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Evaluation of Adverse Drug Properties with Cryopreserved Human Hepatocytes and the Integrated Discrete Multiple Organ Co-culture (IdMOC™) System

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Human hepatocytes, with complete hepatic metabolizing enzymes, transporters and cofactors, represent the gold standard for *in vitro* evaluation of drug metabolism, drug-drug interactions, and hepatotoxicity. Successful cryopreservation of human hepatocytes enables this experimental system to be used routinely. The use of human hepatocytes to evaluate two major adverse drug properties: drug-drug interactions and hepatotoxicity, are summarized in this review. The application of human hepatocytes in metabolism-based drug-drug interaction includes metabolite profiling, pathway identification, P450 inhibition, P450 induction, and uptake and efflux transporter inhibition. The application of human hepatocytes in toxicity evaluation includes *in vitro* hepatotoxicity and metabolism-based drug toxicity determination. A novel system, the Integrated Discrete Multiple Organ Co-culture (IdMOC) which allows the evaluation of nonhepatic toxicity in the presence of hepatic metabolism, is described.

***Key words*:** Human hepatocytes, IdMOC, Hepatocyte cryopreservation, Drug metabolism, Drug-drug interactions, Drug toxicity, Drug development

INTRODUCTION

It is becoming apparent that laboratory animal studies alone are not adequate in the definition of adverse drug effects in the human population. The practice of preclinical safety evaluation is based on the assumption that evaluation of xenobiotic toxicity in multiple animal species should provide data useful for the estimation of human safety. The inadequacy of this assumption is illustrated by findings in the pharmaceutical industry, where, in spite of the compulsory vigorous testing in multiple animal species, clinical trial failure rate is estimated to be 85% or higher (1). Moreover, phase I, II, and III clinical trials are also found not to be adequate as indicated by the numerous post-marketing withdrawals and black-box warnings of marketed drugs (2,7-12).

Species difference in drug metabolism and toxicity. Evidence is being accumulated that the effectiveness of the classical approach of safety evaluation, namely, safety testing in laboratory animals, is hindered by the known species-differences in drug properties, especially ADMET drug properties: absorption, disposition, metabolism, elimination and toxicity (2-4). Species difference is in drug metabolism is a well-established phenomenon. The most important drug metabolizing enzymes belong to the cytochromes P450 isoforms which are localized mainly in the parenchymal cells (hepatocytes) of the liver and are found in other organ such as intestinal epithelium, lung, and kidneys. Of the major isoforms involved in drug metabolism, namely, CYP isoforms 1A, 2B, 2C, 2D, 2E, and 3A, the human isoforms are substantially different from those found in rat, dog, and monkey (Table 1).

Drug metabolism is a key determinant in drug toxicity: a toxicant can be rendered more toxic (metabolic activation)

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Abbreviations: ADMET, Absorption, distribution, metabolism, excretion, toxicity; CHRM, Cryopreserved Hepatocyte Recovery Medium; CYP, Cytochrome P450; DDI, Drug-drug interactions; DILI, Drug induced liver injury; EMA, European Medicines Agency; HCP, Human cell paradigm; HLM, Human liver microsomes; IdMOC, Integrated discrete multiple organ co-culture; PBPK, Physiologically based pharmacokinetic (modeling); US FDA, United States Food and Drug Administration.

Table 1. A comparison of human hepatic P450 isoforms to those of the four commonly used laboratory animal species for safety evaluation (mouse, rat, dog and monkey). Extensive species differences have been established, with P450 1A and 2E found to be the only isoforms that are conserved among the multiple animal species. Species difference in P450 isoforms has led to differences in metabolic stability, metabolite profiles, as well as drug toxicity and efficacy. The human isoforms commonly investigated are in bold.

P450 isoform subfamily	Human	Mouse	Rat	Dog	Monkey
1A	**1A1**, **1A2**	1A1, 1A2	1A1, 1A2	1A1, 1A2	1A1, 1A2
2A	**2A6**, 2A7, 2A13	2A4, 2A5, 2A12, 2A22	2A1, 2A2, 2A3	2A13, 2A25	2A23, 2A24
2B	**2B6**, 2B7	2B9, 2B10	2B1, 2B2, 2B3	2B11	2B17
2C	**2C8**, **2C9**, 2C18, **2C19**	2C29, 2C37, 2C38, 2C39, 2C40, 2C44, 2C50, 2C54, 2C55	2C6, 2C7, 2C11, 2C12, 2C13, 2C22, 2C23	2C21, 2C41	2C20, 2C43
2D	**2D6,** 2D7, 2D8	2D9, 2D10, 2D11, 2D12, 2D13, 2D22, 2D26, 2D34, 2D40	2D1, 2D2, 2D3, 2D4, 2D5, 2D18	2D15	2D17, 2D19, 2D29, 2D30, 2D42
2E	**2E1**	2E1	2E1	2E1	2E1
3A	**3A4**, **3A5**, 3A7, 3A43	3A11, 3A13, 3A16, 3A25, 3A41, 3A44	3A1, 3A2, 3A9, 3A18, 3A62	3A12, 3A26	3A8

or less toxic (detoxification) by biotransformation. Species differences in drug metabolizing enzymes such as cytochromes P450 can lead to species differences in xenobiotic toxicity due to differences in rate of metabolic activation and detoxification, as well as differences in metabolite formation. A clear example is the findings of Easterbrook *et al.* (5) that coumarin to 7-hydroxycoumarin was observed in human but not in rat liver microsomes. The report of Lee *et al.* (6) is a pioneering study using animal and human hepatocytes in the definition of metabolite formation in drug development. The finding that metabolites formed by rat hepatocytes were different from those formed by human hepatocytes suggest that, for the drug candidate evaluated, the rat was not an appropriate experimental for human. This species differences in metabolite formation is now a frequently observed phenomenon in drug development.

Human cell paradigm (HCP) for the prediction of human drug properties. I propose here the HCP for human safety evaluation. With the HCP, human-specific adverse drug properties are obtained using physiologically relevant human cell based *in vitro* experimental systems, followed by prediction of human *in vivo* effects using two methods: 1. Direct extrapolation of *in vitro* results to human *in vivo* using known human *in vivo* parameters (*e.g.* physiologically based pharmacokinetics data; genetic polymorphism; environment factors). This approach has been applied successful in the estimation of hepatic metabolic clearance and drug-drug interactions. 2. Develop *in vitro* animal results using similar cell systems from multiple species for the selection of the most relevant animal species for *in vivo* evaluation and extrapolation of results to humans *in vivo*. This approach is recommended for safety studies such as hepatotoxicity. The key emphasis is that human-specific drug properties obtained using *in vitro* human-based experimental systems are critical to the evaluation of human *in vivo* drug properties (Fig. 1).

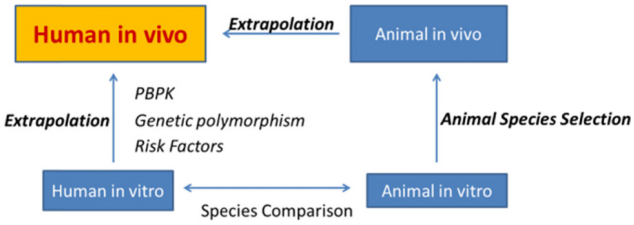

Fig. 1. Human cell paradigm (HCP) for the prediction of human drug properties. With the HCP, emphasis is placed on human-specific drug properties obtained with physiologically relevant human-based *in vitro* experimental systems. *In vitro* results are then extrapolated to *in vivo* based on known human *in vivo* parameters or via the selection of the most appropriate animal species using similar *in vitro* systems, followed by *in vivo* evaluation in the chosen animal species.

Human hepatocyte as a key experimental system for the assessment of adverse human drug properties. As the body's major organ for drug metabolism, the liver plays an important role in drug properties. Biotransformation of drugs determines the duration (metabolic stability) of the drug in the systemic circulation. Hepatic metabolites are known to exhibit toxicological and pharmacological properties which may be different from the parent drugs. Further, drug-drug interactions and liver toxicity are adverse drug properties that have been found to lead to clinical trial failures or withdrawal of marketed drugs (7-12). Human hepatocytes, the parenchymal cells in the human liver, are the key hepatic cells for drug metabolism and when damaged, would lead to hepatic injuries. Severe drug-induced damages to hepatocytes has caused liver failure, leading to a need for liver transplantation and have resulted in deaths (2,10).

Hepatocytes represent the most important cell type for the evaluation of drug metabolism and are generally considered the "gold standard" for *in vitro* drug metabolism and

hepatotoxicity studies. The advantages of hepatocytes over other *in vitro* hepatic experimental systems such as liver homogenate, post-mitochondrial supernatants, and microsomes, are attributed to the various intact cell properties including the intact cell membrane with active transporter functions, complete and uninterrupted metabolic pathways, and metabolic enzymes and cofactors at physiologically relevant concentrations (3,13).

Isolation, cryopreservation, and culturing of human hepatocytes. Human hepatocytes can be isolated from human liver biopsies or whole livers which have been donated, but not used, for transplantation. The application of human hepatocytes in research was limited by the general unavailability of human livers for research and the lack of hepatocyte isolation expertise in most laboratories. Success in human hepatocyte cryopreservation in the recent decade allows the cells to be used routinely for experimentation (14-16). Hepatocytes are generally used as suspension cultures for short-term (hours) studies, and as monolayer cultures for longer-term (days) studies. The loss of polarity of cultured hepatocytes is overcome by culturing the cells in a collagen Matrigel sandwich (culturing of hepatocytes on a collagen-coated surface, followed a thin overlay of Matrigel). The properties (viability; yield; P450 activities) of cryopreserved human hepatocytes are shown in Table 2.

We dedicated extensive effort in our laboratory towards the optimization of hepatocyte isolation, cryopreservation, and post-cryopreservation recovery. A major advancement is the development of a specialized medium (Cryopreserved Hepatocyte Recovery Medium (CHRM^{TM}) which greatly enhances the post-thaw viability of hepatocytes. Upon recovery in CHRM, the viability (based on dye exclusion) is routinely > 85% (Table 1). Furthermore, approximately 50% of the human hepatocyte isolations result in cryopreserved hepatocytes that form monolayer cultures with > 80% confluency (plateable cryopreserved human hepatocytes). We now have extended human hepatocyte cryopreservation to the preparation of cryopreserved human hepatocytes pooled from multiple donors: cryopreserved hepatocytes from individual donors are thawed, cells from multiple donors are pooled, and the cells re-cryopreserved. While individual donors can be used to illustrate individual differences, the pooled cryopreserved hepatocytes can be used for the investigation of drug properties towards a "normalized" human population.

We are also successful in the cryopreservation of nonhuman animal hepatocytes. Cryopreserved hepatocytes from multiple animal species and human can be used in the same experiment for the selection of the animal species most resembling human in the drug property of interest.

The morphology of cultured cryopreserved human and animal hepatocytes is shown in Fig. 2. Morphology of pooled cryopreserved human hepatocytes is shown in Fig. 3.

Application of human hepatocytes in the evaluation of human drug properties. Cryopreserved human hepatocytes are now a universally accepted experimental system for the evaluation of human drug properties. This system is used routinely in pharmaceutical industry for drug discovery and development. Regulatory agencies such as European Medicines Agency (EMA) and United States Food and Drug Administration (US FDA) explicitly recommend drug metabolism and drug-drug interaction evaluations using cryopreserved human hepatocytes. As of this writing, cryopreserved human hepatocytes are available commercially and are routinely used for studies involving drug metabolism, drug-drug interactions such as drug metabolizing

Table 2. Donor demographics, post-thawed viability, yield, and P450 isoform-selective substrate metabolism activities of plateable cryopreserved human hepatocytes. The consistently high viability (majority of lots are > 90%) is a function of the optimized procedures used in the isolation, cryopreservation, and thawing of cryopreserved cells. Universal Cryopreservation Recovery Medium (UCRM) is especially important in thawing and recovery of cryopreserved hepatocytes. Viability was determined by trypan blue exclusion. The metabolic pathways evaluated were: phenacetin 1-hydroxylation (CYP1A2), bupropion hydroxylation (CYP2B6), paclitaxel 6α-hydroxylation (CYP2C8), diclofenac hydroxylation (CYP2C9), dextromethorphan hydroxylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), and testosterone 6β-hydroxylation (CYP3A4), with activity expressed as pmol/million hepatocytes/min (F: female; M: male; C: Caucasian; H: Hispanic; BMI: body mass index).

Lot number	Ethnicity	Gender	Age (years)	BMI	Viability (%)	Yield	CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2D6	CYP2E1	CYP3A4
HH1007	C	F	26	26.9	94.0	5.4	140.0	143.5	3.8	80.5	177.5	240.8	398.3
HH1020	C	F	21	23.2	89.1	4.9	237.5	507.5	2.1	74.3	112.1	229.5	843.2
HH1025	C	F	59	32.8	90.1	5.6	28.0	66.6	0.7	58.8	11.4	120.4	126.4
HH1031	H	M	42	43.6	93.0	5.5	12.7	81.0	2.3	59.8	18.0	38.0	179.2
HH1033	C	F	40	44.9	94.0	6.2	31.1	18.3	1.3	66.0	19.4	35.9	73.9
HH1036	C	M	55	26.0	94.0	7.0	37.5	39.0	1.4	74.6	6.9	49.2	512.8
HH1045	H	M	9	19.5	90.0	9.0	51.9	19.5	1.4	90.3	35.5	38.9	311.4
HH1051	C	M	23	25.3	97.0	5.5	59.5	71.8	0.2	55.0	2.5	34.2	17.9
HH1052	C	M	44	24.1	92.0	7.6	399.0	68.7	0.5	249.9	27.3	35.8	46.3

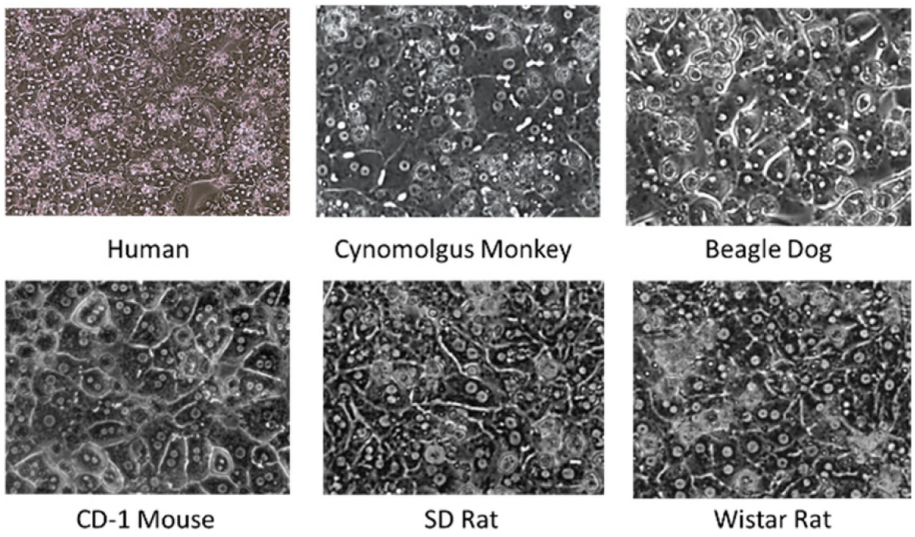

Fig. 2. Morphology of cultured cryopreserved human and animal hepatocytes. The cryopreserved hepatocytes formed highly confluent (near 100%) cultures and exhibited the cobble-stone shape typical of freshly isolated hepatocytes. The successful cryopreservation allows simultaneous experimentation of hepatocytes from human and multiple animal species for the selection of the most appropriate animal species for *in vivo* assessment of human drug properties as described for human cell paradigm (HCP). [Phase-contrast microphotograph (200× magnification)].

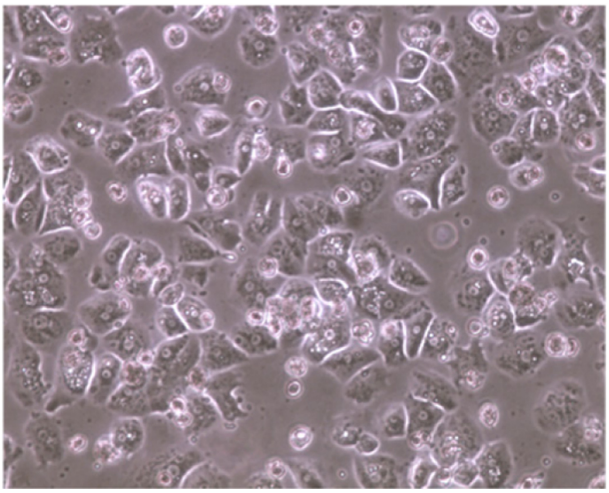

Fig. 3. Morphology of pooled cryopreserved human hepatocytes. Hepatocytes from 5 lots of plateable cryopreserved hepatocytes were thawed, pooled, and cryopreserved. The ability of hepatocytes to be cryopreserved two times to retain viability and plateability demonstrates the effectiveness of cryopreservation technology. [Phase-contrast microphotograph (200× magnification)].

enzyme inhibition and induction, and drug toxicity (3). The most recent application is the assessment of transporter-mediated hepatic uptake (17-20) and efflux (21-23), time-dependent enzyme inhibition (24-28), as well as the evaluation of metabolism-dependent drug toxicity (29,30). The scientific concepts of these adverse drug effects and the human hepatocyte assays developed for their assessment are reviewed here.

Drug-drug interactions (DDI): When a drug affects the efficacy and toxicity of a co-administered drug, the results may have grave consequences. DDI may be due to pharmacological mechanisms, leading to potentiation of efficacy (*e.g.* interacting with different pathway yielding complementary pharmacological effects), or reduction of efficacy of a co-administered drug (*e.g.* antagonistic competition for the same receptors). Pharmacological interactions may lead to serious consequences due to exaggerated pharmacological effects as exemplified by the severe blood pressure drop upon co-administration of sidenafil and nitroglycerine (31). Pharmacological DDI in general are readily managed due to the well-defined pharmacological properties of the marketed drugs.

Serious consequences are usually attributed to pharmacokinetic DDI where one drug significantly alters the metabolic fate of a co-administered drug. The evaluation of DDI potential in general is focused on the major human CYP isoforms, with the most important isoform being CYP3A4, which is known to metabolize > 50% of existing drugs (32). The mechanism of metabolism-based DDI can be inhibitory or inductive.

I. Inhibitory DDI: Inhibition of drug metabolizing enzyme activity by one drug (the perpetrator drug) can lead to diminished metabolism of a co-administered drug (the victim drug) that is mainly cleared by the inhibited pathway. This leads to an increase in systemic burden of the victim drug, which may result in toxic events. A clear example of this type of inhibitory drug-drug interactions is the terfenadine-induced cardiac arrthymia (Torsade des Pointes) upon

co-administration of terfenadine with drugs that are inhibitors of CYP3A4 (33-35). Upon ingestion, terfenadine is rapidly and effectively metabolized by hepatic CYP3A4. Co-administration with CYP3A4 inhibitors such as ketoconazole, itraconazole and erythromycin would lead to elevation of plasma levels of terfenadine to cardiotoxic levels, resulting in severe, sometimes fatal cardiotoxicity. Terfenadine was subsequently removed from the market and replaced by the pharmacological active but nontoxic metabolite, fexofenadine (36).

II. Inductive DDI: Another type of drug-drug interaction is inductive DDI, where the perpetrator drug would accelerate the metabolic clearance of a co-administered victim drug via the induction of a drug metabolism pathway. A major consequence of inductive DDI is the lowered pharmacological activity (loss of efficacy) of the affected victim drug which can have serious consequences. An example of inductive drug-drug interactions is the induction of the metabolism of drugs that are CYP3A4 substrates by the potent enzyme inducer rifampin (37-40). An example of the serious consequence of inductive DDI is the lowered cyclosporine plasma levels in a patient co-administered with rifampin, leading to rejection of a transplanted kidney (41).

III. Transporter-based DDI: It is now well-recognized that many drug transporters can play key roles in drug disposition. Many drugs are not freely permeable to cellular plasma membrane, but would enter or exit a cell as substrates of uptake or efflux transporters, respectively. In the intestinal epithelium, the efflux transporter P-glycoprotein (Pgp) can transport an absorbed drug back into the lumen. In the liver, uptake transporters [*e.g.* organic anion transporter 1 (OAT1) and efflux transporters (*e.g.* multiple drug resistance protein (MDR1)] of the hepatocyte plasma membrane are key determinants of intracellular concentrations, and thereby impact metabolism and toxicity of substrate drugs. Drugs that are transporter inhibitors can cause DDI with drugs that are substrates of the inhibited transporters.

It is now known that clinically significant DDI can occur due to interactions with drug transporters, leading to alterations of cellular uptake and efflux. Clinically significant transporter-based DDI have been found in small intestine, liver and kidney (42-45). Evaluation of transporter-based DDI potential of drug candidates is now recommended by regulatory agencies including US FDA and EMA for marketing approval (46-48).

IV. Human hepatocyte-based assays for metabolism and transporter-based DDI: *In vitro* evaluation of metabolism-based and transporter-based DDI are now routinely used in drug development and are required for regulatory approval (48-51). Human hepatocytes are used routinely for this endeavor.

1. Metabolite profiling and pathway identification: In general, pathway identification assessment of the major drug-metabolizing enzyme pathways involved in the metabolism of the drug in question is the first step of DDI assessment. One of the most useful applications of hepatocytes in DDI evaluation is the generation of a complete metabolite profile, allowing the assessment of the major metabolic pathways (52,53). The observation of oxidative metabolites such as hydroxylated metabolites and the respective conjugates would indicate that phase 1 metabolism is involved, and DDI may occur with drugs that are inhibitors or inducers of the phase 1 pathways such as P450 isoforms. Direct conjugation metabolites would suggest the involvement of phase 2 pathways, and thereby DDI may occur with inhibitors of phase 2 conjugating enzymes. Upon the completion of metabolite profiling, enzyme-selective inhibitors and inducers are used to further identify the specific drug metabolizing enzymes (54). It is to be noted that metabolite profiling using hepatocytes from multiple species is a well-accepted approach to select the most appropriate nonhuman animal species for *in vivo* metabolism studies.

2. P450 inhibition studies: As for metabolic stability, HLM are used routinely for inhibitory DDI evaluation (55). In general, P450 inhibition studies - evaluation of the inhibitory potential of a drug candidate on the major P450 isoforms: CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4, are required for regulatory approval. It is now believed that the use of hepatocytes will provide additional insight on inhibitory potential of a drug based on plasma levels (3). The use of intact hepatocytes allow drug partitioning, with the most important being bioaccumulation due to hepatocytes via uptake transporters. The use of hepatocytes may also generate data on the inhibitory potential of metabolites. For instance, gemfibrozil is a potent inhibitor of CYP2C8 in hepatocytes but not in HLM due to the formation of the potent metabolite inhibitor, gemfibrozil glucuronide, in hepatocytes (56). Procedures for the evaluation of time-dependent P450 inhibition have been established in human hepatocytes (24-26,28). A recent advance is the use of the luminescent substrate luciferin IPA in human hepatocytes to increase throughput for the evaluation of the inhibition of CYP3A4, the P450 isoform that is key to many cases of clinically significant DDI (26,57,58).

3. P450 induction studies: Primary cultured human hepatocytes represent the gold standard for P450 induction studies (14,59). Induction of CYP1A2, CYP2B6 and CYP3A4, each representing the nuclear receptors aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR), respectively, are required by regulatory agencies internationally. Experimental protocols have been developed to improve the efficiency of P450 induction studies via the use of P450 substrate cocktails (60-62) and the use of multi-well plates, including 48- and 96-well formats (63,64). The use of luciferin-IPA as a substrate greatly improves the efficiency of CYP3A induction studies (57,58). While enzyme activity has been regarded as the most relevant endpoint for P450

induction studies, data have been generated suggesting that quantification of gene expression may provide more relevant results, especially for inducers that also inhibit enzyme activities (65,66).

4. Efflux transporter inhibition: Efflux transporters play important roles in drug disposition, with the key organs being the intestine for the uptake of orally-administered drugs, the liver for hepatic uptake of absorbed drugs, and the brain where efflux transporters play a key role in the blood-brain barrier. Experimental approaches have been developed for the evaluation drug-modulation of efflux transporters. While induction has been observed with efflux transporters, clinically-significant DDI are observed mainly with inhibitory effects.

a. Pgp (MDR-1) inhibition studies: As discussed earlier, Pgp inhibition by a drug may increase intestinal absorption via decreased efflux of a co-administered drug that is a substrate of the inhibited transporter. Pgp inhibition is generally studied *in vitro* in cellular models of the intestinal epithelium such as a human colon carcinoma Caco-2 cells, which, when cultured as polarized monolayer culture, are known to express Pgp, and in the Pgp-transfected Madin-Darby canine kidney (MDCK) cells (67,68).

b. Hepatic efflux transporter studies: Inhibition of efflux transporters in hepatocytes may lead to accumulation of a co-administered drug which is a substrate of the inhibited transporter. Furthermore, as hepatic efflux transporters are involved in bile salt excretion, an efflux inhibitor may lead to bioaccumulation of bile salts, leading to hepatotoxicity. Collagen-matrigel sandwiched human hepatocyte cultures are known to express efflux transporters and are used for inhibition studies (22,23,69-75).

5. Uptake transporter inhibition: It is now well-accepted that cryopreserved human hepatocytes can be used routinely to evaluate transporter-mediated drug uptake (20,70). *In vitro* quantification of inhibitory DDI mediated by uptake transporters can be coupled with Physiologically based pharmacokinetic (PBPK) modeling to assess clinical effects (76-81).

Hepatotoxicity. Drug-induced hepatotoxicity is a major challenge in drug development. In spite of the extensive preclinical safety testing in laboratory animals, severe hepatotoxicity remains a major reason for clinical trial failure and withdrawal of marketed drugs (82-84). Extensive efforts are being invested to develop both clinical and preclinical approaches to minimize drug induced liver injury (DILI) (82,85-88). Species difference in sensitivity to drug toxicity and rare incidence due to the requirement of co-occurrence of multiple events are two possible reasons for the difficulty in the preclinical and clinical safety assessment in drug development (2,89).

Hepatic metabolism (90-93) and bile salt efflux transporter inhibition (94-96) are now believed to play critical roles in DILI. Hepatic xenobiotic metabolism may lead to the formation of reactive metabolites, leading to inactivation of critical biochemical pathways and ultimately, toxicity. The hapten hypothesis states that DILI may occur as a result of cytotoxic immune interaction with neoantigens resulting from covalent binding of reactive metabolites to cellular proteins.

Primary cultured human hepatocytes, with near normal xenobiotic metabolic capacity and efflux transporter activity, represent one of the most relevant *in vitro* models for early evaluation of DILI potential of drug candidates. *In vitro* cytotoxicity assays with primary human and animal hepatocytes are now used routinely for *in vitro* screening of hepatotoxic potential during drug development.

1. Cytotoxicity assays: Cytotoxicity can be readily quantified in cryopreserved human hepatocytes cultured as monolayer cultures on collagen-coated vessels, with throughput enhanced via the use of multiwall plates including 96-, 384-, and 1536-well plates (97-103). Via the use of various endpoints, one can assess the mechanism of cytotoxic effects. MTT or MTS metabolism and cellular ATP content are used to quantify cytotoxicity due to mitochondrial impairment. Release of cytoplasmic enzymes such as lactate dehydrogenase, AST, and ALT indicate plasma membrane damage. Caspase activation measures apoptosis. A decrease in reduced glutathione concentration suggests the formation of reactive metabolites. Finally, quantification of reactive oxygen species (ROS) indicates oxidative stress. The use of the endpoints in combination (high-content multi-parameter cytotoxicity assay) has been reported to increase the accuracy of toxicity prediction (87,104,105). Cytotoxicity screening assays with hepatocytes from multiple animal species can aid the selection of nonhuman animal species for *in vivo* safety studies (99,106).

2. Toxicogenomics: The use of microarrays allows the evaluation of the effects of toxicants on a multitude of genes. Human hepatocytes have been used in conjunction with toxicogenomics for the identification of biomarkers of toxicity with some success (107,108). Data mining from both hepatocytes *in vitro* and animals *in vivo* appear to be promising in the identification of gene signatures for hepatotoxicity (109).

3. Metabolism-based cytotoxicity assay: Biotransformation is a key determinant of drug toxicity. Metabolic activation (formation of toxic metabolite from a relatively nontoxic parent) is a well-established phenomenon (110,111). Examples of metabolically-activated toxicants include the environmental toxicant benzo(a)pyrene (112), the food contaminant aflatoxin B1 (29,113), the hepatotoxic drug acetaminophen (114,115), and the anticancer drug cyclophosphamide (116,117). Conversely, metabolic detoxification can lead to lower toxicity (118,119). Definition of the roles of metabolism in drug toxicity may lead to a better understanding of inter-individual differences, which is a major challenge in safety assessment. In our laboratory, assays have been

developed to define the role of metabolism in drug toxicity using metabolic inhibitors as well as a comparative cytotoxicity assay using hepatocytes and a metabolically-incompetent cell line, the mouse 3T3 cells (29). Using a novel co-culture system, the Integrated Discrete Multiple Organ Co-culture (IdMOC™), an *in vitro* assay has been developed to evaluate the role of hepatic metabolism on the cytotoxicity of a drug to hepatocytes and to nonhepatic cells (14,120-124).

Identification of drugs with potential to cause liver failure. One major challenge in the clinical management of drug-induced liver injuries is that the current biomarkers (such as serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) do not always accurately predict the final outcome. Primary cultured human hepatocytes represent an experimental system that may be useful in the identification of biomarkers that can accurately define the onset of serious liver injuries. For instance, in a collaborative study with U. S. FDA National Center for Toxicological Research, our laboratory identified oxidative stress/ATP ratio as an accurate biomarker for drugs that are associated with severe liver injuries (unpublished data), suggesting that oxidative stress-associated products in body fluids may be useful in the assessment of the onset of severe liver injuries. Toxic mechanisms discovered in hepatocytes may aid the detection of occurrence of such events *in vivo*. Transient elevation of serum bile salt for instance, has been correlated with hepatotoxicity due to inhibition of bile salt efflux protein (BSEP) (125). Cultured hepatocytes can also be used to substantiate the mechanistic relevance of promising biomarkers found *in vivo* (86) via correlation of the signal of the biomarkers and hepatocyte cytotoxicity *in vitro*. Toxicogenomics (108), proteomics (126) and metabolic (127) studies with hepatocytes may reveal novel toxic pathways that may lead to discovery of unique biomarkers which can be readily quantified in body fluids as early indicators of liver injuries.

Nonhepatic toxicity and the Integrated Discrete Multiple Organ Co-culture (IdMOC™) experimental system. As described above, hepatocytes represent an effective *in vitro* experimental model for the evaluation of hepatotoxicity. Similar approaches can be used for the evaluation of toxicity with nonhepatic organs such as heart, lung, kidney, intestines, vascular systems, etc., using key cell types from each of the organs to be evaluated. The currently available primary cell systems and the respective organ-specific toxicity that can be evaluated include the following:

- Hepatocytes (hepatotoxicity)
- Renal proximal tubule epithelial cells (nephrotoxicity)
- Vascular endothelial cells (vascular toxicity)
- Neuronal cells, glial cells and astrocytes (neurotoxicity)
- Cardiomyocytes (cardiotoxicity)
- Bone marrow cells (bone marrow toxicity)

The absence of hepatic metabolism is a well-recognized major drawback of the use of nonhepatic cells to evaluate nonhepatic organ toxicity. As described earlier, hepatic metabolism is a key determinant of drug toxicity. A drug can be rendered more toxic (metabolic activation) or less toxic (detoxification) due to hepatic metabolism. The IdMOC experimental system has been developed to allow the incorporation of hepatic metabolism in the evaluation on nonhepatic toxicity.

The IdMOC system involves the "wells-in-a-well" concept. The typical IdMOC plate consists of a chamber within which are several wells (Figs. 4 and 5). Cells of different origins (*e.g.* from different organs) are initially cultured, each in its specific medium, in the wells. When the cells are established, the wells are flooded with an overlying medium, thereby connecting all the wells within each chamber. The multiple cell types now can interact via the overlying medium, akin to the multiple organs in a human body interacting via the systemic circulation. The advantage of the IdMOC system is that one can evaluate differential cytotoxicity of a toxicant towards multiple cell types under near-identical experimental conditions, with interaction among multiple cell types allowed via the overlying medium. As the multiple cell types are physically separated, the effect of a toxicant on each particular cell type can be evaluated individually after experimentation. An IdMOC culture has

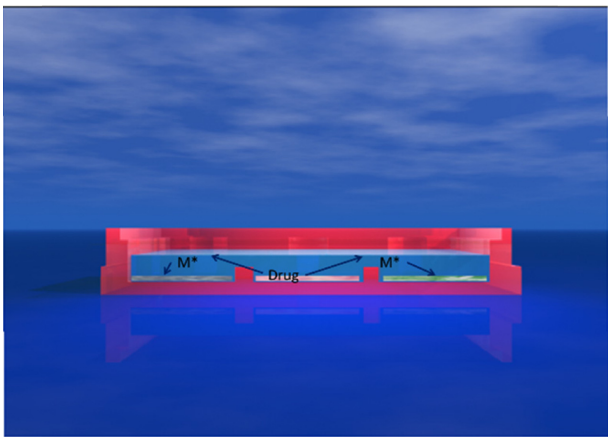

Fig. 4. Schematic representation of the IdMOC experimental system. IdMOC consists of multiple shallow wells within a larger containing well (chamber). Cells are plated firstly into the shallow wells (one cell type per well) to allow attachment and growth. Upon establishment of the cultures, the shallow wells are flooded with a co-culture medium, allowing cells from the difference inner wells to communicate via soluble factors. An important application of IdMOC is to evaluate the toxicity of hepatic metabolites on nonhepatic cells as illustrated here with a drug metabolized by hepatocytes from the center well to form metabolites (M) that are released by the hepatocytes into the medium and ultimately interacting with nonhepatic cells cultured in a different well in the chamber.

Fig. 5. Photograph of an IdMOC multi-well plate with sixteen chambers and six inner wells per chamber (IdMOC-96). The plate has the same footprint of a regular 96-well plate and is compatible with multi-channel pipets.

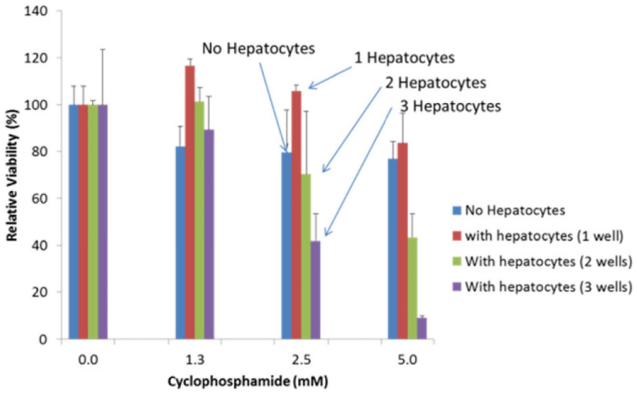

Fig. 6. Cyclophosphamide cytotoxicity in IdMOC with human hepatocytes and 3T3 cells. Each IdMOC chamber is seeded with 3 wells of 3T3 cells co-cultured with 0, 1, 2, and 3 wells of hepatocytes. The chambers are flooded with medium containing 0, 1.25, 2.5 and 5 mM of cyclophosphamide. Dose-dependent cytotoxicity to 3T3 cells was observed only in the presence of 2 and 3 wells of hepatocytes, demonstrating the release of cyclophosphamide metabolites from hepatocytes to cause cytotoxicity in the metabolically-incompetent 3T3 cells. The system can be adopted to evaluate cytotoxicity of hepatic metabolites to any other nonhepatic cell types by simply replacing the 3T3 cells with the cell type of interest (*e.g.* cardiomyocytes for *in vitro* cardiotoxicity studies).

advantages over co-culturing of multiple cell types as mixed cultures in the same cell culture vessel as the cell types evaluated may not have physical contacts *in vivo*, and that it would be very challenging, if not physically impossible, to separate the different cell types after experimentation to evaluate the effects of the toxicants being studied (121,123,124).

Applications of IdMOC include the following:

1. Differential cytotoxicity: Evaluation of the toxicity of a substance on different cell types (*e.g.* cells from different organs) under virtually identical experimental conditions with multiple cell-type interactions.

2. Differential distribution: Evaluation of the differential accumulation/distribution of a substance among multiple cell types. This application is especially useful for the development of cytotoxic anticancer agent with selective affinity towards cancer cells.

3. Incorporation of hepatic metabolism: Via the co-culturing hepatocytes with nonhepatic cells in the IdMOC, one can evaluate the role of hepatic metabolism on the toxicity of a toxicant towards the nonhepatic cells. We have reported the use of IdMOC co-cultures of metabolically hepatocytes and mouse 3T3 cells to demonstrate this major application of IdMOC, namely, the incorporation of hepatic metabolism in the evaluation of cyclophosphamide, a toxicant that is metabolically activated (30) (Fig. 6). Recently, we also have used IdMOC to demonstrate detoxification using aminophenol, a toxicant that is known to be metabolically detoxified.

4. Whole body IdMOC: A new configuration of IdMOC has been developed in our laboratory. We now have IdMOC with 48 and 96 inner wells within each outer well, thereby allowing a larger variety of cells to be co-cultured. An example of an IdMOC-man by co-culturing cells from multiple organs is shown in Fig. 7.

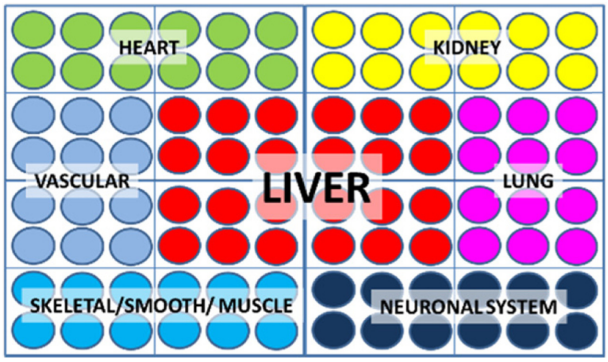

Fig. 7. Next generation IdMOC. This new IdMOC design has a single chamber with 48 wells or 96 wells (as shown), thereby allowing the co-culture of a relatively large number of cell types. An example is shown here with 24 wells of hepatocytes at the center of the plate surrounded by 12 wells for each of the key nonhepatic organs. This configuration allows the culturing of multiple cell types per organ. Each plate thereby models a single animal or human with the selected key organs. This new IdMOC configuration is thereby an *in vitro* system modeling the whole organism (Each of the organs is represented by a different color).

CONCLUSION AND FUTURE DIRECTIONS

For accurate assessment of adverse drug effects, the clas-

sical approach of total reliance on evaluation in laboratory animals is not adequate due to species differences. A new paradigm, HCP, is proposed here in which human-specific data are obtained using *in vitro* human-based experimental systems, and applied in combination with known human *in vivo* parameters (*e.g.* PBPK parameters) or with experimental results from relevant *in vivo* animal species, for a more accurate assessment of human *in vivo* effects.

Human hepatocytes represent an important experimental system for the definition of human-specific drug properties. Successful cryopreservation of human hepatocytes to retain viability and hepatic metabolism allow the cells to be used routinely for experimentation. Cryopreserved human hepatocytes are applied routinely in drug development for the evaluation of metabolic fate, P450 inhibition, P450 induction, *in vitro* hepatotoxicity, metabolism-based toxicity, and IdMOC™ evaluation of hepatic metabolism on nonhepatic toxicity.

Drug-drug interactions using human hepatocytes is now routinely practiced in drug development and is required by government regulatory agencies for new drug approval. Hepatocytes are used for the definition of metabolic pathways, P450 inhibition and P450 induction. Recently, hepatocytes are used to define drug-drug interactions resulting from interaction of a drug with uptake and efflux transporters.

Unlike drug-drug interactions, *in vitro* toxicity evaluation is not yet regarded as a definitive approach to investigate drug safety in drug development. *In vitro* toxicity studies such as hepatocyte cytotoxicity screening are used for the prioritization of compound selection for further development. In some cases, where clinical toxicity is observed, *in vitro* systems are used for mechanistic evaluation to allow a better understanding of the physiological relevance of the findings and to develop approaches for the selection of safe alternatives. A major drawback of *in vitro* systems is the lack of the complex multiple-organ interaction. The IdMOC™ system and microfluidic approaches are steps towards this direction. The ultimate goal of accurate assessment of human drug toxicity will rely on the development of *in vitro* platforms with multiple organs, including the immune system, with each organ represented by multiple cell types and communication among organs achieved using human plasma or equivalent. Current advances in bioengineering and stem cell biology may allow this ultimate goal to be achieved. The development of multiple organ co-culture systems with hepatocytes and immune cells may also facilitate the identification of biomarkers for drug-induced liver injuries.

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