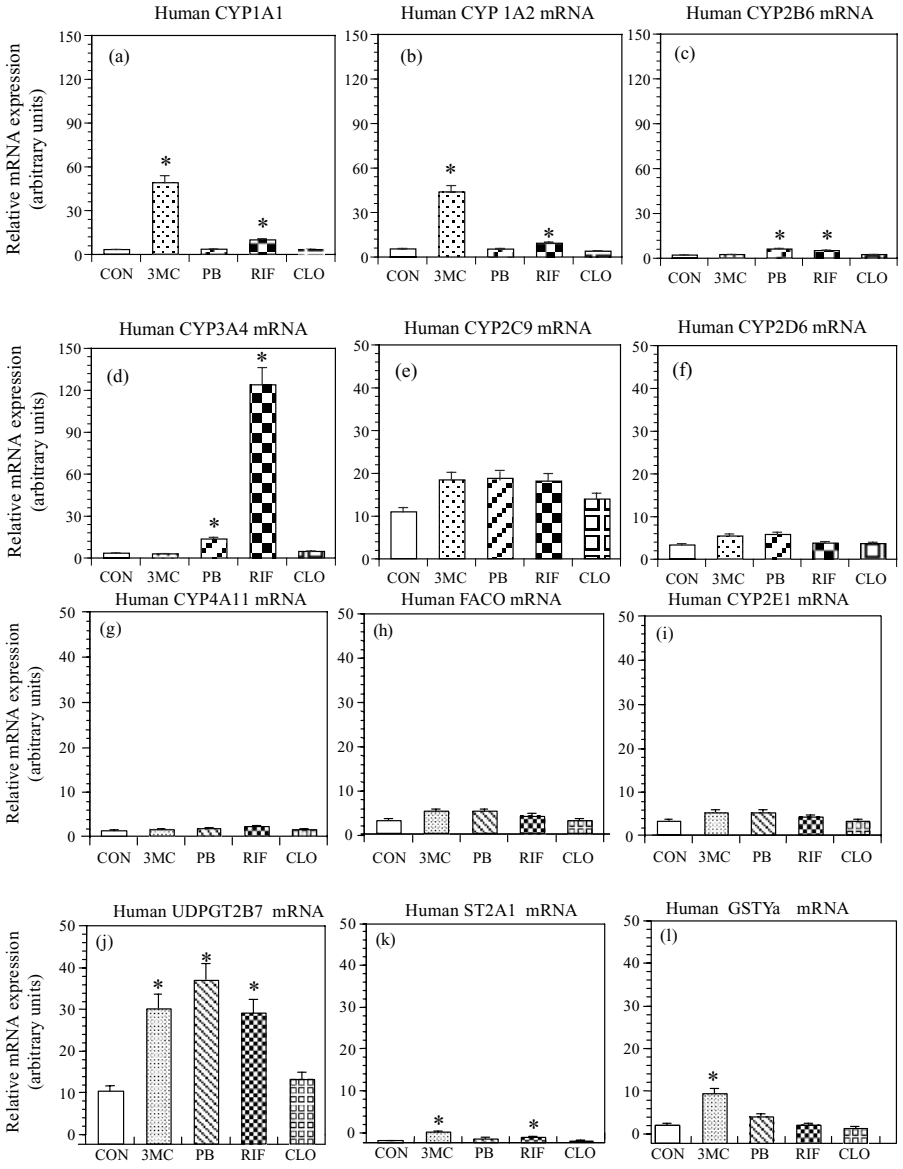

Figure 1.2 *Analysis of mRNA (TaqMan) levels for the analysis of CY450 expression in human hepatocytes. Data represent the results obtained from the analysis of Phase I and II biotransformation enzyme expression in human hepatocytes cultured with diluted Matrigel ® following exposure to prototypical CYP450 inducing agents (3MC, 1 μM; PB, 100 μM; RIF, 10 μM; CLO, 50 μM). The effects of CYP450 enzyme inducers on the expression of CYP1A1 (a) and CYP1A2 (b), CYP2B6 (c), CYP3A4 (d), CYP2C9 (e), CYP2D6 (f), CYP4A11 (g), FACO (h) CYP2E1 (i), UDPGT2B7 (j), ST2A1 (k) and GST-Ya (l) are presented. Data presented are expressed as the mean (± SE) relative mRNA expression levels for each enzyme (fold induction). Data are from one of five separated experiments (N = 3 dishes) using five different donors giving qualitatively similar results*

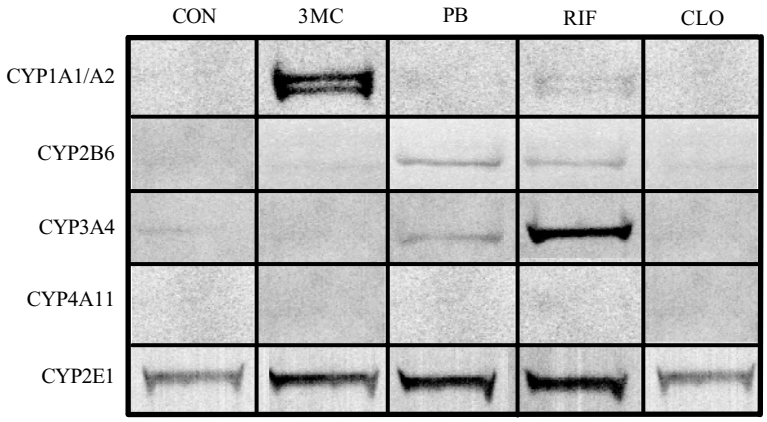

Figure 1.3 *Analysis of CYP450 apoprotein expression in human hepatocytes cultured in the presence of diluted Matrigel ® using Western immunoblotting. Microsomes were prepared from cells exposed to either vehicle (CON, DMEM/0.1 % DMSO) or prototypical CYP450 enzyme inducers (3MC, 1 μM; PB, 100 μM; RIF, 10 μM; CLO, 50 μM) for 48 h. Cells were lyzed and microsomal proteins analyzed for expression of CYP1A1/CYP1A2, CYP2B6, CYP3A4, CYP4A11 and CYP2E1 protein by Western immunoblotting. Data are from one of three separated experiments using three different donors giving qualitatively similar results*

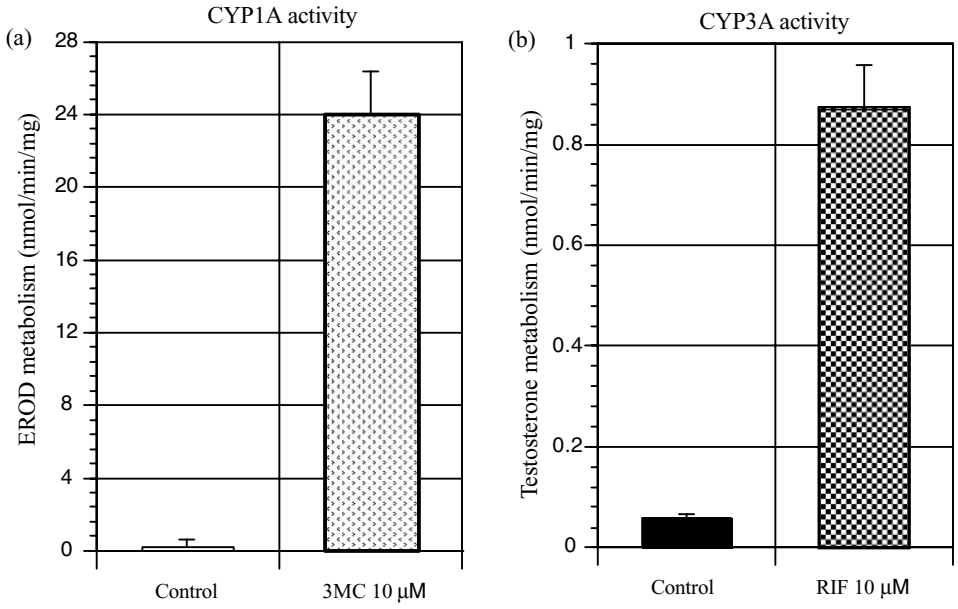

Figure 1.4 *Analysis of CYP1A (a) and CYP3A (b) activity levels by HPLC and fluorometric analysis in primary human hepatocytes cultured with Matrigel® after treatment with RIF and 3MC, respectively*

level in this culture system but not markedly induced by chemical treatment. We found that UDPGT2B7 was inducible by 3MC, PB and RIF, and that SULT2A1 was inducible by 3MC and RIF, and that GSTYa was inducible by only 3MC (Figure 1.2(g–l)). 3MC and PB are known inducing agents of Phase II enzymes in the liver (Eaton and Bammler, 1999; Wormhoudt *et al.*, 1999). Data presented here support the potential utility of this *in vitro* system and RT-PCR for assessing the induction of Phase II conjugating enzymes in human hepatocytes by xenobiotics.

In vivo *and* in vitro *comparison of Phase I and II enzyme mRNA levels.* As part of the validation study, *in vivo* and *in vitro* comparison of Phase I and II enzyme mRNA levels were performed. In these studies, mRNA levels were measured from one hundred human liver tissues and from fourteen separated human hepatocyte cultures (3 day old cells) using real-time PCR. The mean of the target genes was calculated and reported as ΔCt values (see Table 1.5, D). As indicated in Table 1.6, the mRNA basal levels of CYP1A1, CYP1A2, CYP2C9, CYP2D6, CYP2E1, GSTYa and UDPGT2B7 enzymes in primary hepatocytes were highly maintained for three days and comparable to those of liver tissues. However, CYP2B6, CYP3A4, CYP4A11, FACO and ST2A1 mRNA basal levels were found to be decreased but returned back to levels comparable to liver tissues when CYP450 inducers such as rifampicin (RIF, 10 μM), phenobarbital (PB 100 μM) and WY-14,643 (20 μM) were administrated to the cell cultures. The establishment and validation of a cell culture system which mimics drug biotransformation *in vivo* is particularly important when considering the elimination of NCEs that generate toxic or reactive intermediate metabolites early in the development process.

Differential expression of CYP3A by RIF, OMZ, TAM, PCN and HC in rat and human hepatocytes. The last set of validation studies were designed to examine the differential expression of CYP450 enzymes (CYP1A, CYP2B, CYP3A, and CYP4A) in human and

Table 1.6 *In vivo and in vitro comparison of relative CYP450 enzyme (mRNA) levels in human liver (data are presented as ΔCt values)*

Enzyme phase I–II	Δ-Ct *in vivo*	Δ-Ct *in vitro*	Δ-Ct *in vitro* induced cells
1A1	8.9	8.5	—
1A2	4.5	2.5	—
2B6	6.4	8.7	6.2 (Phenobarbital)
2C9	0.9	1.0	—
2D6	2.9	3.4	—
2E1	−2.3	2.0	—
3A4	0.9	5.2	−0.4 (rifampicin)
4A11	4.2	10	3.8 (WY-14,643)
FACO	1	4.6	1.7 (WY-14,643)
ST2A1	1	4	3 (Phenobarbital)
GSTYa	1.8	1.1	
UDPGT2B7	−0.2	0.11	

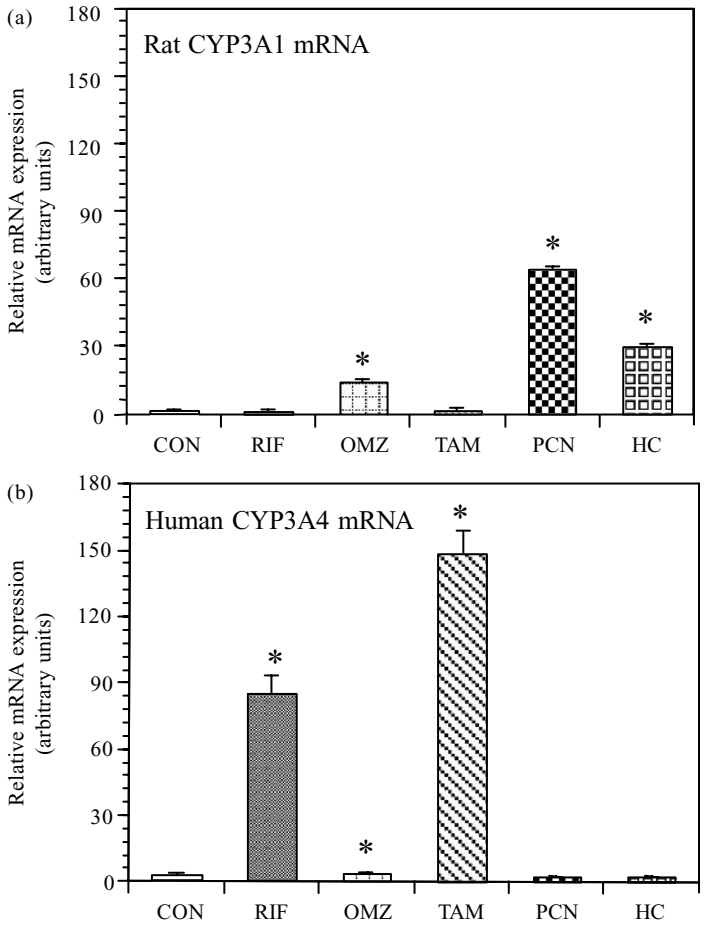

Figure 1.5 *Comparison of CYP3A induction by rifampicin (RIF, 10 μM), omeprazole (OMZ, 20 μM), tamoxifen (TAM, 10 μM), pregnenolone-16α-carbonitrile (PCN, 1 μM) and hydrocortisone (HC, 20 μM) in rat (a) and human (b) hepatocytes using RT-PCR and fluorogenic cDNA probes (TaqMan). Data presented are expressed as the mean (± SE) relative mRNA expression levels for the enzyme (fold induction). Data are from one of three separate experiments using three different donors giving qualitatively similar results*

rat hepatocytes. Results from our studies demonstrate a clear species difference in the induction of these enzymes in rat and human hepatocytes. In particular, CYP3A expression was found to be differentially induced by a number of known CYP3A inducers (Figure 1.5). RIF and TAM were found to markedly induce CYP3A expression in human hepatocytes, whereas OMZ, PC and HC were found to induce CYP3A expression in rat hepatocytes but not in human hepatocytes. These differences in expression of CYP3A, CYP2B and CYP4A enzymes following exposure to xenobiotics have been attributed in part to the

ability of xenobiotics to differentially activate several members of the nuclear hormone receptor (NHR) superfamily such as PXR/SXR, CAR and PPAR, respectively (Giguere, 1999; Peraza *et al.*, 2006; Roberts, 1999; Schuetz *et al.*, 2000; Xie *et al.*, 2000). The quantity or the quality of these nuclear hormone receptors and the presence of a truncated, inactive form of the receptor, such as in the case of PPARα (Gonzalez and Lee, 1996; Peraza *et al.*, 2006; Peters, 2005), have been suggested as potential factors responsible for such species differences in responses to chemicals. In addition, it has been reported that the species origin of the receptor, rather than the promoter structure of the gene, e.g. CYP3A, dictates the species-specific pattern of CYP3A inducibility (Luo *et al.*, 2004; Xie *et al.*, 2000). The ability of these primary rat and human hepatocytes to respond to a variety of chemicals and to detect differences in CYP450s induction suggests the presence in this cell system of multiple regulatory pathways and of transcription factors associated with functional receptors (Li and Chiang, 2006).

Variations in drug metabolism by different species represent a challenge to early metabolism and safety screening. Differential drug-induced expression of liver cytochrome P450 (CYP450) enzymes has been reported to be responsible in part for variations in metabolism and toxicity among species (Hengstler *et al.*, 1999; Kocarek *et al.*, 1995; Lewis *et al.*, 1998). These striking differences in drug-induced CYP450 expression among animal species make it difficult to rely solely on the use of laboratory animals as surrogate species for predicting drug metabolism and toxicity in humans. Therefore, human cell-based systems are important tools in assessing and predicting human drug metabolism and toxicity.

Primary human hepatocytes cultured in a refined cultured condition using Matrigel® provides an attractive screening strategy for testing the qualitative and quantitative induction of human CYP450 enzymes by NCEs and to extend our understanding of the role of these NHRs in regulating CYP450 expression by xenobiotics. Results from these studies also highlight the importance of assessing enzyme induction across species as it relates to the potential for drug–drug interactions and metabolism-mediated toxicity. In conclusion, we have established a 3D-human cell culture system using a Matrigel®–Matrigel® configuration and a quantitative RT-PCR method using fluorogenic cDNA technology (TaqMan) to examine the effects of xenobiotics on the expression of a number of human CYP450 and Phase II conjugating enzymes. We have demonstrated that several Phase I and II liver biotransformation enzymes can be induced by xenobiotics in this system. In addition, we have further demonstrated the need for using human cell culture induction systems to assess the metabolism and toxicity of xenobiotics in the human population.

1.2.2 Stem Cell-Derived Hepatocytes

New platforms for discovery, target validation and predictive toxicity screening in therapeutic areas such as arthritis and inflammation and cardiovascular are needed for a positive identification of new therapeutic agents in preclinical development. Included in the testing scheme is the evaluation of compounds or recombinant gene products on *in vitro* cell systems, such as primary cell cultures, which best reflect the tissue of origin. While primary hepatocytes represent the desired model system for drug metabolism and toxicity, the limited supply of human tissues and the variable quality and interindividual differences hinder its routine use.

Stem cell technology provides unprecedented opportunities not only for investigating new ways to prevent and treat a vast arrays of diseases but also for changing the way we identify new molecular targets, discover and develop new drugs, as well as test them for safety. Because stem cells are self-renewing population of cells, they can be continuously cultured in an undifferentiated state and give rise to more specialized cells of the human body, such as heart, liver, bone marrow, blood vessels, pancreatic islet and nerve cells. Therefore, it offers an important new tool to develop unique *in vitro* model systems for testing drugs and chemicals and potentially predict or anticipate toxicity in humans (Davila *et al.*, 2004; Sinz and Kim, 2006). Recent advances in the isolation and culture expansion of multi-potent stem cells from embryonic or adult tissue offer these cell systems as alternative sources of progenitor cells for wide number of tissue-specific cell types. Although human embryonic stem cells (HESCs) retain the potential to differentiate into all of the major cell types of the body, controversy surrounding their research use has limited their application to date. However, adult stem cells, which can be isolated from non-human embryonic tissue, retain their multipotency in culture, and therefore offer a useful cell culture system for the evaluation of compounds during drug discovery and development.

Significant and continuing advances in stem cell research to generate an reliable supply of fully functional human hepatocytes has the potential to provide a consistent source of normal and functional human cells that would more closely predict the impact of a new drug on human livers in the body. The ability to obtain an unlimited supply of hepatocytes that retain the expression and activity of drug metabolism enzymes would revolutionize toxicity testing, address the largest bottleneck in new drug research and accelerate the drug development process. While the biological qualities of an optimal hepatocyte source could be debated, there are at least four properties that would seem essential to satisfy most of the requirements of stem cell-derived hepatocytes. The stem cell-derived hepatocyte must:

(1) be routinely available;
(2) be easily cryopreserved and restored to culture;
(3) provide cells with a metabolic profile observed in adult hepatocytes;
(4) provide for a diverse genetic background of donors.

Although there are reports of stem cell-derived hepatocyte-like cells, it is safe to say that there are currently no published reports of cells that fulfill all of the above mentioned requirements. The properties of the ideal stem cell must be kept in mind, as certain cell types such as bone marrow, peripheral blood or amnion, would be expected to be routinely available and in sufficient numbers to provide for a wide range of genetic backgrounds; cell types such as embryonic stem cells (ES) may only be available from a far more limited number of individuals. These considerations could be critically important to eventual drug metabolism and toxicology studies with hepatocytes derived from these stem cell sources.

The number of published reports concerning the differentiation of specific cell types into hepatocyte-like cells is too numerous to cite in the context of this limited review. The sources of stem cells reported to differentiate along a hepatic lineage is considerably smaller. By far the most common reports of non-hepatic cells becoming hepatocytes are from the bone marrow, peripheral or placental cord blood or embryonic stem cells of mouse or human origin.

1.2.2.1 Bone Marrow-Derived Hepatocyte-Like Cells

Petersen *et al*. (Petersen *et al.*, 1999) were the first to suggest that bone marrow cells could differentiate to hepatocytes. Because putative hepatic stem or progenitor cells express surface marker antigens such as Thy-1 or c-kit which are also found on hematopoietic cells, it was logical to assume that they might have a common source. This initial report was soon followed by several others confirming and extending the initial observations demonstrating *in vitro* and/or *in vivo* differentiation of bone marrow-derived cells to hepatocytes (Alison *et al.*, 2000; Lagasse *et al.*, 2000; Krause *et al.*, 2001). The initial exuberance was soon followed by stunning reports that most of the hepatic characteristics expressed in transplanted cells were the result of cell fusion with recipient hepatocytes (Wang *et al.*, 2003; Vassilopoulos *et al.*, 2003). Conflicting reports still remain in the literature. A consensus position is that there may be some low level differentiation of bone marrow-derived hematopoietic cells to hepatocytes although it is most likely quite limited (Wagers *et al.*, 2002; Cantz *et al.*, 2004; Yamaguchi *et al.*, 2006). *In vitro* differentiation of bone marrow cells to hepatocytes would seem to be devoid of the 'fusion artifact' and might prove a useful method to derive hepatocyte-like cells from bone marrow. There are now several reports of the generation of hepatocyte-like cells from bone marrow cells propagated with growth factors such as hepatocyte growth factor (HGF) and/or basic fibroblast growth factors (bFGF) (Avital *et al.*, 2001, 2002; Fiegel *et al.*, 2003; Miyazaki *et al.*, 2002, 2004; Kawasaki *et al.*, 2005; Saji *et al.*, 2004). While several hepatic characteristics such as the expression of albumin, alpha-1-antitrypsin, tyrosine aminotransferase, and urea production, were cited as evidence of hepatic differentiation, there are no reports of the production of cells with a complete set of adult hepatocyte characteristics. These initial results are encouraging, and if full hepatic differentiation could be completed from hematopoietic cells *in vitro*, they could be an extremely useful future source of hepatocytes.

It is interesting that the cell type thought to be responsible for fusion with recipient hepatocytes following bone marrow cell transplants are the myelomonocytic cells in the bone marrow (Willenbring *et al.*, 2004). Investigators have tried to induce hepatic differentiation in cultured monocytes (Ruhnke *et al.*, 2005a,b). These authors report albumin secretion and urea production at rates lower than, but similar to, human hepatocytes and ethoxycoumarin-*O*-deethylase activity which was inducible with 3-methylcholanthrene and low but measurable CYP3A4 mediated drug metabolism.

Tissue such as bone marrow is complex and contains many cell types. In addition to the hematopoietic cells, there are other cell types in bone marrow. In general, hematopoietic cells from bone show limited proliferation in culture and grow as a non-adherent cell type. A second cell type found in bone marrow grows as adherent cells and shows much greater potential to proliferate. This adherent fraction can be isolated from bone marrow, umbilical cord blood and even peripheral blood. Although not necessarily the same cell type, the adherent fraction is alternately referred to as stromal cells, fibroblasts or mesenchymal stem cells (MSCs). There are several reports of the differentiation of mesenchymal cells to hepatocyte-like cells, both *in vitro* and *in vivo* (Beerheide *et al.*, 2002; Kakinuma *et al.*, 2003; Kogler *et al.*, 2004; Newsome *et al.*, 2003; Schwartz *et al.*, 2002; Nonome *et al.*, 2005). Markers identified were mainly markers of early hepatic differentiation. In a careful study of MSC-derived hepatocyte-like cells, Ott and coworkers concluded that although the cells expressed some hepatic markers following transplantation into the liver, the MSC-derived

cells did not express several crucial hepatic markers such as cytokeratin expression (Sharma *et al.*, 2005). The lack of cytokeratin expression would suggest that the cells remained mesenchymal and did not fully differentiate into an epithelial cell type such as a parenchymal hepatocyte. However, more recent publications keep the idea of mesenchymal stem cell-derived hepatocytes alive (Aurich *et al.*, 2006). More information on the expression mature liver genes such as the CYP450 genes and adult hepatocyte metabolic activities are needed to determine the actual state of differentiation of these hepatocyte-like cells (Hengstler *et al.*, 2005; Teramoto *et al.*, 2005)

1.2.2.2 Embryonic Stem Cell-Derived Hepatocytes

It might seem that the derivation of hepatocytes from embryonic stem (ES) cells might be the major focus of researchers trying to generate hepatocytes from stem cells. However, this is not the case. More publications report hepatic differentiation of stem cells from sources other then ES cells. There is good preliminary data to suggest that hepatocyte-like cells can be derived from ES cells (reviewed in Teramoto *et al.*, 2005, and Lavon and Benvenisty, 2005). While most reports concern mouse ES cells, there are reports of human ES cell-derived hepatocyte-like cells (Lavon *et al.*, 2004; Rambhatla *et al.*, 2003; Shirahashi *et al.*, 2004; Soto-Gutierrez *et al.*, 2006). Each of the reports show the expression of early markers of hepatic differentiation as well as some more differentiated functions such as some limited drug ammonia metabolism or urea production. There are no reports of robust drug metabolism by any ES-derived hepatocyte-like cells. While quite preliminary, these initial results suggest that at least some of the ES cells are able to differentiate along a hepatic lineage. It is not entirely clear from the published work if differentiation is being directed by the experimental design or if spontaneous differentiation of ES cells is occurring in the cultures. More work will be needed to fully characterize the ES-derived hepatocytes and to optimize and direct the differentiation process. Results reported with pluripotent cells like ESs is complicated by the possibility of differentiation along other pathways. Hepatic marker genes such as albumin, alpha fetoprotein and transthyretin are not only expressed in developing liver, they are also expressed in yolk sac. Yolk sac performs many liver-like functions during fetal life. Thus, it is difficult to determine if the expression of hepatic marker genes is evidence of hepatic differentiation or simply differentiation to visceral endoderm of the yolk sac. A recent report by Asahina *et al.* (Asahina *et al.*, 2004) suggests that the expression of CYP enzymes such as CYP7A1 was the only definitive marker of actual hepatic differentiation and could distinguish between differentiation of ES cells to visceral endoderm (yolk sac) and the definitive endoderm from which the liver and pancreas eventually are derived. By this measure, the expression of CYP7A1 in stem cell-derived hepatocyte-like cells indicated authentic hepatic differentiation.

1.2.2.3 Hepatocyte-Like Cells Derived from other Sources

There are a number of reports to suggest that hepatocyte-like cells can be derived from other tissue sources. Hepatocyte-like cells were generated from pancreatic tumor cells following long-term exposure to dexamethasone (Tosh *et al.*, 2002). A number of liver-enriched genes we detected included glucose-6 phosphatase, carbamoylphosphate synthetase (CPS I) and glutamine synthetase (GS), as well as some CYP450 and Phase II enzymes. If high levels of mature liver enzymes could be induced, the transdifferentiation of other cell types into

hepatocytes might prove to be a useful cell source. Miki *et al.* (Miki *et al.*, 2005) reported that cells with characteristics of pluripotent stem cells could be identified and isolated from human amnion. The amnion-derived stem cells could be induced to differentiate into cell types from all three germ layers, including cells with hepatocyte characteristics. The expression of many CYP450 genes and metabolic activity consistent with the expression and induction of CYP1A family proteins was reported (Davila *et al.*, 2004). A significant observation was that CYP7A1 was expressed in amnion-derived hepatocyte-like cells, suggesting that the culture conditions used in the experiments induced differentiation of the stem cells to definitive endoderm and along an authentic hepatic lineage.

1.2.2.4 Hepatic Stem or Progenitor Cells

It is far beyond the scope of this discussion to thoroughly review the topic of hepatic stem cells. Recent reviews have updated this area (Forbes *et al.*, 2002; Walkup and Gerber, 2006). Recent reports suggest the identification and isolation of putative hepatic stem or progenitor cells (Dan *et al.*, 2006; Nowak *et al.*, 2005; Schmelzer *et al.*, 2006). The expression of some mature liver functions and the ability to expand the cells in culture suggest that the hepatic stem or progenitor cells identified in these studies could be a useful source of human hepatocytes (Dan *et al.*, 2006; Nowak *et al.*, 2005).

1.2.2.5 Summary and Suggestions for Future Studies

In here are a number of sources of cells which all give rise to cells with characteristics of hepatocytes. However, to date none of the stem cell sources, including those derived from the liver, have been shown to produce cells with full mature liver functions. Thus, additional effort is warranted with each stem cell type with the goal to improve hepatic differentiation, *in vitro*. A number of hepatic genes have been used in the studies cited above as markers of hepatic differentiation. In most cases, the reports identified markers of early liver development, such as the expression albumin and alpha 1-antitrypsin, cytokeratins or human hepatocyte antigen. While these markers are useful to show differentiation along a hepatic lineage, the hepatocyte-like cells acquire the expression of these gene products early in the differentiation process. The expression of later markers of mature liver function, such as CYP450 gene expression and metabolic activity, are rarely reported. In addition, in most studies when mature liver genes were reported they were merely detected by qualitative methods such as immunohistochemistry or RT-PCR. For the field to move forward it will be necessary to get quantitative estimates of the expression of a number of mature liver functions in the stem cell-derived hepatocytes which can be compared to authentic adult human hepatocytes. Although not hepatocyte-specific, the CYP450 genes are expressed at higher levels in liver than most other tissues. In addition, because the CYP450 gene products are critically involved in drug metabolism and toxicology, the expression of these genes will be central to the utilization of the stem cell-derived hepatocytes for basic research. The important drug metabolizing genes of the cytochrome P450 (CYP) families are expressed relatively late in hepatic development and, as such, we propose that they are useful markers of hepatic maturation.

There are marked differences in the expression of the CYP enzymes between immature and mature hepatocytes. These differences in the expression of specific CYP450 genes can be used as a gage of hepatic maturation. It is believed that the CYP enzymes expressed early in hepatic development are needed and are responsible for the metabolism and elimination

of many endogenous compounds, such as steroids prostaglandins and retinoic acid. Both fetal and adult liver express genes of the CYP450 1A, 2C, 3A and 7A families; however, the expression of these genes even in fetal liver is generally in the range of a few percentage of normal adult levels. For most CYP enzymes there is a dramatic increase in expression in the postnatal period. In addition, the expression of specific family members differs in characteristic ways between fetal and adult liver which makes the expression of these genes diagnostic for the degree of hepatic differentiation. For example, Fetal liver expresses little of the adult form CYP1A2 (Maenpaa *et al.*, 1993) but expresses high levels of CYP1A1 (Hines and McCarver, 2002; Omiecinski *et al.*, 1990; Shimada *et al.*, 1996; Yang *et al.*, 1994). Adult liver expresses little CYP1A1 as its expression is slowly extinguished in the postnatal period. The expression of CYP1A2 increases after birth, however; it is one of the last of the CYP enzymes to develop in the postnatal period. Recent studies suggest that CYP1A2 levels may only reach 10 % of adult levels in the first postnatal month and may remain at or below 50 % of the adult levels out to one year (Sonnier and Cresteil, 1998; Tateishi *et al.*, 1997). We propose that the expression of CYP1A2, is an indication of 'true' hepatic maturation and the ratio of expression of CYP1A1 to CYP1A2 provide a measure of the maturation process. A similar expression profile exists for members of the CYP3A family. Fetal liver expresses high levels of CYP3A7 (> 98 %) and little CYP3A4 (< 2% of total 3A) (Schuetz *et al.*, 1994; Wrighton *et al.*, 1988; Yang *et al.*, 1994) while CYP3A4 is the major CYP enzyme expressed in adult liver. The expression of CYP3A7 is extinguished in most individuals over the first 6 postnatal months (Hines and McCarver, 2002; Stevens *et al.*, 2003). Some CYP3A7 expression remains in 10–20 % of individuals but even in these CYP3A7 would only be expected to account for < 20 % of total CYP3A expression in that individual. Even after birth, CYP3A7 is not immediately extinguished such that the ratio of CYP3A4/3A7 may not equal 1 until 2–5 years of age. There is a change in the ratio of expression of CYP3A4 to 3A7 during development with the ratio of 3A4/3A7 extremely low during fetal development and high in adults.

We suggest that the ratios of CYPs 3A4 to 3A7 and 1A2 to 1A1 will provide an estimate of the degree of hepatic differentiation of the stem cells. In addition to mRNA measurements, the metabolic activity of the individual CYP enzymes should be evaluated with probe drugs, and Western Blots should be run for individual CYP proteins. In this manner, direct comparisons can be made between stem cell-derived hepatocytes and authentic human hepatocytes for each endpoint studied. If implemented, suggestions like these will add quantitative analysis to this area of stem cell research.

The development of a stem cell-derived hepatocyte system to examine the potential of new chemical entities (NCEs) to cause hepatotoxicity by a metabolism-mediated process early during the discovery process will provide a competitive advantage within the Pharmaceutical Industry. This novel cell-based system will provide an efficient means of aiding rational drug design and selection, selecting species differences in their responses to xenobiotics and, most importantly, for reducing the probabilities of causing unexpected adverse reaction in the liver when the compound reaches the market place.

In addition to primary hepatocytes cultured in a sandwich configuration and stem cell-derived hepatocytes, several recent developments in biological engineering and cellular biology have enabled a variety of new models for studying hepatotoxicity *in vitro*. Genetically engineered cells, as well as so called 'three-dimensional (3-D) hepatocyte bioreactors', have been established recently and are just now being applied to further our understanding of

toxic events in the liver. In the case of engineered cells, these systems seek to provide a basis for more mechanistic insight into our current understanding of drug-induced hepatotoxicity, whereas the 3-D perfused models attempt to re-establish the *in vivo* microenvironment as well as build and improve upon some of the limitations of existing primary cell models.

1.2.3 Cell Lines and Genetically Engineered Cells

Advances in molecular biology have revolutionized our ability to investigate and dissect complex mechanisms of hepatotoxicity. Although primary hepatocytes remain the 'gold standard' for investigating the potential of a novel therapeutic agent to elicit a toxic event, several cellular models can be applied to address similar predictions of hepatotoxicity. Cell lines are available for studying various mechanisms of toxicity and these cells and cell-derived systems are becoming better defined and understood as new data emerge from the laboratories and into the literature. Some systems have gained application in the field of hepatotoxicity as they derived from liver-based, cancer cell systems (e.g. HepG2, WIF-B9 cells), whereas some systems have been engineered/created to study specific mechanisms of hepatotoxicity where aspects of a given mechanism are understood (e.g. CYP-engineered cells, BSEP-expressing cells or vesicles). The utility and predictability of these systems relies heavily upon understanding how each system is related to the intact liver *in vivo*. Although each system may not express the full complement of proteins responsible for overall hepatic function, we are learning now that each system may have merit for predicting hepatotoxic events very early on in the drug-discovery process.

1.2.3.1 WIF-B9 Cells

A sub-clone of the WIF 12-1 cell line, WIF-B9 cells are derived from Fao rat hepatoma cells fused with WI38 human fibroblasts. As a result of their multi-species origin, these cells co-express many rat and human liver-specific proteins (Decaens *et al.*, 1996; Ihrke *et al.*, 1993; Shanks *et al.*, 1994). These cells are capable of synthesizing albumin and were shown recently to express an array of rat and human P450 isoforms, some of which are inducible (Biagini *et al.*, 2006a; Decaens *et al.*, 1996). In addition to metabolic capacity, WIF-B9 cells are polarized, such that they establish functional canalicular spaces into which the cells are capable of transporting bile acid-like molecules (Bravo *et al.*, 1998). Interestingly, these cells do not express the major human protein responsible for the uptake of bile acids into the cell (NTCP; sodium-taurocholate co-transporting polypeptide). However, they may express functional rat Ntcp and/or human OATPs (organic anion transporting polypeptides) that also have been shown to uptake bile acids (Konieczko *et al.*, 1998). As in the case of NTCP, WIF-B9 cells do not express the human isoform of BSEP (bile salt export pump), the transport protein responsible for canalicular excretion of bile acids across the canalicular membrane. Therefore, due to the functional expression of rat Ntcp and rat Bsep (demonstrated by the vectorial excretion of a fluorescently labeled bile acid in these cells), the ability of this model to predict transporter-mediated hepatotoxicity (e.g. drug-induced cholestasis) would be limited to predictions based upon the rodent genes (Bravo *et al.*, 1998; Gradilone *et al.*, 2005). Although, this system may better reflect transporter-mediated toxicity relative to that in the rat, WIF-B9 cells have shown promise in the prediction of hepatic toxicity, as measured by general cytotoxicity measurements (Biagini *et al.*, 2006b). Specific evaluation of WIF-B9 cells for determination of alcohol-induced

liver injury indicated that CYP2E1 was functional and that alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) activity was intact and similar to that observed in isolated hepatocytes (Schaffert *et al.*, 2004). Moreover, the recent demonstration of aquaporin expression and localization suggests that this cell line may have utility in the prediction of toxic events that result in retarded bile formation in the liver (Gradilone *et al.*, 2005).

1.2.3.2 HEPG2 Cells

Probably the most studied cell line with respect to hepatotoxic endpoints is the human hepatoma HEPG2 cell line. HEPG2 cells were first identified and isolated from a Caucasian adolescent over two decades ago (Aden *et al.*, 1979; Knowles *et al.*, 1980). Since their discovery in 1980, these cells have been utilized to examine various mechanisms of hepatotoxicity from ethanol-induced liver damage to most recently a high-content-screening approach for predicting hepatotoxicity due to drug administration (Neuman *et al.*, 1993; O'Brien *et al.*, 2006). One characteristic of HEPG2 cells that makes them attractive for use in hepatotoxicity studies is the expression and inducibility of relevant drug metabolizing enzymes. Expression and function of the key Phase I enzymes CYP1A, CYP2B, CYP3A, and CYP2E as well as several Phase II enzymes (e.g. UDPGT and GST) has been demonstrated and several of these enzymes can be induced by prototypical enzyme inducers (Knasmuller *et al.*, 1998, 2004). However, it should be noted that not all drug metabolizing enzymes are present in HEPG2 cells; those that are may not be expressed at levels similar to those observed *in vivo* and may not respond to inducers similar to that in primary cells (Smith *et al.*, 2005). Nevertheless, some investigators have transfected metabolic enzymes into this cellular system to establish a more realistic model for hepatotoxicity. HEPG2 cell transfection with CYP3A4, followed by administration of compounds known to form toxic metabolites, resulted in P450-specific formation of reactive species, leading to measured cytotoxicity (Vignati *et al.*, 2005). Furthermore, following treatment with acetaminophen, HEPG2-CYP2E1 cells generated reactive metabolites that formed protein adducts and damaged mitochondria, hence suggesting that introduction of mechanism-specific enzymes known to cause toxicity into HEPG2 cells may yield a predictive model for evaluating novel molecular entities (Bai and Cederbaum, 2004). In addition to drug metabolizing enzymes, expression of nuclear transcription factors in HEPG2 cells has provided insight into mechanisms of hepatotoxicity. 'Upregulation' of adipose differentiation-related protein through PPARγ was demonstrated in HEPG2 cells, offering insight into the mechanism of hepatic steatosis (Motomura *et al.*, 2006). Furthermore, TCDD was able to upregulate CYP1A1 (as an indicator of oncogenesis) in HEPG2 cells through the aryl hydrocarbon receptor (Zhang *et al.*, 2006). However, one must interpret these data carefully as CYP1A1 induction in HEPG2 cells is much more sensitive to that observed in primary hepatocytes and may not translate to the *in vivo* response. Much like the P450 enzymes, nuclear receptor transfection into HEPG2 cells also has been utilized to understand the involvement of these molecular factors in various mechanisms of hepatotoxicity (McCarthy *et al.*, 2004; Vignati *et al.*, 2004).

HEPG2 cells also show promise for prediction of phospholipidosis, mitochondrial toxicity and oxidative stress (Bova *et al.*, 2005; Kessova and Cederbaum, 2005; Sawada *et al.*, 2005). Although these experimental systems have been optimized to predict specific hepatotoxic endpoints, the idea that HEPG2 cells can predict overall hepatotoxicity from a

mechanism-independent perspective is now becoming accepted. Evaluation of multiple endpoints through the use of high content screening (see Section 1.3 below) in HEPG2 cells suggests that this cellular system can detect human hepatotoxicity with 80% sensitivity and 90% specificity (O'Brien *et al.*, 2006). These results show promise for this cell line to predict overall hepatotoxicity and underscore that even after more than 25 years of research with this cell line, we continue to uncover the underlying biology of the system and develop means by which to exploit the cells to predict *in vivo* adverse events.

1.2.3.3 BSEP-Expressing Cell Lines

Modulation of normal bile salt export pump (BSEP) activity (e.g. through inhibition) has been identified as a major molecular mechanism responsible for drug-induced cholestasis observed in the clinic (Fattinger *et al.*, 2001; Funk *et al.*, 2001). Several methodologies have been created to assess the potential for novel therapeutic agents to interact with the BSEP and cause a clinical manifestation of cholestasis. Of the systems available, BSEP-transfected cells and vesicles derived from these cells show a high degree of concordance with clinical observations of cholestasis. One of the first and simplest experimental models involved the expression of a rodent BSEP using an Sf9 insect cell-based system, followed by formation of vesicles from the BSEP-expressing cells (Stieger *et al.*, 2000). The addition of the bile acid taurocholate, as well as ATP, to the vesicle system resulted in functional BSEP-mediated transport into vesicles. In addition, several known inhibitors (e.g. cyclosporine A, glibenclamide and rifampicin) were able to inhibit the transport of taurocholate by the BSEP. Although the cell lines expressing the rodent isoforms of the BSEP were generated very soon after the full-length clone was isolated, due to the presence of a cryptic bacterial promoter in the BSEP cDNA, the human isoform of the BSEP was much more difficult to clone and express. However, two groups were able to clone and express functional human BSEP into Sf9 cells and demonstrate a high degree of functional similarity of this protein to the rodent isoform (Byrne *et al.*, 2002; Noe *et al.*, 2002). Following the functional expression of the human BSEP, several groups have utilized these Sf9-derived vesicles to explain the observed hepatotoxicity of therapeutic agents such as nefazadone, ritonavir, and saquinavir (Kostrubsky *et al.*, 2006; McRae *et al.*, 2006). Most recently, the transfection of the human BSEP (coupled with human NTCP) into LLC-PK1-polarized cells has enabled the study of vectorial/polarized transport of bile acids across a cell monolayer (Mita *et al.*, 2006). Undoubtedly, as our understanding of the role of specific transporter proteins in various mechanisms of hepatotoxicity continues to expand, the more predictive these systems will be in assessing the potential for a toxic event resulting from the administration of a novel therapeutic agent.

1.2.3.4 CYP-Engineered Cells

In some instances, hepatotoxicity may not be related to the original molecular entity administered to the patient. Rather, the formation of a highly reactive metabolite by CYP450 may contribute to an observed clinical adverse event. To predict the potential for CYP450 involvement in toxicity, cells have been engineered to express each of the major P450 isozymes responsible for the metabolism of xenobiotics. Immortalization of liver epithelial cells with an SV40 T antigen virus followed by transfection with human CYP450 genes yielded a THLE-CYP cell line that has been applied to address metabolism-mediated toxicity (Pfeifer *et al.*, 1993). These transfected cells have been shown to express upwards of

20-fold higher levels of CYP450 and have low basal expression levels of P450 enzymes, making them an ideal system to study isolated mechanisms of metabolism. The most studied compound in this system, Aflatoxin B1, is able to form DNA adducts that are modulated by the presence of the CYP enzymes (Mace *et al.*, 1997). Moreover, these cells were able to identify the oxidative pathways for diclofenac and derive the contribution of each P450-mediated process to the overall metabolism *in vivo.* (Bort *et al.*, 1999a). Specific CYP isozymes have been expressed in other cell lines. When exposed to 1,3-dichloropropanol or cyclophosphamide, NIH-3T3 cells or V79 cells engineered to express individual CYP isoforms displayed a higher degree of toxicity than the control cells (Bull *et al.*, 2001). This increased sensitivity was hypothesized to be due to the ability of the CYP-expressing cells to form reactive metabolites of these two compounds, thereby enhancing the cytotoxicity. Although these cells have promise for predicting the impact of Phase I metabolism on the hepatotoxic endpoint, these reagents often lack Phase II enzymes, overexpress the P450 of interest and do not respond to inducers of metabolism pathways. As a result, ultimate *in vitro/in vivo* correlation of these systems remains a challenge for their widespread use and adoption for assessing hepatotoxicity.

1.2.3.5 Other Cell Lines and Engineered Cellular Systems

In addition to the cellular systems mentioned above, several other cell lines have been used, although not as widespread, to address mechanisms of hepatotoxicity. These reagents include cell lines such as HUH-7, HepaRG and HEP3B cells (Aninat *et al.*, 2006; Le Vee *et al.*, 2006; Manov *et al.*, 2002; Xu *et al.*, 1997). Continued evaluation and testing of these cell lines will be a critical step for the acceptance of these as viable reagents to predict toxic events in the liver.

1.2.4 Three Dimensional (3D) Hepatocyte Bioreactors

Outside of using the intact organ *ex vivo*, one of the major limitations of current *in vitro* models of liver function has been the static nature of most *in vitro* systems. Due to the static nature of these experimental models, it is often difficult to re-establish the microenvironment that cells experience under normal *in vivo* conditions, and thus mimic *in vivo* liver physiology. It has been well established that flow through the liver sinusoids imparts shear stress onto liver cells that dictates cellular gene expression and function (Hara *et al.*, 2003; Sato *et al.*, 1997, 1999). Moreover, extensive cell-to-cell contact, establishment of cell polarity and the impact of non-parenchymal cells all can be important for re-establishing *in vivo* hepatic function (Hamilton *et al.*, 2001; Hoebe *et al.*, 2001; Hoffmaster *et al.*, 2004; LeCluyse *et al.*, 2005; Saad *et al.*, 1993). Static systems have been used extensively, and albeit to some degree of success have shown promise for predicting some incidences of hepatotoxicity in the clinic. In contrast to the more frequent use of static cultures of primary hepatocytes and hepatic cell lines to predict hepatotoxicity as mentioned above, little measure of hepatoxic endpoints in these complex, perfused, 3D models of liver function have been performed. Given the *in vivo* phenotype observed in many of these novel systems, one can only imagine that the predictive nature of each of these models may far exceed our current ability to assess hepatotoxic events before entering the clinic with a promising new chemical entity. Although the application to the field of hepatotoxicity has not yet been

exploited with these emerging technologies, the background and rationale for several of the leading 3D perfused models is described briefly below.

1.2.4.1 MIT Liverchip

One of the most promising 3D models for recapitulating liver function has been developed by fabricating a series of small channels into a scaffold where primary hepatocytes can be seeded and subsequently perfused with a user-defined cell culture medium. Developed at the Massachusetts Institute of Technology (MIT), this unique microfabricated bioreactor design enables morphogenesis of 3D tissue structures, optimal oxygen transfer to the established tissue and physiologic shear stresses to be imparted upon the cells cultured in the device (Powers *et al.*, 2002a,b). As a result, these *in vitro* cultures of cells yield morphology similar to that observed in the intact liver, expression of liver-specific proteins and mRNA near physiologic levels and metabolic function for several key Phase I enzymes in line with formation rates and metabolic profiles observed in freshly isolated hepatocytes or native, intact tissue (Sivaraman *et al.*, 2005). For example, whereas cytochrome P450 levels decline rapidly in primary hepatocytes shortly after isolation, the expression of Cyp2c and Cyp3a was maintained in the 'Liverchip', and further supported by formation of specific testosterone metabolites of these CYP isoforms. Testosterone hydroxylation rates in these studies also were similar to rates observed *in vivo* (Sivaraman *et al.*, 2005). One key advantage of this approach is the ability to scale the system and adapt the system to a high-throughput configuration. Since the functional unit of the reactor is a single channel, the mass of tissue in the device can be controlled directly through fabrication of larger or smaller scaffolds containing more or less individual channels. In addition, the device has been recently adapted to a multi-well configuration amenable to higher-throughput and automated liquid handling – ideal design criteria for screening new chemical entities in a drug discovery environment (unpublished data). Some challenges remain before this technology gains wide application in the field of toxicology. As the majority of data generated to date reflects experiments conducted with rat hepatocytes, the introduction and success of human hepatocytes in the 'Liverchip' will be very important for translating data from this system to understand the clinical manifestations of hepatotoxicity. The further characterization of cellular function in the device (e.g. bile acid transport, mitochondrial function, etc.), coupled with specific experiments designed to assess different mechanisms of hepatotoxicity will help demonstrate the overall utility of this system. Nevertheless, the results and data generated to date suggest strongly that this system offers superiority over currently available static culture systems for evaluating liver-specific function.

1.2.4.2 Hollow Fiber Reactor

Originally designed as an extracorporeal bridging device for liver transplant patients, the multi-compartment woven fiber bioreactor has shown that hepatocytes spontaneously form 3D structures once implanted into the device (Gerlach *et al.*, 2003). These structures display morphology similar to that observed *in vivo*, and cells can be maintained upwards of seven weeks in culture, without undergoing major histological changes (Gerlach *et al.*, 1994; Gerlach *et al.*, 1995). The fundamental principal behind this technology is the incorporation of artificial capillaries providing perfustate flow that promote the formation of 3D structures. When cultured for two weeks, freshly isolated cells were capable of maintaining liver-specific function such as urea synthesis, albumin production, P450 function and glucose

metabolism (Zeilinger *et al.*, 2000, 2002). Recent data also suggests that non-parenchymal cells are able to organize into functional structures complementary to the hepatocytes, and cells may have some proliferative capacity when cultured in the device (Zeilinger *et al.*, 2004).

1.2.4.3 Zonation-Based Bioreactor

A flat-plate reactor that incorporates both flow and the concept of oxygen gradients over the length of the reactor chamber has been fabricated (Allen and Bhatia, 2003; Allen *et al.*, 2005). These researchers constructed a model based upon the concept of zonation of the liver *in vivo*, and have recreated this environment by varying the degree of oxygen tension over a flat plate of hepatocytes with or without co-culture with fibroblasts (Allen and Bhatia, 2003; Jungermann, 1986; Pang *et al.*, 1994). Once this gradient is established in culture, regional heterogeneity of drug metabolizing enzymes (e.g. CYP3A, CYP2B) can be seen, similar to the patterns observed in intact liver. Cell viability can be maintained in this device for at least five days and P450 enzymes can be induced by prototypical inducing agents such as dexamethasone. The zonation-based bioreactor is one of the few novel perfusion based system where specific toxicity studies have been designed and conducted. Treatment of the bioreactor with a range of acetaminophen concentrations resulted in zonal toxicity of the hepatocytes in the device; toxicity was higher in the low oxygen portion of the culture (Allen *et al.*, 2005). These data are supported by the hypothesis that low oxygen concentrations promote glutathione depletion, an important detoxification pathway for the toxic NAPQI acetaminophen mediated metabolite. However, enzymes other than CYP3A responsible for acetaminophen metabolism (e.g. CYP2E, UGT, SULT, etc.) have yet to be characterized in the reactor system.

1.2.4.4 Microvascular-Based MEMS Bioreactor

The highly branched vasculature of the liver represents a challenge for reconstruction in an *in vitro* system. Nevertheless, using principles based on photolithography and silicon micromachining, a group of scientists has machined successfully a vascular network onto a silicon wafer using micro-electromechanical system (MEMS) techniques (Fidkowski *et al.*, 2005; Kaihara *et al.*, 2000). Transfer of this network onto a biodegradable polymer (the wafer acts as a micro-mold) and subsequent removal results in the formation of a two-dimensional polymer scaffold similar to the vascular and capillary networks of the liver. The resultant vascular channels can be seeded with endothelial cells; hepatocytes are seeded into an adjacent parenchymal compartment. These compartments are separated by the presence of a highly permeable membrane that allows the movement of small molecules but isolates the cell types within the device. Oxygen exchange can also happen across this membrane and although the cells remain physically separated, inter-cellular communication through soluble factors is accommodated in this design. When multiple monolayers of hepatocytes are layered and adhered to each other, these ‘sheets’ of hepatocytes recreate a three-dimensional tissue structure that is highly vascularized (Kulig and Vacanti, 2004). The cells remain viable for several days in culture and are able to maintain liver-specific function (e.g. albumin synthesis, urea production, etc.). As the initial application of this technology focused on the fabrication of an implantable human liver, the incorporation of biodegradable and biocompatible polymers has been the recent focus for the development of the technology. However, researchers have realized quickly that this model system shows

significant promise for the evaluation and screening of potential hepatotoxicants in a human cell-based system prior to moving into the clinic. Experiments are just now being designed to address and exploit this opportunity to better understand the potential for hepatotoxicity in this more complex, more physiologic model system.

1.3 Applications of *in Vitro* Models

For *in vitro* models to be considered relevant to *in vivo* situations, several criteria have to be satisfied: (1) the models are well-characterized and understood; (2) the concentrations of the test articles used are considered relevant *in vivo*; (3) the measured outcomes are considered relevant *in vivo*; (4) the underlying mechanisms are considered relevant *in vivo*. Representative applications of cell-based assays currently used to study DILI are summarized in Table 1.2. Although enzyme leakages (ALT, AST, LDH leakages) can be easily measured both *in vivo* and *in vitro*, they do not provide any insight into the underlying mechanisms of damage. Recently, high-content analysis (HCA) has emerged as a versatile technology platform with several distinctive advantages and applications. HCA is essentially a multi-parametric analysis of data that often include both biochemical and cytometric measurements. The HCA readouts may be kinetic on live cells, or single time-point readouts on fixed cells. High-resolution analysis of sub-population of cells in each well, as well as the morphometric measurements of subcellular organelles, is often part of the HCA readouts. These image cytometric measurements compliment the traditional biochemical readouts such as enzyme leakage, ATP content, albumin synthesis or urea synthesis. High-content screening (HCS) is an abbreviated version of HCA, by selecting a few parameters from the HCA that were deemed to be most informative, predictive and robust. By selecting only a subset of the most informative parameters to measure, HCS can significantly increase the throughput of HCA to meet today's drug discovery needs.

1.3.1 High-Content Screening for Human Hepatotoxicity Potential

Recent studies have demonstrated that the clinical occurrence of drug-induced human hepatotoxicity is highly concordant with *in vitro* cytotoxicity when assessed in a cell-based model with a novel combination of critical features and using high content screening (HCS; (O'Brien *et al.*, 2006)). In contrast, concordance was low for previous cytotoxicity assays applied to drugs that produce human hepatotoxicity, because of poor assay sensitivity (Xu *et al.*, 2004). Whereas these previous assays were only effective with severely hepatotoxic chemicals and drugs, they had high specificity, with positive test results being more than 90 % indicative of hepatotoxicity potential.

HCS technology applies epifluorescence microscopy and image analysis to live cells incubated in microtiter plates under physiological conditions. It rapidly and automatically photomicrographs and quantitates fluorescence of multiple dyes at different subcellular locations (Haskins *et al.*, 2001). This enables sensitive detection and following of the progression of key cytomorphologic and intracellular biochemistry effects of hepatotoxic drugs, such as on cell proliferation, nuclear and mitochondrial DNA, mitochondrial mass and activity, intracellular calcium, lysosomal mass, oxidative stress, and cell structure (Figures 1.6 and 1.7).

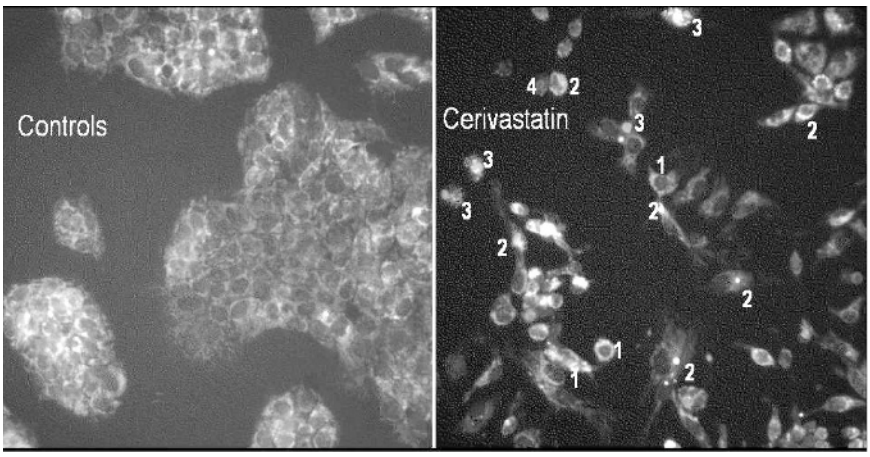

Figure 1.6 *Detection of human hepatotoxicity potential with high-content screening. Composite images of hepatocytotoxic changes produced by incubation of HepG2 cells with cerivastatin (25 μM) for three days. 1, increased mitochondrial potential; 2. increased ionized calcium; 3, permeabilized plasma membrane with increased Ca and decreased mitochondrial membrane potential; 4, ruptured cell*

There are several critical features of the cell model used with HCS that produce high assay sensitivity and concordance with human toxicity, in contrast to past cytotoxicity models (O'Brien *et al.*, 2006; O'Brien and Haskins, 2006). These include duration of treatment, multiparametric monitoring of individual and live cells, drug metabolic competency and testing at concentrations relevant to efficacious concentrations. Most critical was the need for sufficient time to allow expression of the cytotoxicity (O'Brien *et al.*, 2006; Slaughter *et al.*, 2002; Xu *et al.*, 2004). Three days of incubation of cells was effective for more than 95 % human hepatotoxic drugs, whereas a single day of incubation was frequently ineffective (O'Brien *et al.*, 2006) or produced cytotoxicity at a much higher concentration (O'Brien *et al.*, 2006; Slaughter *et al.*, 2002; Xu *et al.*, 2004).

Next most important for predictivity was the number and choice of parameters measured. Early, sublethal effects on cell proliferation, cell morphology and mitochondria occurred consistently and ubiquitously with toxicity and when used collectively were most diagnostic. The occurrence and timing of effects on intracellular calcium concentration, lysosomal mass, oxidative stress or plasma membrane permeability frequently provided additional information indicative of mechanism of toxicity. For example, in Figure 1.7, phospholipidosis from cationic amphiphilic drugs, mitochondrial DNA depletion by nucleoside reverse transcriptase inhibitors that also inhibit mitochondrial DNA polymerase gamma and redox cyclers that produce reactive oxygen species are demonstrated. In addition, in Figure 1.6, the complex mechanism of statin-induced toxicity is demonstrated with early sublethal effects on apoptosis, mitochondrial function and calcium homeostasis (Diaz and O'Brien, 2006).

Use of human hepatocytes with potential for drug metabolism is also important for assessment of hepatotoxicity as numerous drugs produce this effect by their hepatic metabolites. Drugs producing idiosyncratic hepatotoxicity and/or toxicity by their metabolites

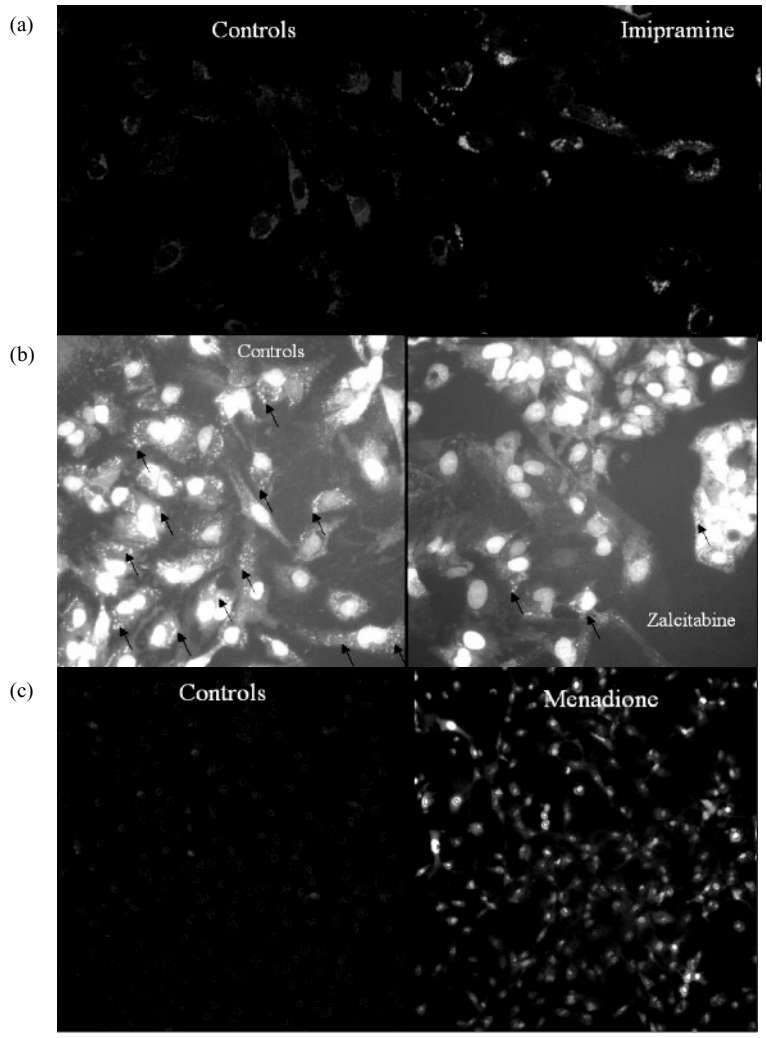

Figure 1.7 *Mechanisms of human hepatotoxicity identified in HepG2 cells by high-content screening. (a) Phospholipidosis was produced by incubating cells with 12.5 μM imipramine for three days and then staining them with Hoechst 33342 for DNA, Mitotracker Far Red for mitochondrial mass and Lysotracker Green for lysosomal mass, as indicated. (b) Mitochondrial DNA depletion by nucleoside reverse transcriptase inhibitors is demonstrated by incubation of HepG2 cells for three days with 1.6 μM zalcitabine and staining nuclear and mitochondrial DNA (arrows) with Picogreen. (c) Oxidative stress produced by menadione. HepG2 cells were treated with 100 μM menadione and stained with Hoechst 3342 and dihydroethidium. Selection of objects for assessment is indicated by the circles and is based on Hoechst 3342 staining of nuclear DNA. Ethidium forms as a result of oxidative product of dihydroethidium, binds nuclear DNA and fluoresce with a different wavelength than the Hoechst. This is seen in the photomicrograph as increased whiteness of the encircled areas (right)*

(Kalgutkar *et al.*, 2005; Kaplowitz, 2005) were detected with HepG2 cells as effectively as drugs producing toxicity directly (Table 1.7). This high concordance contrasted remarkably with the 15 % concordance of seven other conventional assays in which a 50 % effect at 30 μM was considered positive for changes in any of the seven readouts: DNA synthesis, protein synthesis, glutathione depletion, superoxide secretion, caspase-3 activity, membrane integrity and mitochondrial reductive activity. The basis for this difference in sensitivity was not determined but may relate in part to induction of metabolic competence over the three days of exposure in the HCS assay. Finally, assay effectiveness also depended on measurement at the single-cell level in order to allow exclusion of extracellular staining or artifact or dead cells.

Determination of the concentration producing cytotoxicity in the HCS sublethal cytotoxicity assay was assessed by 12-point dose response curves (Figure 1.8). A toxic effect was defined as the point when values for the parameter departed from the baseline and negative controls by more than two coefficients of variation. In Figure 1.8, typical dose – responses are illustrated in which all eight parameters measured were affected. The pattern and sequence of changes in the different parameters frequently reflected the mechanism of toxicity. For fenofibrate (Figure 1.8), there was nuclear swelling and inhibition of cell proliferation, followed by mild increases in intracellular calcium with some loss of mitochondrial membrane potential and an increase in membrane permeability, followed by overt oxidative stress with mitochondrial biogenesis. This pattern contrasted with that of cerivastatin (Figure 1.6), where there was first nuclear shrinkage and increased mitochondrial membrane potential, followed by increased intracellular ionized calcium. At higher concentrations, the calcium progressively increased, the mitochondrial potential progressively fell and membrane permeability increased.

Virtually all drugs and chemicals cause toxicity at high enough concentrations. Thus, it is critical to assess toxicity at concentrations relevant to those that are used for drug efficacy. Efficacious concentration, as defined as the maximal serum concentration of drug used for treatment (C_{max}), is highly variable, ranging 10 000 000-fold in one study of 187 marketed human drugs from 100 pM to 2 mM, with 90 % values less than 100 μM, 60 % less than 10 μM, 37 % less than 1 μM and 12 % less than 100 nM (Figure 1.8). Most human hepatotoxic drugs (94 % of 102 tested) are cytotoxic in the sublethal HSC cytotoxicity assay at concentrations less than 100-fold C_{max}, whereas most non-toxic drugs (96 % of 23 tested) are cytotoxic in this assay at concentrations more than 100-fold C_{max} (O'Brien *et al.*, 2006) (Figure 1.9).

Hormesis, in which compensatory adaptive changes precede and occur at lower doses than degenerative changes, was detected for half of the toxic drugs for cell proliferation, cell morphology and mitochondria (O'Brien *et al.*, 2006). It is demonstrated for cerivastatin in Figure 1.6 for mitochondrial membrane potential and for fenofibrate in Figure 1.8 for mitochondrial mass and nuclear area. Hormesis could not be assessed for parameters that normally have low values, such as intracellular calcium measured by fluo4 or membrane permeability measured by toto-3, because assay methods were not sufficiently sensitive. However, for calcium, more sensitive dyes, with calcium dissociation constants closer to the physiologic concentration of ionized calcium, have detected biphasic effects on resting calcium (O'Brien *et al.*, 1990).

In the sublethal HCS assay, sensitivity and specificity for identification of human hepatotoxicant drugs were 94 % and 96 %, respectively, when testing only hepatotoxicant drugs

Table 1.7 *Concordance of HCS assay results with idiosyncratic or drug metabolite-implicated human hepatotoxicity (based on O'Brien et al., 2006). Drugs are compared with how they tested in seven conventional cytotoxicity assays (column 2) and how they tested in the HCS assay (columns 3–7). Results for the HCS assay are tabulated for lowest observed adverse effect level (LOAEL) concentrations (in μM) causing effects on cell number, mitochondrial membrane potential, intracellular ionized calcium concentration, membrane permeability and nuclear area, respectively. The maximal plasma total concentrations of drugs associated with efficacy (C_{max}) are indicated in column 8. The ratio of the lowest cytotoxic concentrations from columns 3–7 to C_{max} from column 8 is indicated in column 9 (TI). The percentage of drug that is bound to plasma proteins is also indicated (column 10) and where known the cellular mechanism of cytotoxicity (column 11). The sensitivities of the conventional assays and the HCS assay are indicated in the bottom row. The sensitivity of the HCS assay was determined using a TI cutoff of 100 in column 9. Tacrine is the only drug in this panel that falls just outside of the 100 cutoff. It is of interest to note that tacrine only causes asymptomatic elevations of liver enzymes that do not progress to frank liver injury despite continued use of the drug*

	Drug[a]	Conventional Tests[b]	Cell Number[c]	Mitochondrial potential[c]	Ca	Membrane Pote ntial	Nuclear area	C_{max} (uM)	TI	PPB (%)	Mechanism[d]
1	[i,r]Acetaminophen	–	500↑4000↓	4000	—	—	7800	130	4	8	OS
2	[r]Chloramphenicol	–	260	—	—	—	—	57	5	53	OS
3	[r]Danazol	–	13	50↑100↓	25	50	13	0.16	81	—	—
4	[i,r]Diclofenac	–	16↑126↓	250	—	126	126↑	4.2	4	99	OP, Ap
5	[i,r]Felbamate	–	315	160↑	—	—	315	42	4	24	IM
6	[r]Flutamide	–	50	50	50	50	—	6	8	90	M
7	[i,r]Hydralazine	ND	67	135↑	270	—	75	5	13.5	87	IM
8	[r]Ibuprofen	–	50	—	—	—	—	250	0.2	99	IM, M
9	[r]Indomethacin	–	190	47	—	—	2↑	6	0.3	90	IM, OS
10	[r]Imipramine	–	0.8↑50↓	100	50	50	50	0.6	1	100	IM, M
11	[r]Isoniazide	–	—	50↓	—	—	—	40	1	0	IM, OS
12	[i,r]Leflunomide	+	25	—	—	—	—	340	0.07	99.3	IM
13	[i,r]Methyldopa	ND	330	330	330	330	330	11	30	15	—

14	[i]Minocycline	−	13	25	—	—	50↑	8	2	76	IM, M
15	[i]Nitrofurantoin	−	12	50	50	50	0.7↑	6	0.1	62	IM, OS, M
16	[r]Piroxicam	−	5	160	—	—	5↑	5	1	99	—
17	[r]Procainamide	ND	630	630↑	630	—	320	12	27	18	IM
18	[i]Rifampicin	+	50	—	100	100	13↑	9	1	81	—
19	[i]Sulindac	−	285	140	—	—	570↑	19	8	94	IM, OS
20	[r]Sulfamethoxazole	−	1100↑	—	—	—	—	217	5	62	IM, M
21	[r]Tacrine	−	25	50↑100↓	50	25	13↑50↓	0.1	130	55	OS, M
22	[r]Tamoxifen	+	100↑	25	—	25	0.4↑25↓	0.4	1	98	M
23	[i,r]Terbinafine	ND	3	6	6	6	13	4	0.8	99	—
24	[i,r]Valproate	−	1000	4000↑	—	4000	8000	540	2	93	OS, M
Sensitivity		***15 %***					***96 %***				

[a] Superscript 'i', idiosyncratic heptatotoxicity; Superscript 'r', reactive metabolite.
[b] ND, not determined.
[c] ↑signal increased;↓ signal decreased.
[d] OS, oxidative stress; Ap, apoptosis; IM, imune-mediated; M, mitochondrial.

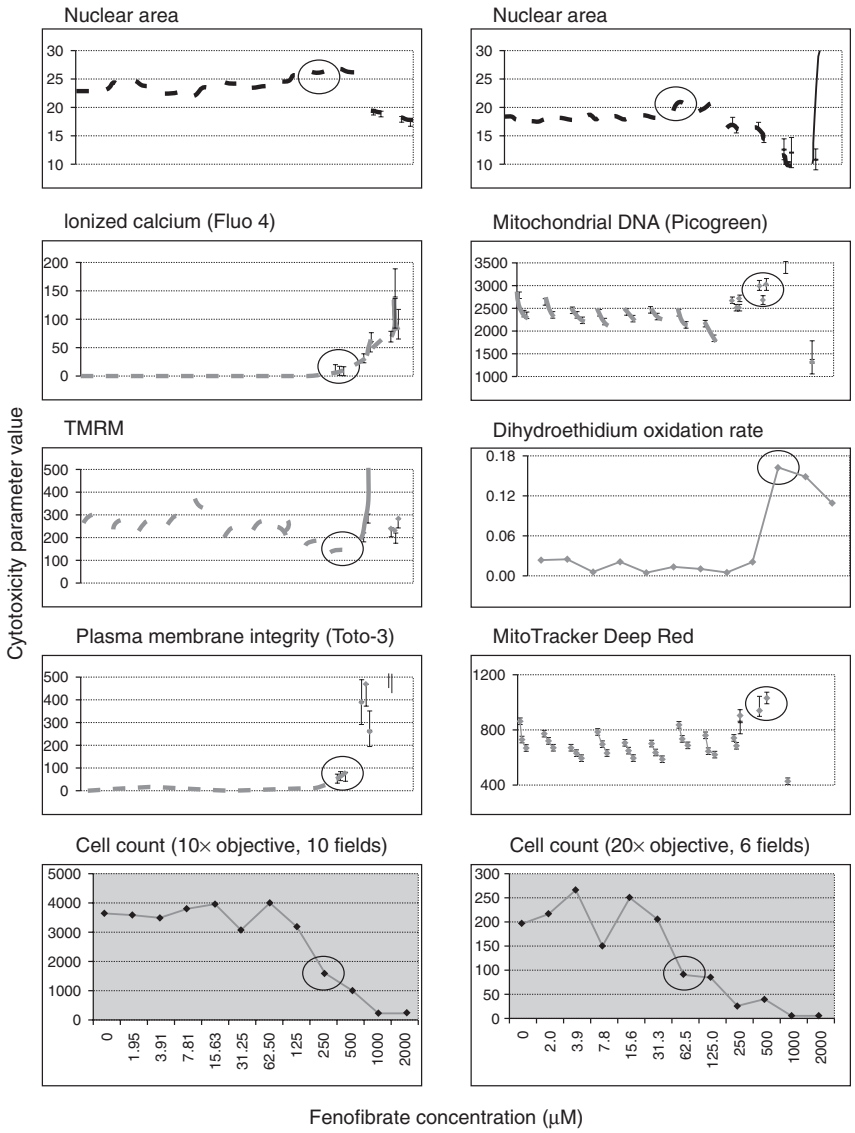

Figure 1.8 *Use of high-content screening for quantitative determination of dose–response relationships for drug-induced, human hepatocellular toxicity (based on O'Brien et al., 2006). Fenofibrate (25 μM) was assayed in HepG2 cells at doubling concentrations from 2 to 2000 using two different combinations (columns 1 and 2) of fluorescent probes. For both assays, Hoechst 33342 was used to determine nuclear area and cell count. Ionized calcium was assayed using Fluo4, mitochondrial membrane potential using TMRM, plasma membrane integrity using TOTO-3, mitochondrial DNA using picogreen, mitochondrial mass using MitoTracker Deep Red and reactive oxygen species using dihydroethidium. The circles in each dose–response curve indicate the lowest observed adverse effect level (LOAEL) values which were used to tabulate the LOAEL concentrations shown in Table 1.7*

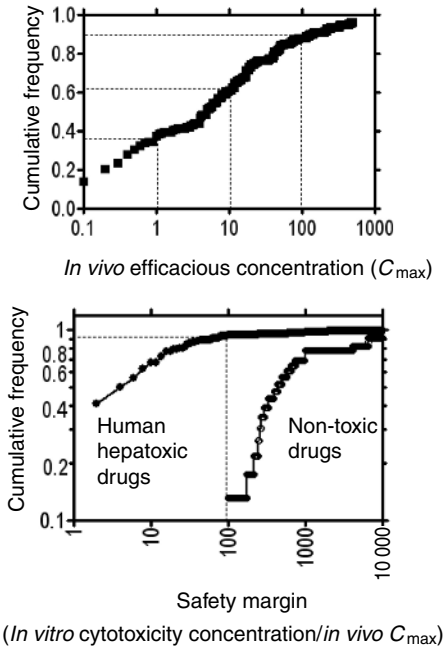

Figure 1.9 *Safety margins for HCS sublethal cytotoxicity assay concordant with human hepatotoxicity. A frequency histogram (a) for the* in vivo *efficacious concentration in humans (C_{max}) of 187 marketed drugs demonstrates a wide range and indicates the need to assess cytotoxicity in the context of efficacious concentration. A frequency histogram (b) of the safety margin (*in vitro *HCS cytotoxicity concentration/C_{max}) for 102 human hepatotoxicant drugs and the 23 drugs for which this ratio was determined indicates that a safety margin of 100 is the most effective discriminator of human hepatotoxicity potential (based on O'Brien et al., 2006)*

and non-toxic drugs (O'Brien *et al.*, 2006). However, when other drugs that produce other organ toxicities (e.g. kidney, heart, bone marrow, muscle and pancreas) were tested their cytotoxic effects were not distinguishable from those of drugs causing hepatotoxicity. Thus, cytotoxicity in the HCS assay was concordant with human toxicity but not specific for liver toxicity. An additional caveat in the use of the assay is that it did not detect cholestatic effects of drugs. Nor did it detect other organ toxicities produced by drugs' effects on proteins not found in hepatocytes.

1.3.2 Primary Hepatocytes and Liver-Specific Toxicities

While hepatic cell lines have several advantages over primary cells, it is still prudent to take a selected subset of compounds into primary hepatocytes. The reasons are mainly the following. primary hepatocytes are more differentiated cells compared to cell lines. They maintain higher levels of metabolic activity, normal p53 status and cell cycle regulation and more normal levels of transporter protein expression (Le Vee *et al.*, 2006; Wilkening *et al.*, 2003). For example, primary hepatocytes are more suitable than HepG2 to study metabolism-mediated liver toxicants, such as benzo[a]pyrene, dimethylnitrosamine (DMN),

2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Wilkening *et al.*, 2003), acetaminophen, diclofenac, cyclophosphamide, disulfiram (Bort *et al.*, 1999b; Wang *et al.*, 2002) and nimesulide (Rainsford *et al.*, 2001). High-content analysis of primary hepatocytes has also been used to study more liver-specific toxicities, such as steatosis, steatohepatitis, cholestasis and reactive metabolites.

Steatosis, or accumulation of fatty acids (i.e. 'fatty liver'), can be caused by a variety of drugs including amiodarone, perhexiline maleate, 4,4′-diethylaminoethoxyhexestrol (DEAH), tetracycline, valproic acid and several antiviral nucleoside analogues, such as fialuridine (Fromenty and Pessayre, 1997). One of the major mechanisms involved in steatosis is the inhibition of beta-oxidation of long-chain fatty acids, either by direct inhibition or indirect inhibition, such as CoA sequestration or mitochondria DNA damage (Fromenty and Pessayre, 1995; Jaeschke *et al.*, 2002b). The resulting fatty acid accumulation can be detected and quantified by staining primary hepatocytes with neutral lipid stains such as Oil red O and performing HCA under the microscope (Amacher and Martin, 1997; McMillian *et al.*, 2001). This is different from using phospholipids stains such as NBD-PC or NBD-PE to study phospholipids accumulation in the cytoplasm (i.e. phospholipidosis) (Gum *et al.*, 2001). In addition, the aldehyde products of lipid peroxidation, 4-hydroxynonenal and malondialdehyde (MDA), can be measured by biochemical readouts (Berson *et al.*, 1998a,b). MDA is a known stimulator of inflammatory responses. Therefore, repeated challenge by steatotic drugs and the resulting lipid peroxidation represent an important mechanism of drug-induced steatohepatitis (Ivanov *et al.*, 1992). While simple steatosis was regarded as benign, research in the past decade suggested that mitochondria dysfunction and reactive oxygen species generation are important factors to differentiate simple steatosis from nonalcoholic steatohepatitis (NASH) (Fromenty *et al.*, 2004). Using primary cultured hepatocytes, it was found that amiodarone, perhexiline maleate and DEAH increased the mitochondrial formation of reactive oxygen species and caused lipid peroxidation (Berson *et al.*, 1998b). Based on these findings, it would be important for high content assays to measure mitochondria function and oxidative stress in addition to simple neutral lipid accumulation.

Intrahepatic cholestasis, defined as impairment in bile formation and/or bile flow, is another common manifestation of drug-induced liver disease. In humans, intrahepatic cholestasis most often occurs in the elderly (Feuer and Di Fonzo, 1992). As the population ages and the occurrence of multiple drug therapy in geriatric patients increases, it is expected that jaundice and/or drug-induced intrahepatic cholestasis will become even more prevalent. Bile formation is dependant on the specific transporter proteins in hepatocytes. The expression and the appropriate membrane sorting of these transporters are highly dependent upon the differentiated phenotype of the cells (Hoffmaster *et al.*, 2004; LeCluyse *et al.*, 1994). Sandwich-cultured primary hepatocytes are currently the most well-characterized *in vitro* model to study the disposition of taurocholate (Liu *et al.*, 1999), bilirubin and its glucuronide (Lengyel *et al.*, 2005) and drug-induced cholestasis (Kostrubsky *et al.*, 2003). As expected, inhibition of important hepatobiliary transporters, such as the bile salt export pump (BSEP), can result in drug-induced cholestasis (Kostrubsky *et al.*, 2006). Although in the cases of rifampicin (Li and Chiang, 2006), it was found to induce bile acid and bilirubin detoxification (Marschall *et al.*, 2005) via PXR activation and this drug has been successfully used to treat cholestatic liver disease. This example highlights the importance of using longer-term primary hepatocyte cultures (> 3 days) that express differentiated and

polarized liver functions as opposed to the short-term BSEP inhibition experiments (< 1 h), to provide a more complete picture of *in vivo* outcome.

It is well known that many hepatotoxic agents can be metabolized to reactive metabolites that can either be detoxified or react with glutathione, enzymes, nucleic acids, lipids or proteins (Knowles *et al.*, 2000; Pessayre, 1995). These reactive intermediates are electrophilic metabolites or free radicals that are generated during the metabolism of a broad range of functional groups. Reactive metabolite formation and/or oxidative stress induced by drugs are also considered important factors in immune-mediated idiosyncratic drug hypersensitivity (Park *et al.*, 2001). There are several rapid *in vitro* methods to detect and measure the generation of such reactive intermediates. For example, high-throughput assays for identifying pharmaceutical compounds that produce reactive metabolites have been developed. These methods involve incubating drug candidates with a liver microsomal drug metabolizing enzyme system in the presence of glutathione and detecting glutathione conjugates via tandem mass spectrometry (Chen *et al.*, 2001; Haroldsen *et al.*, 1988; Pearson *et al.*, 1988). However, it is now recognized that reactive metabolite formation *per se* is not sufficient to cause tissue injury (Park *et al.*, 2005; Sanderson *et al.*, 2006). Hence, cell-based models to evaluate the toxicological consequences of the formation of reactive intermediates (or lack thereof) are becoming increasingly important. In cells, the reduced form of glutathione (GSH) is known to interact with electrophilic compounds/metabolites and free radicals to play a key role in the detoxification of such reactive molecules. Depletion of the reduced form of glutathione was reported to be a marker of hepatotoxicity (e.g. Fouin-Fortunet *et al.*, 1984), suggesting its evaluation is important in toxicological studies. For example, monochlorobimane and chloromethylfluorescein diacetate (CMFDA) have been successfully used to monitor cellular GSH levels by epifluorescence in isolated hepatocytes (Lilius *et al.*, 1996; Thompson *et al.*, 1998). The fluorometric dye, 2′,7′-dichlorodihydrofluorescein (H_2DCF) can be used to detect oxidative stress induced by various oxidative radicals in human cells including hepatocytes (Lautraite *et al.*, 2003; LeBel *et al.*, 1992; Wang and Joseph, 1999). The diacetate form of H_2DCF freely enters the cell and is hydrolyzed by intracellular esterases to liberate H_2DCF, which then reacts with oxidizing species to produce the highly fluorescent DCF. These cell-based assays are amenable to high-throughput evaluation of chemical compounds and their reactive metabolites and/or oxidative species in drug discovery (Lautraite *et al.*, 2003).

1.3.3 RNAi Technology

Cell-based systems are currently being coupled with new technologies such as RNA interference (RNAi) as a potential tool to identify genes with predictive functions significant to drug development. RNAi represents an evolutionary conserved mechanism developed by nature to protect the genome against endogenous and exogenous stress insults, and regulate gene expression. There are numerous excellent reviews on the use of RNAi as a research tool and as therapeutic agents (Cejka *et al.*, 2006; Cullen, 2006; Dillon *et al.*, 2005; Dykxhoorn and Lieberman, 2005; Dykxhoorn *et al.*, 2006; Hannon and Rossi, 2004; Lee and Sinko, 2006; Shankar *et al.*, 2005; Sledz and Williams, 2005). Briefly, endogenous mammalian RNAi is mediated by small-interfering RNAs (siRNAs) produced from long double-stranded hair pin RNA known as micro RNA (miRNA) through the action of an endonuclease of the ribonuclease-III type, called ‘drosha’ and ‘dicer’. These siRNAs

or small double-stranded RNAs are incorporated into the RNA-inducing silencing complex (RISC), which contains RNAase activity, to become small single-stranded RNAs. The single-stranded RNA guides RISC to mRNA that has a complementary sequence and mediates gene silencing by targeting RNA. This silencing is caused by either translational repression if there is a mismatch in the sequence of the siRNA and target mRNA or by RNA cleavage if there is an exact match between the siRNA sequence and the target mRNA. Thus, the synthesis of the protein encoded by the mRNA targeted by the siRNAs is prevented, and that protein is selectively depleted from the cell. The enzymatic machinery required to process siRNA is ubiquitously expressed in most eukaryotic cells and can be co-opted by exogenous RNAs to direct the sequence-specific gene silencing. Therefore, siRNA can be chemically synthesized and transfected into cells, tissues and animals after being integrated into specialized delivery systems, such as liposomes. Alternatively, plasmids or viral vectors can be used to express shRNA (short hairpin RNA that structurally mimics a miRNA precursor). Successful 'knockdowns' of specific genes allow the investigation of potential drug targets, as well as the genetic basis of physiological and disease processes in mammalian systems.

RNA interference (RNAi) technology is widely used *in vitro* for modulating specific targets and assessing biologic pathways; in addition, this novel technology has the potential to be used for addressing drug-induced liver injury (DILI). Toxicological effects observed upon treatment with a given drug in the presence or absence of a specific RNAi molecule can be evaluated in cell-based systems to determine whether the toxicity associated with chemical entities is mediated by interaction with that specific target. For example, Hep2 cells stably expressing specific small interfering RNA directed against the activating signal cointegrator-2 (ASC-2) have been successfully used to demonstrate that ASC-2 protein is likely to participate in acetaminophen-mediated hepatotoxicity as a transcriptional coactivator of the xenobiotic nuclear receptor CAR *in vivo* (Choi *et al.*, 2005); mouse hepatocytes silenced against Fas expression were found to be protected from cytotoxicity caused by actinomycin D (Song *et al.*, 2003); Moreover, RNAi technology will further demonstrate participation of the specific cytochrome P450 (CYP450) enzyme in the DILI process. This was illustrated when CHL-3A4 cells with 3A4 siRNAs significantly diminished the cytotoxicity of cyclophosphamide and ifosfamide (Chen *et al.*, 2006). RNAi technology has also been utilized to understand the role of transport proteins such as Mrp1-3, Mdr1 and Bcrp (Lee and Sinko, 2006; Sahi, 2005; Tian *et al.*, 2004; Xu *et al.*, 2005), and the functional analysis of nuclear receptors such as the Farnesoid X receptor and androgen receptor (Plass *et al.*, 2002; Wright *et al.*, 2003) and their co-regulators, such as SRCs, p300, NcoR and SMRT (Debes *et al.*, 2002; Yoon *et al.*, 2003; Zhou *et al.*, 2003) in DILI.

At present, the prediction of metabolism-dependent hepatotoxicity is difficult or even impossible because there are no suitable experimental *(in vivo/in vitro*) models and we do not understand the basic mechanism involved in the toxicity when it does occur in man. Cell-based models coupled with RNAi technology could be useful systems for predicting metabolic activity of drugs *in vivo*. *In vivo* delivery is still the greatest challenge to the effective use of this technology in target characterization and investigating adverse pharmacology and chemically mediated toxicity. However, recent advances in *in vivo* delivery and vector technology make it probable that RNAi can be effectively used for *in vivo* hypothesis-driven research (Aligner, 2006; Behlke, 2006; Lu *et al.*, 2006).

1.4 Conclusions

We have reviewed various *in vitro* models currently used or under development to better enable the evaluation of drug-induced liver injury (DILI). Continuing advances in stem cells research, new immortalized cell lines and genetically engineered cells, 3D-hepatocyte bioreactors and our enhanced ability to predict outcomes from primary cells have allowed a better understanding of the mechanisms of toxicity, metabolism of drugs and the species differences in expression of toxicity. Each model described in this review has significant advantages and it is possible to find an appropriate system for any particular toxicological question asked. Further research should be directed towards the refinement of existing methodologies and the development of new alternatives and testing paradigms for human relevancy. This approach coupled with the establishment of new technologies (e.g. HCS, RNAi and 'omics') will improve our ability to assess DILI earlier in the drug discovery process and would allow us to identify compounds with decreased risk of hepatotoxicity at later stages of development for eventual human health risk assessment. To fully establish the credibility and relevance of *in vitro* toxicity evaluation of drugs, it is essential that *in vitro/in vivo* correlations of toxicity of NCEs or drugs be determined by toxicologists and cell culture scientists.

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