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Review

Application of cryopreserved human hepatocytes in drug development: metabolism, drug-drug interactions, and drug toxicity

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ABSTRACT

Successful cryopreservation of hepatocytes, especially human hepatocytes, is one of the major reasons for the recent routine application of this experimental system in drug development. Cryopreserved human hepatocytes retain viability and metabolic capacity and are used extensively as suspension cultures to evaluate the metabolic fate (metabolic stability and metabolite profiling) of new chemical entities (NCE) during drug development. Pooled cryopreserved human hepatocytes, i.e., hepatocytes cryopreserved from several individual donors that have been thawed, pooled, and re-cryopreserved, represent the most commonly used system for routine metabolism studies. One major issue with cryopreservation, namely, the loss of the ability of the cells to be cultured, has been overcome. Now hepatocytes from both animals and humans can be cryo-preserved to retain their ability to form monolayer cultures (known as "plateable" cryopreserved hepatocytes). The use of "plateable" cryopreserved hepatocytes enhances experimental efficiency by providing an immediate supply of easily stored cells and eliminating the centrifugation steps to remove test articles after treatment (e.g. uptake and time-dependent inhibition studies), which is required for suspension cultures. Plating extends their use in applications that involve culturing for a prolonged period (multiple days), such as evaluating metabolic stability of slowly-metabolized compounds, P450 induction, efflux transport, and hepatotoxicity. A significant advancement in the application of plateable cryopreserved hepatocytes is the evaluation of the role of metabolism-based drug toxicity on extrahepatic organs in a single test system, especially the novel Integrated Discrete Multiple Organ Co-culture (IdMOC™) system.

KEYWORDS: cryopreserved hepatocytes, drug metabolism, drug toxicity, drug development, drug-drug interactions, drug-transporter interactions, IdMOC

INTRODUCTION

Overcoming species differences is a major challenge for drug development. Drug development in general employs the classical paradigm of the selection of safe and efficacious drug candidates based on results obtained from laboratory animals, followed by clinical trials in humans. It is now known that the effectiveness of this classical approach is hindered by the known species differences affecting drug properties, especially ADMET drug properties: absorption, disposition, metabolism, elimination and toxicity [1-3]. The end result is the high incidence of clinical trial failure, mainly due to unacceptable efficacy and safety issues, which is estimated to be 85% or higher [4]. Proposed solutions include redesigning drug evaluation strategies, including early clinical trials and reducing or refining tests performed in animals [5].

A prudent and efficient approach to overcome the challenges of species differences is the early

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assessment of human drug properties using human-based experimental models, which has been applied successfully in drug metabolism and pharmacokinetic studies. An often-cited statistic is the dramatic decrease in clinical trial failure due to pharmacokinetics, which is accredited to the use of *in vitro* human drug-metabolism test systems. Mechanistic understanding of drug metabolism science has helped to achieve successful *in vitro-in vivo* extrapolation approaches using human-derived *in vitro* test systems to evaluate human drug metabolism. A significant development is that regulatory agencies such as the Food and Drug Administration (FDA) of the United States now requires *in vitro* human drug metabolism results for the assessment of drug-drug interactions in lieu of animal data.

Despite the acceptance of human *in vitro* test systems as a predictor of drug metabolism information, the safety assessment of an Investigative New Drug (IND) application for FDA approval is completely driven by laboratory animal studies. An inevitable outcome is that candidates with human-specific drug toxicity would fail in clinical trials.

Classical toxicologists are reluctant to accept *in vitro* results with human experimental system for safety assessment due to the inadequacy of *in vitro* systems to model the complex whole organism. The one aspect of *in vitro* results accepted by toxicologists is the concept of metabolism-dependent drug toxicity - that a drug can be rendered less toxic (detoxification) or more toxic (metabolic activation) by biotransformation. Comparison of the metabolic fate of a drug candidate in *in vitro* experimental systems from multiple animal species and human is the accepted approach to justify the relevance of a certain laboratory animal species as an *in vivo* model for the evaluation of human safety. This is an important conceptual and practical advancement which hopefully will lead to further advances. Species differences in toxicological mechanism, such as molecular targets, pathways and repair are yet to be elucidated to aid the major challenges in drug development. Through mechanistic understanding of drug toxicity triggers and with advancements in mechanistically-relevant *in vitro* experimental systems and *in vitro-in vivo* extrapolation approaches, drug safety can be readily evaluated using *in vitro* systems, as in the disciplines of drug metabolism, pharmacokinetics, and drug-drug interaction.

Hepatocytes as a key *in vitro* experimental system for drug development

For a drug to be effective, it needs to be readily bioavailable (preferably via oral administration), be metabolically stable to allow a reasonable administration regimen, efficacious, safe, and without significant drug-drug interaction and toxicity potential. The liver plays a key role in these drug properties with the exception of bioavailability, which is mainly a function of intestinal absorption. Biotransformation determines the duration (metabolic stability) of the drug in the systemic circulation. Metabolic drug-drug interactions may lead to toxicity or loss of efficacy. Hepatic metabolites are known to exhibit toxicological and pharmacological properties which may be different from the parent drugs. Drug-drug interactions and toxicity are adverse drug properties that have been found to lead to clinical trial failures or withdrawal of marketed drugs [6-11].

While laboratory animals are routinely used for the evaluation of ADMET studies, the results may not be relevant to humans due to species differences. *In vitro* human-based hepatic systems are now routinely used to evaluate human-specific drug metabolism. These *in vitro* human test systems include liver post-mitochondrial supernatant (S9 or S10), liver microsomes, recombinant P450 isoforms, and hepatocytes. Hepatocytes, especially human hepatocytes, are generally considered the "gold standard" for *in vitro* drug metabolism and hepatotoxicity studies. The superiority of hepatocytes over the other *in vitro* systems is attributed to the various 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 [2, 12]. Furthermore, hepatocytes can be used for the evaluation of hepatotoxic potential of a drug. Hepatocytes (or parenchymal cells in the liver) are the target cells for hepatotoxic drugs. Drug-induced damage to hepatocytes is known to lead to liver failure [1, 9].

Isolation, cryopreservation and culturing of human hepatocytes

Human hepatocytes can be isolated from human liver biopsies or from whole livers that have been donated for transplantation but found to be unsuitable for that purpose. A two-step collagenase digestion procedure initially developed to isolate hepatocytes from laboratory animals is used. Success in hepatocyte cryopreservation in the recent decade allows human hepatocytes to be used routinely for experimentation [13-15]. It is now known that cryopreservation of hepatocytes does not significantly alter hepatocyte properties including P450 and non-P450 metabolism and drug transport. A significant recent advancement of hepatocyte cryopreservation is the retention of the cells' abilities to be cultured (i.e., plateable cells), akin to freshly isolated hepatocytes. This advancement is made possible via the use of a recovery medium that minimizes cell damage during the thawing process (commercially available as Cryopreserved Hepatocytes Recovery Medium (CHRM™) from APSciences Inc., Columbia, MD, and as Universal Cryopreservation Recovery Medium (UCRM) from In Vitro ADMET Laboratories LLC, Columbia, MD). With this medium, the post-thaw viability of cryopreserved human hepatocytes is consistently near 90%, as measured by the trypan blue exclusion method. (Table 1). In our laboratory, we are able to cryopreserve rodent and monkey hepatocytes to yield plateable hepatocytes routinely. For dog and human hepatocytes, the ratio of suspension grade (not plateable) to plateable hepatocytes is approximately 1:1. The morphology of plateable cryopreserved hepatocytes is similar to that of freshly isolated cells (Figure 1).

Another advancement of human hepatocyte cryopreservation is the preparation of hepatocytes pooled from multiple donors. Hepatocytes cryopreserved from individual donors are thawed, pooled (usually from 5 or 10 donors), followed by refreezing. In our laboratory, we have developed a QuickRefreeze™ procedure which allows re-cryopreservation of hepatocytes with minimal damage to the cells. The metabolic properties of the QuickRefreeze™ pooled hepatocytes are shown in Table 2.

Hepatocyte culturing

Cryopreserved human hepatocytes are routinely used for studies involving drug metabolism, drug-drug interactions such as drug-metabolizing enzyme inhibition and induction, and drug toxicity [2]. The most recent applications are the assessment of transporter-mediated hepatic uptake [16-19] and efflux [20-22], time-dependent enzyme inhibition [23-27], as well as the evaluation of metabolism-dependent drug toxicity [28, 29]. The culturing procedures and the associated applications are listed below:

1. Suspension culture

This is one of the most widely used methods for drug metabolism [30] and uptake [19] studies. With this culturing format, the hepatocytes are simply suspended in an isotonic buffer or culture medium for the evaluation of drug metabolism either in a water bath or incubator maintained at 37 ºC. One disadvantage of this procedure is that hepatocytes in suspension have a relatively short life span. Experience in our laboratory is that, on average, 50% of cell viability is lost for every 6 hrs of incubation. This limitation would not affect drug uptake studies due to their short incubation durations (minutes). For drug metabolism studies with human hepatocyte suspensions, incubations are generally performed for a maximum duration of 4 hrs. Suspension-grade cryopreserved hepatocytes, especially pooled cryopreserved human hepatocytes, are routinely used as suspension cultures.

2. Collagen matrigel sandwich (CMS) culture

CMS culture is the most widely used format for monolayer culturing of human hepatocytes. The hepatocytes are first allowed to attach onto culture vessels pre-coated with collagen. After 4 hours of attachment, the plating medium is changed to medium containing a basement membrane-derived protein mixture commercially known as Matrigel [31], usually at a concentration of 0.25 or 0.5 mg/mL. After prolonged culturing of the hepatocytes as CMS culture (e.g. 5 - 7 days), the cells can develop mature bile canaliculi. CMS cultured hepatocytes are used routinely for P450 induction [32] and drug efflux studies [22]. A major challenge with CMS cultures is that while

Table 1. Viability of human hepatocytes after recovery from cryopreservation. Results of 29 consecutive cryopreserved hepatocyte preparations from our laboratory are shown (each from a whole liver donated but not used for liver transplantation). Age is in years. Viability was determined by trypan blue exclusion assay. The cryopreserved hepatocytes were thawed in a 37 ºC water bath, followed by recovery via centrifugation in the Universal Cryopreservation Recovery Medium (UCRM). The mean viability is 87% $\pm$ 7%, with a cell number per vial of 6 $\pm$ 1 million viable hepatocytes. The results illustrate that human hepatocytes can be readily cryopreserved to retain high viability. Successful cryopreservation of the human hepatocytes is instrumental for the wide application of the cells in drug development. The uniformity of cell viability and cell number per vial allows the cells to be used readily as a laboratory reagent. (M: Male; F: Female; C: Caucasian; H: Hispanic).

Lot number	Gender	Race	Age	Viability	Yield per vial (million viable cells)
HH1006	M	H	35	82%	4
HH1007	F	C	26	94%	5
HH1008	F	C	60	93%	4
HH1009	F	C	47	99%	5
HH1012	M	C	22	89%	5
HH1016	F	H	15	93%	7
HH1018	M	C	17	55%	10
HH1019	M	C	21	87%	7
HH1023	F	C	48	81%	5
HH1024	M	C	59	87%	8
HH1019	M	C	17	87%	7
HH1021	F	C	21	84%	5
HH1022	F	C	21	84%	5
HH1023	F	C	21	81%	5
HH1024	M	C	48	87%	8
HH1025	F	C	59	80%	5
HH1027	F	C	59	88%	5
HH1028	M	H	60	98%	4
HH1029	M	C	61	87%	4
HH1031	M	H	63	92%	7
HH1032	F	C	64	92%	7
HH1033	F	C	65	92%	6
HH1034	M	C	66	89%	7
HH1035	F	C	67	88%	4
HH1036	M	C	68	94%	5
HH1037	M	C	69	96%	5
HH1038	M	C	70	83%	6
HH1039	M	C	71	83%	5
HH1040	F	C	72	90%	4

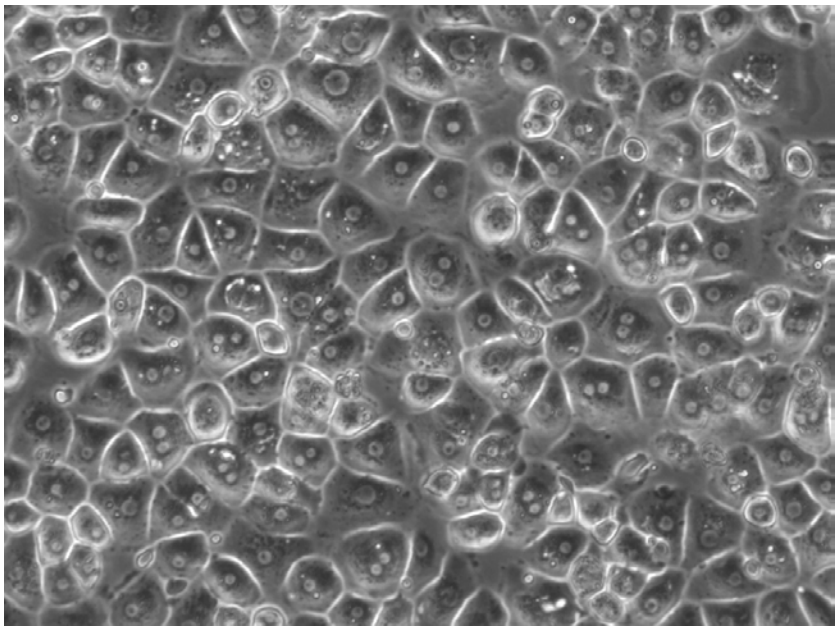

Figure 1. Morphology of cultured cryopreserved human hepatocytes (Lot HH1031; from a 63 year old female Hispanic donor). The confluency (near 100%) and cobble-stone epithelial cell morphology are hall marks of high quality, plateable cryopreserved hepatocytes. (From In Vitro ADMET Laboratories Archives, Columbia, Maryland, with permission).

the bile canaliculi would develop, thus increasing efflux transporter activities, most P450 and uptake transporter activities tend to decrease with culture duration (at a rate of approximately 50% per day). In our laboratory, we have developed a novel medium, the Li's Differentiation Maintenance Medium (LDMM) that can maintain most uptake and P450 gene expression in addition to increased efflux transporter gene expression. The utility of the LDMM is now being further investigated.

3. Co-culture of hepatocytes with nonhepatic cells

Hepatocytes have been co-cultured with other cell types to extend their utility in research applications. Examples are as follows:

a. Hepatocyte-Kupffer cell co-culture

Kupffer cells are known to have both protective and damaging effects in the liver. Kupffer cells are essential for scavenging bacterial endotoxins such as lipopolysaccharide (LPS) derived from the gut flora, thereby preventing hepatic damage. However, it is now also known that Kupffer cells are the major source of proinflammatory cytokines [33], which would cause down-regulation of P450 activities [34] and may exacerbate drug-induced liver injuries [35]. Hepatocytes-Kupffer cell co-cultures have been used to model inflammation-drug interactions [36].

b. Hepatocyte-nonhepatocyte co-culture to maintain differentiation

Hepatocytes are known to lose differentiation function with culture duration. This is illustrated by the rapid decrease in P450, uptake and efflux transporter gene expression within 48 hrs of plating. Co-culturing of hepatocytes with liver epithelial cells has been reported to maintain certain hepatic functions such as albumin production [37]. This observation generated optimism in the field, suggesting that the apparent inevitable dedifferentiation of primary hepatocytes in culture may eventually be overcome via modification of culturing conditions. One of the most recent developments is the micropatterned co-culture with islands of hepatocytes being cultured on a lawn of mouse 3T3 fibroblasts to allow prolonged culturing with applications in metabolism and toxicity studies [38, 39].

Table 2. Post-thawed viability, yield, and P450 isoform-selective activities of "pooled" cryopreserved human hepatocytes. Cryopreserved human hepatocytes from 10 individual donors were thawed, combined ("pooled"), and re-cryopreserved. In spite of being cryopreserved twice, the cells retained relatively high viability and normal P450 activities. Pooled cryopreserved human hepatocytes are now routinely used for metabolic studies to minimize individual differences. The preparation of pooled cryopreserved hepatocytes was performed using a patent-pending QuickRefreeze™ process developed by In Vitro ADMET Laboratories.

Post-thaw viability and cell quality assessment			
Thawing medium used	**Optimal centrifuge conditions**	**% Viability**	**Viable cell yield per vial**
UCRM™	100 x g for 10 min	81%	5.5 x 10^6 cells

Metabolic activity assessment				
P450	**Substrate**	**Concentration**	**Incubation (min.)**	**Metabolic Activity (pmol/ 10^6 cells/min.)**
1A2	Phenacetin	100 µM	15	53.4
2B6	Bupropion	500 µM	15	3.2
2C8	Paclitaxel	20 µM	15	1.8
2C9	Diclofenac	25 µM	15	345.2
2C19	S-Mephenytoin	250 µM	30	9.7
2D6	Dextromethorphan	15 µM	15	22.4
3A4	Testosterone	200 µM	15	446.7
ECOD	7-Ethoxycoumarin	100 µM	30	167.8
7-HCG	7-Hydroxycoumarin	100 µM	30	611.5
7-HCS	7-Hydroxycoumarin	100 µM	30	156.1

c. Hepatocyte-nonhepatocyte co-culture for metabolism-dependent drug toxicity

In vitro toxicity evaluation with nonhepatic primary cells or cell lines have the drawback of the lack of hepatic metabolism which may lead to metabolic activation or detoxification. The novel Integrated Discrete Multiple Organ Co-culture (IdMOC™) system which allows hepatocytes and nonhepatocytes to be co-cultured as physically separated cells but interconnected by a common overlying medium, represent one of the efforts to overcome this challenge and has been applied towards the definition of metabolism-dependent toxicity [29, 40, 41]. Microfluidic systems for similar applications have also been developed [42]. The IdMOC™ plate is shown in Figure 2.

The various culture formats for hepatocytes and their respective applications are shown in Table 3.

Human hepatocyte assays for drug development

1. Metabolic stability evaluation with human hepatocytes

One important drug property is its duration in the human body after administration. Hepatic metabolic clearance represents a major determinant of the half-life of a drug in the systemic circulation. During drug development, new chemical entities are routinely screened for metabolic stability - the rate of hepatic metabolic clearance. Metabolic stability routinely is performed using human liver microsomes (HLM) with NADPH as cofactor, thereby evaluating mainly phase 1 oxidation pathways such as P450 metabolism [43, 44]. Technical advances have been made, especially in mass spectrometry, to improve the throughput of

Figure 2. The Integrated Discrete Multiple Organ Co-culture (IdMOC™) experimental system. IdMOC uses a wells-in-a well concept, where cells from different organs are cultured in the inner wells, with interconnection achieved via flooding the inner wells with an overlying medium. The IdMOC thereby models the human *in vivo*, with multiple organs that are physically separated (discrete) but interconnected (integrated) by the systemic circulation. An IdMOC plate with the footprint of a 96-well plate is shown. The IdMOC plate is compatible with routinely used laboratory equipments such as the multichannel pipette shown here. (From In Vitro ADMET Laboratories Archives, Columbia, Maryland, with permission).

HLM metabolic stability screening [45-49]. The universal application of HLM metabolic stability screening in drug development has led to the accumulation of NCEs that are stable towards microsomal oxidative pathways, however, some are seen to exhibit unacceptable metabolic stability *in vivo* due to non-microsomal or non-NADPH-dependent biotransformation pathways, such as phase 2 conjugation. As a result, human hepatocytes, especially cryopreserved human hepatocytes pooled from multiple donors, are also used either as a primary screen or secondary screen (after HLM screening) to assess overall hepatic metabolic stability [48, 50-52]. It has been reported that intrinsic clearance results with both HLM and human hepatocytes can be accurately extrapolated to human *in vivo* for chemicals that are mainly metabolized via phase 1 oxidation [53]. Di *et al.* [54], suggest that comparison of intrinsic clearance values between HLM and human may provide valuable information to guide drug design. For instance, for compounds that are predominately metabolized by phase 1 oxidation such as cytochrome P450 (CYP) metabolism, the intrinsic clearance values from HLM and human hepatocytes would be similar. However, for compounds with non-CYP pathways, such as uridine-dependent glucuronosyl transferase (UGT) and aldehyde oxidase (AO), intrinsic clearance would be higher (more rapidly cleared) in hepatocytes than in HLM [54].

One challenge with the use of human hepatocytes for clearance studies is that hepatocytes in suspension are known to show a decrease in viability with time in culture (T1/2 of approximately 6 hrs). Due to this limitation, incubation duration in general is limited to a maximum of 4 hrs. To overcome this limitation, a relay method has been established by Di *et al.* [55], in which the test articles are incubated for 4 hrs, with the media separated from the hepatocytes by centrifugation, followed by re-incubation with a new hepatocyte suspension. Using this method, an incubation of 20 hrs can be achieved by five 4-hr incubations. This relay method is useful for compounds with low hepatic intrinsic clearance.

One advantage with monolayer cultures of human hepatocytes is that the cells, upon attachment to a substratum (e.g., cell culture dishes or multi-well plates), would remain viable for multiple days. In our laboratory, we routinely use monolayer cultures of human hepatocytes (using plateable cryopreserved hepatocytes) for metabolic stability studies. We have modified the relay method of Di *et al.*, using plated hepatocytes with the relay incubation step after 24 hrs of incubation. Linear time-dependent disappearance of parent compounds is observed up to 96 hrs of incubation.

Metabolic stability results are useful in the estimation of *in vivo* hepatic intrinsic clearance (CL_{int}) using the observed time for 50% hepatocyte-metabolism dependent disappearance

Table 3. Culture formats and applications for hepatocytes. The application of hepatocytes in drug development is enhanced by new development in culture technologies. While suspension cultures remain widely used for short-term incubations (hours), monolayer single cell type and co-cultures, allow the application of hepatocytes to evaluate drug properties that requires a longer incubation duration (days). Microfluidic and IdMOC™ cultures allow the evaluation of metabolism-dependent drug toxicity, a major challenge for *in vitro* evaluation of drug toxicity.

Culturing format	Application	Example	Reference
Suspension	Short-term (hours) metabolism studies	Prediction of metabolic clearance	[169]
Suspension	Drug uptake	Evaluation of role of uptake transporters on provastatin drug-drug interactions	[170]
Monolayer culture on collagen-coated substratum	*In vitro* toxicity	High throughput screening of hepatotoxicity	[146]
Collagen-matrigel sandwich culture	P450 induction	Multiple endpoint (activity, protein, mRNA) evaluation of CYP1A2, CYP2B6 and CYP3A4 induction	[171]
Collagen-matrigel sandwich culture	Drug uptake	Development of screening for uptake transporter-based drug interaction potential	[172]
Collagen-matrigel sandwich culture	Drug efflux	MRP2 inhibition by HIV protease inhibitors in rat and human hepatocytes as a possible mechanism of hepatotoxicity	[118]
Micropatterned hepatocyte-3T3 cell co-culture	Drug metabolism; drug toxicity	Screening of drugs with clinical hepatotoxicity	[38]
Microfluidic hepatocyte culture	Drug metabolism; drug toxicity	Prediction of *in vivo* hepatic clearance of model compounds	[173]
Hepatocyte spheroids	Drug metabolism; drug toxicity	Evaluation of drug-induced liver injuries	[174]
Hepatocyte bioreactors	Drug metabolism; drug toxicity	"Long term" drug metabolism and drug toxicity	[175, 176]
Integrated discrete multiple organ co-culture (IdMOC)	Metabolism-dependent toxicity	Classification of toxicants based on the role of hepatic metabolism and site of toxicity	[29]

of the compound ($T_{1/2}$). Parent drug concentration is plotted versus incubation time, followed by a plot of ln2 (% remaining) versus time (min). CL_{int} is then calculated using the following equations:

$T1/2 = [ln2/-slope]$

$CL_{int} = ln2 \times (T1/2)^{-1} \times (\text{cell concentration})^{-1} \times \text{cellularity} \times (\text{liver weight/body weight})$

Units:

T1/2: minutes; cell concentration: million cells/mL in the suspension culture; cellularity: million cells per g of liver; liver weight: g; body weight: kg.

The commonly used constants for CL_{int} calculations for animals and human are shown in Table 4.

Table 4. Hepatocyte parameters used for calculation of intrinsic hepatic clearance *in vivo* from data obtained with hepatocytes *in vitro*.

Species	Hepatocellularity (million cells per g of liver)	Liver weights (g liver per kg body weight)
CD-1 mouse	135	54.9
SD Rat	110	33.6
Beagle dog	169	32
Cynomolgus monkey	120	32
Man	120	21

2. Metabolite profile evaluation with human hepatocytes

Identification of hepatic metabolites is an important activity in drug development. The identities of the metabolites provide clues on key metabolic pathways, therefore allowing structural modification to improve stability as well as estimation of possible drug-drug interaction potential. Another application of metabolite profiling data is the selection of animal species with biotransformation pathways similar to human for the chemical in question.

With the rapid advancement in liquid chromatography/mass spectrometry (LC/MS) technologies, metabolite identification can be readily performed in most analytical chemistry laboratories. The study is performed by incubating the chemical in question with an *in vitro* metabolism system for an extended time period followed by LC/MS identification of metabolites.

As described for metabolic stability, HLM studies would provide information for metabolites formed by microsomal drug-metabolizing enzymes such as CYP metabolites, while information about hepatic metabolites formed by both microsomal and non-microsomal pathways can be obtained with human hepatocytes. A comparison of results from HLM and hepatocytes provides useful insight on the key pathways of metabolism [56]. We were one of the first laboratories to report metabolite profiling with hepatocytes to illustrate species-difference in metabolism between human and rat [57]. Metabolic profiling, especially the identification of reactive metabolites and their conjugates (e.g. L-glutathione conjugates) is routinely used to aid elucidation of potential drug toxicity, especially hepatotoxicity [58-64].

Metabolic profiling results allow the identification of biotransformation pathways (reaction phenotyping [65]) which is one of the key steps in the estimation of drug-drug interaction potential. For this purpose, human hepatocytes represent the most appropriate experimental system. Metabolite profiles in human hepatocytes provide information on the relative contribution of phase 1 and phase 2 metabolic pathways. If phase 1 metabolites and their conjugates are found to be the major metabolites, CYP metabolism is likely to be involved. The follow-up studies would then use HLM, in combination with isoform-selective CYP inhibitors or expressed CYP isoforms, to define the key CYP isoforms involved. Identification of these key isoforms would thereby allow the identification of potential victim or perpetrator drugs in relation to the NCE [2, 66-69]. The application of human hepatocytes in the definition of drug-drug interaction potential is further described below.

3. Drug-drug interactions

Polypharmacy, the treatment of a patient with multiple drugs either for the treatment of multiple ailments or a single ailment, is commonly practiced. It is necessary to ensure that co-administration of multiple drugs in a patient would not lead to undesirable drug interactions. Exacerbated pharmacological effects may occur when two drugs affect either the same pathway or unrelated pathways, but have similar physiological effects. An example is the severe hypotension in patients

co-treated with sildenafil for the treatment of erectile dysfunction and nitrate for the treatment of chest pain [70]. In general, pharmacological-based drug-drug interactions can be readily managed as the molecular mechanisms of action of most drugs are well-defined. On the other hand, a drug may interfere with the metabolic clearance of a co-administered drug, leading to unintentional alteration of drug exposure. Such pharmacokinetic or metabolism-based interactions cannot be defined empirically as experimental evaluation of a drug with all possible co-administered drugs would be a monumental task. A practical approach is to evaluate the effects of a drug on key metabolic pathways followed by estimation of the potential interactions with known substrates of the affected pathways. This mechanism-based experimental assessment of drug-drug interaction potential is generally practiced in drug development and is required by the U.S. FDA for new drug applications.

I. Metabolism-based drug-drug interactions (DDI)

When one drug significantly alters the metabolic fate of a co-administered drug, serious consequences may occur. 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 [71]. The mechanism of metabolism-based DDI can be inhibitory or inductive.

a. 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, leading 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 (Torsades de Pointes) upon co-administration of terfenadine with drugs that are inhibitors of CYP3A4 [72-74]. 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 [75].

Most inhibitory DDI is due to reversible P450 inhibition which can be alleviated by removal of the perpetrator drug from being administered with the victim drug. Another form of inhibition, time-dependent inhibition or mechanism-based inhibition is due to irreversible inactivation of metabolizing enzymes by highly reactive drug metabolites. In this case, the metabolic capacity of the patient will return to normal after replacement of the inactivated enzyme molecules with molecules that are newly synthesized after the removal of the perpetrator drug.

b. Inductive DDI

Another type of drug-drug interactions is inductive drug-drug interactions 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 [76-79]. 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 [80].

II. Transporter-based DDI

Besides metabolism-based DDI which is a result of the inhibition or induction of drug metabolizing enzymes by the perpetrator drugs, it is now known that clinically significant DDI can occur due to interactions with drug transporters, leading to alterations in cellular uptake and efflux. Clinically significant transporter-based drug-drug interactions occur in many organs, including the small intestines, liver and kidney [81-84]. Evaluation of transporter-based DDI is now recommended by international regulatory agencies. [85-87].

Examples of clinically significant transporter-based DDI are as follows:

1. Inhibition of intestinal P-glycoprotein (P-gp) mediated efflux

Inhibition of drug efflux at the intestinal mucosa epithelium leads to increased net absorption and higher systemic exposure. Digoxin, a P-gp substrate that is not metabolized by CYP3A4, is used routinely as an *in vivo* probe for P-gp inhibition. The known P-gp inhibitors that are found to increase digoxin exposure in human patients include ritonavir [88] and grapefruit juice [89].

2. Inhibition of hepatic uptake

Inhibition of hepatic uptake transporters by a drug has been found to lead to elevated plasma levels of the affected drug due to its decreased hepatic clearance, leading to toxicity. Inhibitors of uptake transporters with clinically-observed drug interactions include inhibition of OATP1B3 by lopinavir and ritonavir, resulting in the elevation of bosentan exposure [90]; cyclosporin inhibition of OATP1B1 and OATP1B3-mediated hepatic uptake of cerivastatin, leading to elevated plasma concentrations and resulting in rhabdomyolysis [91-94]; and OATP1B1 inhibition by a single dose of rifampin, leading to increased glyburide exposure [95].

3. Inhibition of renal excretion

Drugs that are inhibitors of renal uptake transporters (e.g. organic cation transporter 2 (OCT2), organic anion transporter1 (OAT1), multiple drug and toxin extrusion 1, 2 (MATE1, MATE2) can have DDI with substrates of the transporters. Clinically observed renal transporter-mediated DDI include cimetidine inhibition of metformin excretion by OCT2 [96, 97] and probenecid inhibition of renal tubule secretion of ciprofloxacin and gemifloxacin [98, 99].

In vitro evaluation of metabolism-based and transporter-based DDI are now routine in drug development and are required for regulatory approval [87, 100-102]. Human hepatocytes are used routinely for the evaluation of DDI potential due to their extensive capabilities regarding metabolism and transporter applications.

III. Human hepatocyte-based assays for metabolism and transporter-based DDI

1. Pathway identification

Pathway identification of the major drug-metabolizing enzyme pathways of a drug candidate, in general is the first step of DDI assessment. As discussed earlier under metabolite profiling, the most useful application of hepatocytes in DDI evaluation is the generation of a complete metabolite profile, allowing the assessment of the major metabolic pathways [103, 104]. The observation of oxidative metabolites such as hydroxylated metabolites and their respective conjugates would indicate that phase 1 metabolism is involved, and DDI may occur when co-administered with drugs that are inhibitors or inducers of those 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. After the completion of metabolite profiling, enzyme-selective inhibitors and inducers can be used to further identify the specific drug metabolizing enzymes [67].

2. P450 inhibition studies

P450 inhibition studies are performed via incubation of the compound in question with an *in vitro* hepatic metabolism system and an isoform-specific P450 substrate. After an appropriate incubation time, the reaction is stopped via the addition of an organic solvent (e.g. acetonitrile) followed by quantification of substrate metabolism. In general, P450 inhibition studies (evaluation of the potential of a drug candidate to inhibit the major P450 isoforms: CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4) are required for regulatory approval. As for metabolic stability, HLM are used routinely for inhibitory DDI evaluation [68]. It is now believed that the use of hepatocytes will provide additional insight on inhibitory potential of a drug based on plasma levels [2]. The use of intact hepatocytes allow drug investigations regarding partitioning, with the most important being uptake transporter mediated bioaccumulation in hepatocytes. 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 [105]. Procedures for the evaluation of time-dependent P450 inhibition have been established in human hepatocytes [23-25, 27]. A recent advance is the use of luminescent substrate luciferin-IPA to increase throughput for CYP3A4 inhibition in human

hepatocytes, which is the key mechanism of many cases of clinically-significant DDI [25, 106, 107].

3. P450 induction studies

Primary cultured human hepatocytes represent the gold standard for P450 induction studies [13, 108]. Induction assessment of CYP1A2, CYP2B6 and CYP3A4, each representing induction via the three major nuclear receptors: aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR), respectively, is 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 [109-111], and the use of multi-well plates including 48-, and 96-well formats [112, 113]. The use of luciferin-IPA as a substrate greatly improves the efficiency of CYP3A induction studies [106, 107]. While enzyme activity has been regarded as the most pertinent endpoint for P450 induction studies, data have been generated suggesting that quantification of gene expression may provide a more accurate estimation of clinical results, especially for inducers with enzyme inhibitory activities [114, 115].

4. Efflux transporter inhibition

Efflux transporters play important roles in drug disposition, with the key organs being the intestines, blood brain barrier, kidney, and liver. Experimental approaches have been developed to evaluate drug-modulation of efflux transporters. While induction has been observed with efflux transporters, clinically-significant DDI are observed mainly with inhibitory effects.

a. P-gp (MDR-1) inhibition studies

As discussed earlier, P-gp inhibition by a drug may increase intestinal concentration via decreased efflux of a co-administered drug that is a substrate of the inhibited transporter. P-gp inhibition in general is studied *in vitro* in cellular model of the intestinal epithelium such as a human colon carcinoma Caco-2 cells which, when cultured as polarized monolayer culture, are known to express P-gp, and in the P-gp-transfected Madin-Darby canine kidney (MDCK) cells [116, 117].

b. Hepatic efflux transporter studies

Inhibition of efflux transporters in hepatocytes may lead to accumulation of a co-administered drug that 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 [21, 22,118-124].

5. Uptake transporter inhibition

It is now well-accepted that cryopreserved human hepatocytes can be used routinely to evaluate transporter-mediated drug uptake [19, 119]. *In vitro* quantification of inhibitory DDI mediated by uptake transporters can be coupled with physiologically-based pharmacokinetics (PBPK) modeling to estimate clinical effects [125-130].

Drug toxicity evaluation in human hepatocytes

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 [131-133]. Extensive efforts are being invested to develop both clinical and preclinical approaches to minimize drug induced liver injury (DILI) [131, 134-137]. There are many reasons for the difficulties experienced when trying to predict drug toxicity during preclinical and clinical safety assessments, including species' differences in drug toxicity sensitivities and the rarity of concurrent events required to elicit a toxic response [1, 138].

Hepatic metabolism [139-142] and bile salt efflux transporter inhibition [143-145] are now believed to play critical roles in DILI. Primary cultured human hepatocytes, with near normal 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.

- Type I: Direct-acting (Tamoxifen): Organ-specific toxicity dependent on exposure to parent toxicant

- Type II: Metabolically activated, localized toxicity (Aflatoxin-B1): Organ-specific toxicity dependent on site of metabolic activation

- Type III: Metabolically activated, diffusible toxic metabolites (Cyclophosphamide): Organ-specific toxicity dependent on metabolite distribution

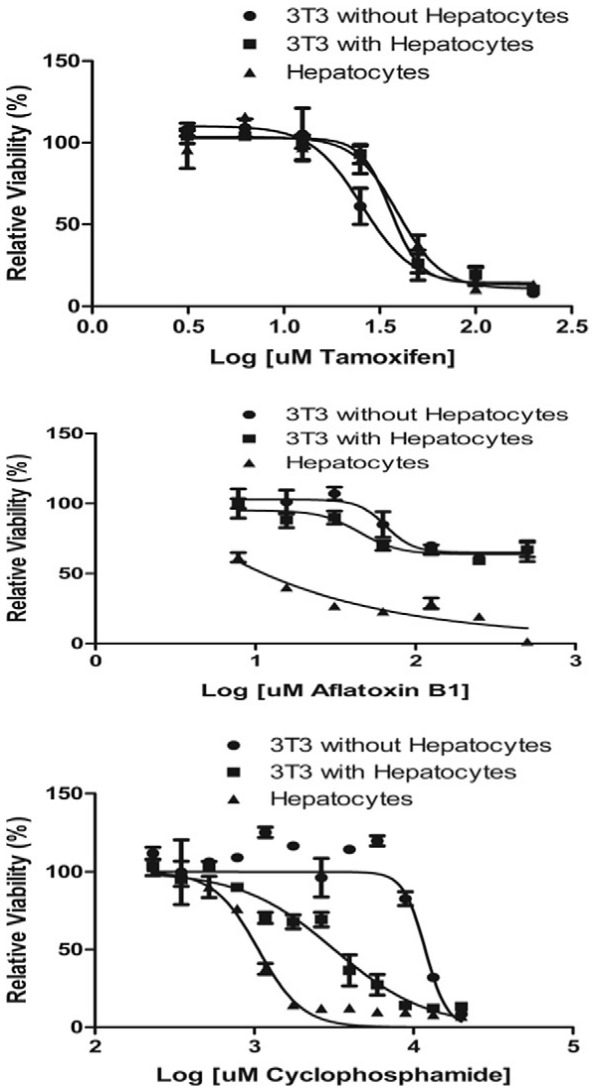

Figure 3. The use of IdMOC in the metabolic classification of cytotoxicants. Three model toxicants: the direct acting tamoxifen caused similar cytotoxicity in hepatocytes and the co-cultured 3T3 cells as well as in the control culture of 3T3 cells without hepatocytes (top panel); the metabolism-dependent toxicant aflatoxin which caused higher cytotoxicity in hepatocytes than in 3T3 cells, with the highly reactive toxic metabolites causing toxicity mainly in the hepatocytes; and the metabolism-dependent toxicant which is metabolized by the hepatocytes to form diffusible, cytotoxic metabolites that are cytotoxic to the co-cultured 3T3 cells (with no cytotoxicity in the control 3T3 culture without hepatocyte co-culture). (Modified and adapted from [29]).

1. Cytotoxicity assays

Cytotoxicity can be readily quantified in cryopreserved human hepatocytes cultured as monolayer cultures on collagen-coated vessels, with the throughput enhanced via the use of multi-well plates including 96-, 384-, and 1536-well plates [50, 64, 146-150]. Through the use of various endpoints, one can assess the mechanism of cytotoxic effects. For instance, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (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 for plasma membrane damage, caspase activation for apoptosis, and cellular reduced

glutathione for the formation of reactive metabolites. The use of the endpoints in combination with high-content multi-parameter cytotoxcity assay has been reported to increase the accuracy of toxicity prediction [136, 151, 152]. Cytotoxicity screening assays with hepatocytes from multiple animal species can aid the selection of the most appropriate animal species for *in vivo* safety studies [148, 153].

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 [154, 155].

3. Metabolism-based cytotoxicity assay

Biotransformation is a key determinant of drug toxicity. Metabolic activation, the formation of toxic metabolite from a relatively nontoxic parent is a well-established phenomenon [156, 157]. Examples of metabolically-activated toxicants include the environmental toxicant benzo(a)pyrene [158], the food contaminant aflatoxin B1 [28, 159], the hepatotoxic drug acetaminophen [160, 161], and the anticancer drug cyclophosphamide [162, 163]. Conversely, metabolic detoxification can lead to lower toxicity [164, 165]. Definition of the roles of metabolism in drug toxicity may lead to a better understanding of individual differences, 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 [28]. The Integrated Discrete Multiple Organ Co-culture (IdMOC™) has been developed to evaluate the role of hepatic metabolism on the cytotoxicity of a drug to hepatocytes and to nonhepatic cells [13, 40, 41, 166-168]. The IdMOC system and its application to define the role of hepatic metabolism on drug toxicity are shown in Figures 2 and 3.

CONCLUSION

Because of species difference, drug properties observed in laboratory animals can differ greatly from those observed in man. Knowledge of human-specific drug properties before the performance of clinical trials is critical to successful drug development. The concept that *in vitro* results obtained with human-based experimental systems can accurately predict *in vivo* human drug properties is now generally accepted in drug metabolism and drug-drug interaction studies. This acceptance is a result of research findings in the validation of the *in vitro* systems, including objective elucidation of limitations and advantages of the various systems, and the development of robust assays and *in vitro-in vivo* extrapolation approaches. Of the various *in vitro* systems for the evaluation of drug metabolism, human hepatocytes are now generally considered to be the “gold standard”, as they can adequately model the key hepatic processes: passive diffusion, transporter-mediated uptake, biotransformation by P450 and non-P450 pathways, and transporter-mediated efflux. Successful cryopreservation allows human hepatocytes to be used routinely, and the availability of high quality cryopreserved human hepatocytes is a major reason for the wide application of this valuable experimental system in drug development.

CONFLICT OF INTEREST STATEMENT

The author, Dr. Albert P. Li, is the president and CEO of In Vitro ADMET Laboratories LLC (IVAL), Columbia, MD and Malden, MA. IVAL is a commercial vendor of freshly isolated and cryopreserved human and animal hepatocytes and is a provider of contract research services.

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