



# Human Hepatocytes as an Effective Alternative Experimental System for the Evaluation of Human Drug Properties: General Concepts and Assay Procedures

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## Summary

*Human-based in vitro hepatic experimental systems are now used routinely in drug development. The rationale for the use of human-based in vitro systems is based on the known species-species differences in drug properties. Human-specific drug properties, by definition, cannot be defined using nonhuman experimental animals, and therefore can only be assessed in the preclinical phase of drug development using in vitro human-based approaches. A widely applied human-based in vitro experimental system for preclinical evaluation of drug properties is human hepatocytes. Our laboratory was one of the first to successfully isolate highly viable and functional hepatocytes from human livers, and we recently developed cryopreservation procedures to retain high viability and high attachment efficiency of the isolated hepatocytes. Successful cryopreservation of human hepatocytes greatly enhances the utility of this valuable in vitro experimental system, allowing storage, transport, convenient scheduling of experimentation and repeat experimentation using hepatocytes isolated from the same donors. Effective assays have been developed with cryopreserved human hepatocytes using multiwell plates for the evaluation of critical drug properties, including metabolic stability, drug-drug interaction potential, and drug toxicity. Human hepatocytes represent an alternative experimental system that plays a significant role in the 3Rs – the reduction, refinement, and replacement of the use of animals in preclinical drug development research.*

*Zusammenfassung: Humane Hepatozyten als effektives alternatives Testsystem für die Evaluierung von Substanzwirkungen auf den Menschen: allgemeine Konzepte und Testmethodik Human-basierte in vitro Modelle der Leber werden inzwischen routinemäßig in der Entwicklung von Medikamenten verwendet. Das ursprüngliche Konzept, human-basierte in vitro Systeme zu verwenden, basiert auf bekannten Spezies-Spezies-Unterschieden. Human-spezifische Wirkqualitäten können per Definition nicht mittels nichthumaner Labortiere untersucht werden und können daher in der präklinischen Phase der Entwicklung von Medikamenten nur unter Nutzung von human-basierten in vitro Ansätzen evaluiert werden. Ein weit verbreitetes human-basiertes in vitro System für präklinische Evaluierung der Wirkung von Substanzen ist das humane Hepatozytensystem. Unser Labor war eines der Ersten, die erfolgreich lebensfähige und funktionelle Hepatozyten aus menschlichen Lebern isoliert haben, und wir haben jüngst Kryokonservierungsmethoden entwickelt, durch die die hohe Überlebensfähigkeit und Anheftungsfähigkeit der isolierten Hepatozyten erhalten werden. Erfolgreiche Kryokonservierung humaner Hepatozyten verbessert die Anwendbarkeit dieses nützlichen experimentellen Systems deutlich und erlaubt Lagerung, Transport, bequeme Planung von Experimenten und Wiederholung von Experimenten mit den Zellen des gleichen Spenders. Effektive Tests sind unter Nutzung von kryokonservierten Hepatozyten in Multiwellschalen entwickelt worden, um kritische Substanzeigenschaften, u.a. metabolische Stabilität, Substanzinteraktionspotential und Substanztoxizität während der Substanzentwicklung festzustellen. Humane Hepatozyten stellen ein alternatives experimentelles System dar, das eine wichtige Rolle in der Reduktion, der Verbesserung und dem Ersatz von Tierversuchsmethoden in präklinischer Medikamentenforschung spielt.*

**Keywords:** human hepatocytes, drug metabolism, drug-drug interactions, cryopreservation

## 1 Introduction

One major challenge in the selection of drug candidates for clinical trials is that, due to species-species differences in

drug properties, human specific drug effects cannot be detected using nonhuman animal experimental systems. The high rate of clinical trial failures has been attributed to this species-species difference

(DeMasi et al., 2003). One of the reasons for species-species differences in drug properties is the occurrence of species-specific xenobiotic metabolism pathways. Species-differences in P450-dependent monooxygenases, a major group of enzymes responsible for drug

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metabolism, are well established (Guengerich, 2006).

*In vitro* experimental systems with human-specific properties represent an attractive tool for the assessment of human-specific drug properties. *In vitro* experimental systems derived from the human liver, namely, human hepatocytes and human liver tissue fractions, are now used routinely for the assessment of human drug metabolism. The combined use of human *in vitro* hepatic systems and relevant nonhuman animal models is believed to be responsible for the reduction in the contribution of pharmacokinetics as a major factor in human clinical trial failures from approximately 40% in 1991 to approximately 10% in 2000 (Kola and Landis, 2004).

The parenchymal cells of the liver, commonly known as hepatocytes, contain the majority of, if not all, hepatic xenobiotic biotransformation enzymes. The drug metabolic activities of the hepatocytes therefore represent those of the liver as an organ. Furthermore, the hepatocytes are often the cells damaged by hepatotoxic drugs, leading in some cases to severe liver damage, including organ failure. Hepatocytes isolated from the liver have long been believed to be a relevant experimental system for the evaluation of drug properties (e.g. Fry, 1982), a view that continues to be held by the scientific community (e.g. Li, 2007; Gomez-Lechon et al., 2007). The use of hepatocytes in the evaluation of drug metabolism, drug-drug in-

teraction potential, and drug toxicity is now routine practice in both academic and industrial laboratories (Li, 2005).

There are many reviews, including those by this author, on the general concepts of the use of human hepatocytes in drug metabolism and toxicology research (Li, 2004a; 2004b; 2007; Gomez-Lechon et al., 2007; Hewitt et al., 2007). The emphasis of this review is to provide a practical, state-of-the art overview of the use of human hepatocytes in the evaluation of drug properties during drug development.

## 2 Human hepatocytes for the evaluation of human drug properties

A major challenge in the prediction of human drug properties using the conventional approach, namely, testing in laboratory animals, is that because of species-species differences, human-specific drug properties cannot be detected. Species-differences in drug properties are mainly a result of differences in drug metabolizing activities. As illustrated in Table 1, the isoforms of the major family of drug metabolizing enzymes, the P450-dependent monooxygenases, are different between laboratory animals and humans. The different isoforms may lead to differences in rates of metabolism and formation of different metabolites from a chemical entity, resulting in species-species differences in metabolic fate and toxicity. A

clear example of species-differences in metabolism is the formation of 7-hydroxycoumarin in humans but not in rodents (Easterbrook et al., 2001).

Hepatocytes isolated from human livers would retain human-specific hepatic metabolism activities and therefore represent a valuable preclinical experimental system for the early assessment of human-specific drug properties.

### 2.1 Isolation of human hepatocytes

The general procedures for the isolation of hepatocytes from all animal species, including humans, are essentially similar, involving firstly the perfusion of the liver or liver fragment with an isotonic, divalent ion-free buffer containing the calcium chelator EGTA to remove blood, dissolve clots, and to loosen cell-cell junctions. This is followed by perfusion with a collagenase-containing isotonic buffer with the divalent ions calcium and magnesium, which are required for collagenase activity. The collagenase serves as an enzyme to dissociate the hepatocytes from the liver parenchyma into single cell suspension. Our laboratory represents one of the first to isolate and cryopreserve human hepatocytes and a detailed procedure for human hepatocyte isolation based on the original method of Berry and Friend (1969) was previously reported by our laboratory (Li et al., 1992) for human liver fragments. The procedures have now been modified for large scale hepatocyte isolation from the whole human liver (Li, 2007).

Procurement of human livers for research as well as human hepatocyte isolation are activities that are not commonly available to most laboratories, and had represented the major hindrance to research with human hepatocytes when this experimental system was initially introduced to the scientific community. This major hindrance to the use of human hepatocytes is now circumvented by the cryopreservation of the hepatocytes.

### 2.2 Cryopreservation of human hepatocytes

Our laboratory was one of the first to report successful cryopreservation of human hepatocytes (Loretz et al., 1989), as well as

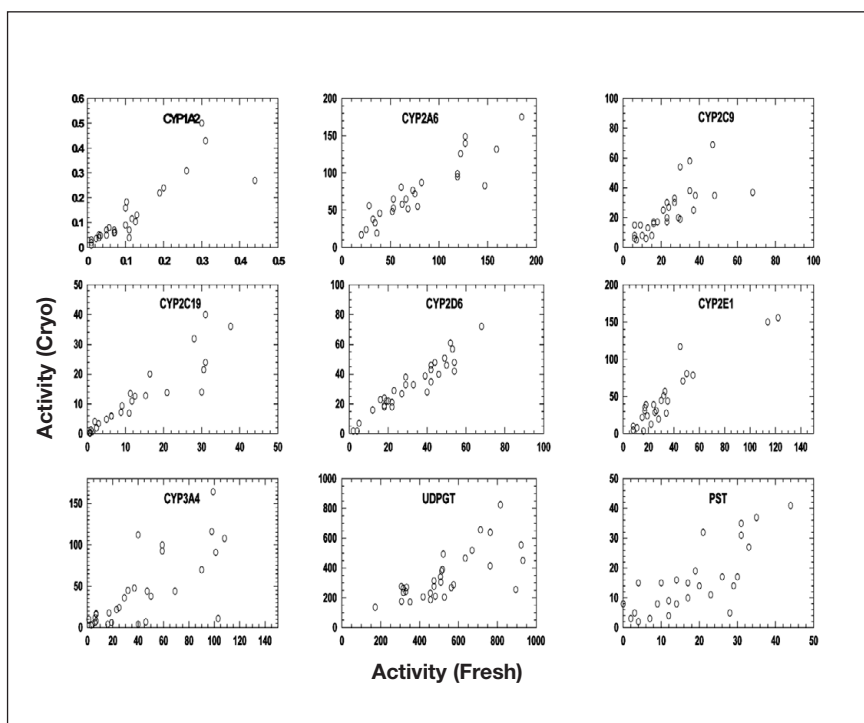
**Tab. 1: Predominant P450 isoforms in various animal species**

The P450-dependent monooxygenases are the major xenobiotic metabolizing enzymes in the liver. The table here illustrates one of the scientific bases for species-species differences in drug properties. Species-specific P450 isoforms may lead to different affinities and rates of metabolism of xenobiotics, leading to species-differences in metabolic fate and/or toxicity. For the five animal species shown here, species-differences in P450 isoforms are present for all P450 families except for CYP1A and CYP2E.

P450 Family	P450 Isoforms				
	Human	Mouse	Rat	Dog	Monkey
CYP1A	1A1/2	1A1/2	1A1/2	1A1/2	1A1/2
CYP2A	2A6	2A5	2A5	2A13/25	2A23/24
CYP2B	2B6	2B9/10	2B1	2B11	2B17
CYP2C	2C8/9/19	2C29/37/38/40/44	2C6/7/11	2C21/41	2C20/43
CYP2D	2D6	2D22	2D1	2D15	2D17
CYP2E	2E1	2E1	2E1	2E1	2E1
CYP3A	3A4/5	3A11/13	3A1/2	3A12/26	3A8

the first to show similar drug metabolizing enzymes between cryopreserved and freshly isolated human hepatocytes, and the development of assays for metabolic stability, drug-drug interactions, and cytotoxicity using cryopreserved human hepatocytes (Li et al., 1999a, 1999b). The similarity between freshly isolated and cryopreserved human hepatocytes in drug metabolizing enzyme activities is now generally accepted by the scientific community (Li et al., 1999a; 1999b; Li, 2007; Hewitt et al., 2007; Jouin et al., 2006). A comparison of drug metabolizing enzyme activities between cryopreserved and freshly isolated human hepatocytes is shown in Figure 1.

Until recently, cryopreserved hepatocytes in general would lose their ability to be cultured as attached, monolayer cultures, presumably due to the unavoidable membrane damage occurring during the cryopreservation and subsequent thawing processes. It has been projected in the past that one out of 10 to 20 human hepatocyte isolations would lead to “plateable” cryopreserved hepatocytes. A focused research effort was initiated in our laboratory in 2005 to overcome this deficiency in hepatocyte cryopreservation. Our research resulted in the development of highly optimized hepatocyte isolation, cryopreserva-



**Fig. 1: A comparison of the drug metabolizing enzyme activities of freshly isolated human hepatocytes versus the cryopreserved cells from the same isolations**  
Activities of the major human P450 isoforms, UDP-dependent glucuronosyl transferase (UDPGT) and phenol sulfotransferase (PST) are shown. The results show that while there are individual to individual variations in activities, which are expected, there is in general a 1:1 ratio of the activities of the hepatocytes before and after cryopreservation. The data suggest that cryopreserved human hepatocytes can be used in lieu of freshly isolated cells for drug metabolism studies involving these drug metabolizing enzymes. Graph adopted from Li et al. (1999b).

**Tab. 2: Reagents used with the application of cryopreserved human hepatocytes in drug metabolism, drug-drug interactions, and cytotoxicity studies**

These are the reagents used in the author's laboratories and may be replaced with similar reagents from other manufacturers.

Reagent	Application	Manufacturer	Catalog Number
Trypan blue solution (0.4%)	Viability determination	Sigma-Aldrich (www.sigmaaldrich.com)	T8154
Cryopreserved Hepatocytes Recovery Medium (CHRM)	Recovery of hepatocytes after thawing	APSciences Inc. (www.apsciences.com)	MB7002
Hepatocyte Suspension Medium (HSM)	Medium for the resuspension of thawed cryopreserved hepatocytes for viability and yield determination	APSciences Inc. (www.apsciences.com)	MB7004
Hepatocyte Metabolism Medium (HMM)	Medium for metabolism studies such as metabolic stability, metabolite profiling. The medium is also used for the evaluation of P450 substrate metabolism for P450 inhibition and induction studies.	APSciences Inc. (www.apsciences.com)	MB7010
Hepatocyte Plating Medium (HPM)	Medium for the plating of hepatocytes for culturing as attached monolayer cultures	APSciences Inc. (www.apsciences.com)	MB7006
Hepatocyte Induction Medium (HIM)	Medium for enzyme induction studies	APSciences Inc. (www.apsciences.com)	MB7012
Collagen-coated Cell Culture Plates	Plates for the culturing of hepatocytes as monolayer cultures	APSciences Inc. (www.apsciences.com)	CCP-24 (24-well plates) and CCP-96 (96-well plates)
ATP Reagent	Quantification of cellular ATP content for cytotoxicity studies	Perkin Elmer (www.perkinelmer.com)	6016736

tion, and recovery procedures (Li, 2007).

One practical achievement of our research with hepatocyte cryopreservation is the development of a Cryopreserved Hepatocyte Recovery Medium (CHRM), which greatly enhanced the quality of the human hepatocytes when thawed from cryopreservation.

Key reagents used in our laboratory for the thawing, recovery and application of cryopreserved human hepatocytes described in this paper are listed in Table 2.

The following are the procedures for the thawing and recovery of cryopreserved human hepatocytes using CHRM:

1. Place a 50-ml tube of CHRM in a water bath at 37°C and allow approximately 2 hours for the CHRM to reach 37°C.
2. Remove a vial of cryopreserved human hepatocytes from the liquid nitrogen freezer and immediately immerse the vial in a 37°C water bath for thawing.
3. Continuously shake the vial in the water bath until the ice crystal totally disappears.
4. Quickly pour the thawed hepatocytes into the pre-warmed 50-ml tube of CHRM.
5. Gently invert the CHRM to allow even distribution of the hepatocytes in the medium.
6. Centrifuge at 100 x g for 10 minutes to pellet the hepatocytes.
7. Discard the supernatant and resuspend the hepatocytes in 2 ml of Hepatocyte Suspension Medium (HSM) for cell concentration and viability evaluation.

Viability of thawed hepatocytes is usually quantified using a hemocytometer based on trypan blue exclusion. A common procedure is as follows:

1. Add 100 µl of the hepatocyte suspension into 700 µl of culture medium.
2. Add 200 µl of trypan blue solution. Invert to mix. Wait approximately 5 minutes at room temperature to allow dye penetration into damaged hepatocytes.
3. Load the mixture into a hemocytometer for counting.

Hepatocyte viability is expressed as the percent of trypan-blue excluding cells:

Viability (%) = Number of trypan blue-excluding cells / Total number of cells x 100%

Cell concentration is determined from the hemocytometer counts using the following equation:

Cells / ml = number of viable cells per square x 10,000 x Dilution Factor

The initial 10,000 factor is a correction for the liquid volume of each square of the hemocytometer ( $10^{-4}$  ml). The dilution factor using the above dilution scheme is 10. (A cell count of 25 trypan-blue excluding hepatocytes per square, for instance, would result a cell concentration of 2.5 million hepatocytes per ml, or 5.0 million viable hepatocytes per vial.)

The major achievement of our laboratory is that the post-thaw viability is consistently over 85%, and that over 50% of our cryopreserved human hepatocyte lots attach and form monolayer cultures after thawing (Li, 2007). The viability and yield of cryopreserved cells from 18 human hepatocyte isolations are shown in Table 3. The morphology of cultures established from plateable cryopreserved human hepatocytes is shown in Figure 2.

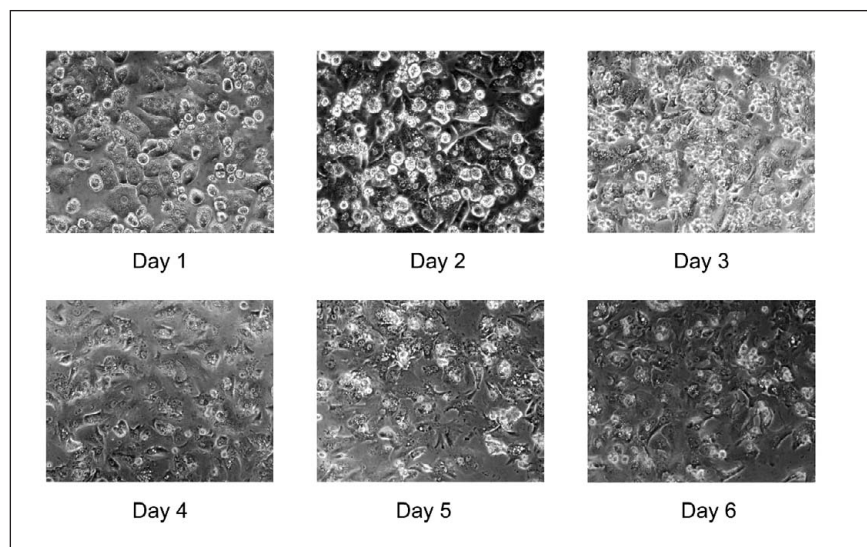
Besides the retention of high viability and plateability, human hepatocytes after cryopreservation have been shown to retain human drug-metabolizing enzyme activities, including the activities of P450 isoforms, UDP-dependent glucuronosyl transferase activity (UGT), and sulfotransferase activity (ST) (Li et al., 1999a; 1999b). The origi-

nally proposed applications of cryopreserved hepatocytes in drug metabolism studies (Li et al., 1999a; 1999b) have been generally accepted by the scientific community at large (Jouin et al., 2006; Brown et al., 2007). As described later, plateable cryopreserved human hepatocytes can also be used for enzyme induction studies (Kafert-Kasting et al., 2006). One of the latest findings with plateable cryopreserved human hepatocytes is that they form functional bile canaliculi and therefore can be applied towards the evaluation of hepatobiliary excretion (Bi et al., 2006).

Human hepatocyte cryopreservation is an enabling technology for the use of cryopreserved human hepatocytes. The advantages of cryopreserved hepatocytes over freshly isolated cells include long-term storage, ease of experimental scheduling, choice of pre-characterized lots for experimentation, and repeat experimentations with hepatocytes from the same donors.

### 3 Applications of human hepatocytes in drug development

The following are the current routine applications of human hepatocytes in drug



**Fig. 2: Morphology of primary cultures of previously cryopreserved human hepatocytes**

The cryopreserved hepatocytes were thawed, recovered in the Cryopreserved Hepatocytes Recovery Medium (CHRM), and plated into collagen-coated 24-well plates at a cell density of 350,000 cells per well. Phase contrast photomicrographs of the human hepatocytes at days 1-7 after culturing are shown. The results show that cryopreservation did not affect the ability of the cells to attach and form monolayer cultures with the cobble-stone morphology typical of hepatocytes.





development. Cryopreserved human hepatocytes are routinely used for these assays. While the general scientific principles of the *in vitro* screening methodologies have been previously reviewed (Li, 2001; 2004a; 2007), the specific procedures for each assay are described here.

### 1. Metabolic stability screening

An important “drug-like” property for new chemical entities (NCE) is appropriate metabolic stability to allow a practical frequency of drug administration. In the past, liver microsomes were used routinely for metabolic stability screening. However, as

liver microsomes contain mainly enzymes such as the P450 isoforms for Phase I oxidation, the assay would only yield metabolic stability towards microsomal oxidative enzyme metabolism, while in humans *in vivo*, the chemicals studied may be cleared via nonmicrosomal enzyme pathways such as conjugating enzyme pathways. A comparison of the drug metabolizing enzyme pathways between hepatocytes, liver microsomes, and liver post-mitochondrial supernatant (also commonly designated as S-9 or S-10) is shown in Table 4.

Intact hepatocytes therefore represent a more relevant experimental system for metabolic stability evaluation than liver microsomes (Lavé et al., 1997; Li, 2001; 2004a; 2007; Jouin et al., 2006). We have developed a simple assay, the Hepatocyte Metabolic Stability Assay, for a relatively high throughput screening for metabolic stability.

The procedures for the Hepatocyte Metabolic Stability Assay are as follows:

- Plating of human hepatocytes (35,000 cells in 50 µl per well) in 96-well plates in Hepatocyte Metabolism Medium (HMM).
- Addition of 50 µl per well of HMM containing 2x concentration of the test article to be evaluated. A concentration that is routinely used for metabolic stability evaluation is 1 µM.
- Incubation at 37°C for multiple time points (for the determination of  $T_{1/2}$ , the time period leading to disappearance of 50% of the parent test article) or, for screening purposes, one single time point (e.g. 30 minutes).
- Addition of 100 µl acetonitrile (ACN) to terminate metabolism.
- Centrifugation to remove hepatocytes and cellular macromolecules from the supernatant containing the remaining test article.
- LC/MS/MS quantification of the parent test article concentration after incubation.

Results are generally expressed as % of the parent test article remaining after incubation.

% Remaining = (Concentration after incubation) / (Concentration before incubation) x 100%

**Tab. 3: Viability (based on trypan blue exclusion), yield (per vial), and plateability of cryopreserved human hepatocytes from 18 isolations**

The cryopreserved human hepatocytes were thawed and recovered using the Cryopreserved Human Hepatocyte Recovery Medium (CHRM) or using DMEM/F12 medium. Substantial gain in viability was observed using CHRM without any significant decrease in yield. Of the 18 isolations, 11 were plateable (can be cultured as attached monolayer cells with over 50% confluency).

Lot Number	Viability (%)		Yield (million viable cells)	
	CHRM	DMEM-F12	CHRM	DMEM-F12
4001	80	61	4.5	4
4004	80	62	6	5
4010	77	64	6.2	5
4018	91	70	5.5	5
4027	92	76	5.9	5
4032	99	92	4.8	5
4054	97	81	8.1	7
4000(plateable)	93	79	7	7
4006(plateable)	92	90	5	5
4013(plateable)	92	65	7	5
4029(plateable)	90	81	6	7
4037(plateable)	98	91	6	5.9
4039(plateable)	94	90	3.7	6
4040(plateable)	95	91	4.2	7
4049(plateable)	98	88	4.4	7
4053(plateable)	91	85	4.8	5
4059(plateable)	82	77	4.2	6
4022(plateable)	91	75	5.5	5
Average	90.7	78.8	5.5	5.7
sd	6.6	10.8	1.2	1.0

**Tab. 4: A comparison of hepatocytes, liver post-mitochondrial supernatant (S9) and liver microsomes in drug metabolizing enzyme activities**

The drug metabolizing enzymes are P450 isoforms for phase 1 oxidative metabolism, UDP-dependent glucuronosyl transferase (UGT) and sulfotransferase (ST) for phase 2 conjugation, monamine oxidases (MAO), and glutathione transferase (GST). The hepatocytes contain all these important xenobiotic metabolizing enzymes as undisrupted pathways, with both the enzymes and the required co-factors at physiological levels. The liver S-9 contains both cytosolic and microsomal enzymes, but lacks mitochondrial enzymes such as MAO. The liver microsomes lack MAO well as cytosolic enzymes such as ST, GST. The hepatocytes therefore represent the most physiologically relevant experimental model for xenobiotic metabolism, while the cell free systems such as liver S9 and microsomes are useful for the evaluation of specific pathways.

	P450 Isoforms	UGT	ST	MAO	GST
Hepatocytes	Yes	Yes	Yes	Yes	Yes
Liver S9	Yes	Yes	Yes	No	Yes
Liver Microsomes	Yes	Yes	No	No	No

*In vivo* hepatic intrinsic clearance can be further calculated from the  $T_{1/2}$  values as an initial estimation of the rate of human *in vivo* hepatic clearance of the NCE in question (Jouin et al., 2006; Li, 2007). The laboratory of Lu et al. (2006) have shown that the correlation between *in vitro* human hepatocytes and human *in vivo* results can be improved by considering not only the rate of metabolism, but also protein binding and intracellular uptake.

Results for metabolic stability are illustrated in Figure 3 for two model chemicals, testosterone and propranolol. The results show that the metabolically less stable testosterone can be readily distinguished from the metabolically more stable propranolol, thereby illustrating the application of this assay for the selection of drug candidates with the most appropriate metabolic stability for further development.

## 2. Metabolite profiling and species-comparison

The identification of metabolites formed from the parent drug (metabolite profiling) is important to drug development, as it allows the design of chemical structure to improve metabolic stability or to decrease cytotoxicity (see below). Metabolite identification is also important for the determination of the key drug metabolizing enzyme pathways (e.g. oxidation or conjugation) as part of the program to understand drug-drug interaction potential. Lastly, metabolite profiling allows the selection of laboratory animal species most relevant to humans for *in vivo* experimentation. An animal species which forms metabolites found in humans would be more relevant than one with metabolites different from those formed in humans. This species-comparison is routinely performed using *in vitro* systems such as hepatocytes (e.g. from human, rat, mouse, guinea pig, dog, monkey).

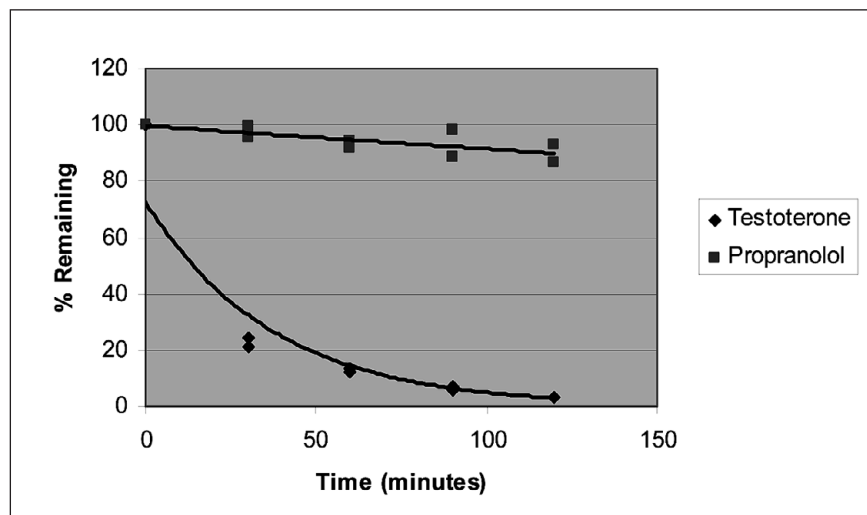
The procedure for the Hepatocyte Metabolite Profiling Assay is as follows:

- Plating of human or animal hepatocytes (250,000 cells in 0.25 ml of HMM per well) in 24-well plates.
- Addition of 0.25 ml of HMM containing 2x of the drug to be evaluated.
- Incubation at 37°C for 2 hours.
- Addition of 1 ml ACN to terminate reaction.

e. Centrifugation to remove cellular macromolecules.

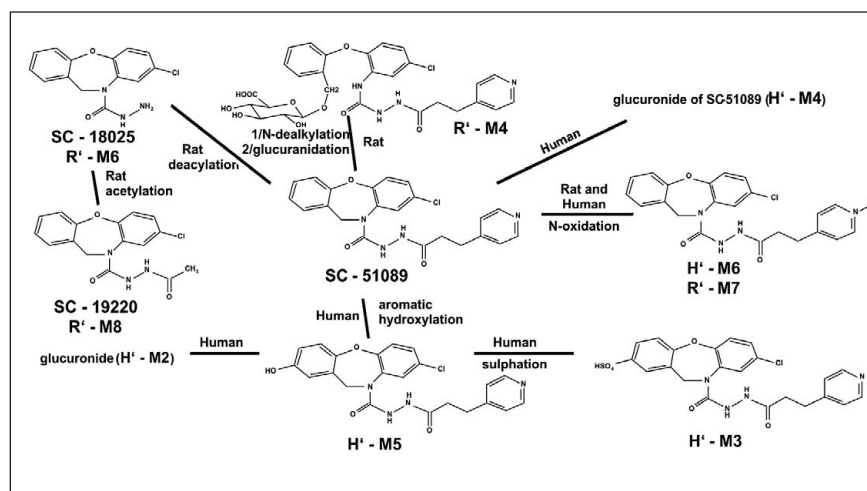
f. LC/MS/MS quantification and identification of metabolites.

The metabolites are in general identified based on mass to charge ( $m/z$ ) ratio, and with the identity ascertained based on differences in  $m/z$  ratio to the parent. For in-



**Fig. 3: Hepatocyte metabolic stability assay**

Typical results for metabolic stability studies with human hepatocytes as illustrated with two model chemicals that are known to be subjected to hepatic metabolic clearance, testosterone and propranolol. The chemicals were incubated with cryopreserved human hepatocytes in Hepatocyte Metabolism Medium at a substrate concentration of 10  $\mu$ M and hepatocyte concentration of 0.5 million cells per ml. Duplicate incubations were performed for the time points of 0, 30, 60, 90 and 120 minutes. The amount of the parent compounds remaining at each time point was quantified by HPLC. Based on the results, the  $T_{1/2}$  values were determined to be 26 minutes for testosterone and >120 minutes for propranolol. The results illustrate how the metabolic stability assay can be used to distinguish between metabolically stable and less stable compounds.



**Fig. 4: Hepatocyte metabolite profiling assay**

The metabolite profiling assay evaluates the site of metabolism and the pathways of metabolism via structural identification of the metabolites. One major application is to aid the determination of the most appropriate animal species for *in vivo* experimentation (e.g. pharmacokinetics and safety studies). The results here with the drug candidate SC-51089 show that almost all the metabolites formed by rat hepatocytes (R'-M) were different from those formed by human hepatocytes (H'-M), thereby suggesting that the rat is not a relevant experimental model for the prediction of human metabolically-based drug properties such as metabolic stability and toxicity. Results adopted from Lee et al. (1994), data from Li et al., 1999b..

stance, a+16 change in m/z would indicate the addition of oxygen, suggesting the formation of a hydroxylated metabolite. The recent advances of mass spectrometry, including machines with accurate mass measurement and software for metabolite identification, have greatly facilitated the metabolite profiling process.

Our laboratory was one of the first to apply the Hepatocyte Metabolite Profiling Assay in drug development. We have shown that minor structural modifications can have profound effects on species-differences in metabolite profiles (Lee et al., 1994). There are now plentiful reports on the use of isolated hepatocytes from multiple animal species and humans for the selection of the most appropriate animal species as an *in vivo* experimental model to predict human metabolism and pharmacokinetic properties (Zhang et al., 2007). This assay is now an FDA requirement for Investigative Drug Application (FDA, 2006).

Results based on our first publication on the Hepatocyte Metabolite Profiling Assay (Lee et al., 1994) are shown in Figure 4.

### 3. Drug-drug interaction evaluation

A major adverse drug property with fatal outcome is drug-drug interaction. A drug may inhibit the metabolic clearance of a co-administered drug, leading to toxicity due to high systemic exposure to the affected drug (inhibitory drug-drug interactions).

Conversely, a drug may enhance the metabolic clearance of a co-administered drug, leading to inefficacy due to lower than optimal systemic exposure (inductive drug-drug interactions). Inhibitory drug-drug interactions are caused by the inhibition of drug metabolizing enzyme activities. Inductive drug-drug interactions are caused by the induction of drug metabolizing enzyme activities. Both types of drug-drug interactions can be evaluated with human hepatocytes.

a. Hepatocyte P450 Inhibition Assay (evaluation of inhibitory drug-drug interaction):

- Add 125 µl of HMM containing 4x concentration of the drug to be evaluated into each well of a 24-well plate.
- Add 125 µl of HMM containing 4x concentration of the drug metabolizing enzyme substrate into the same well.
- Add 250 µl of HMM containing 250,000 human hepatocytes.
- Incubate for 30 minutes at 37°C.
- Add 1 ml of ACN to terminate reaction.
- Centrifuge to remove cellular macromolecules.
- LC/MS or HPLC quantification of metabolites

The P450 isoform-specific substrates used routinely for the inhibitory drug-drug interaction assay and isoform-specific inhibitors which can be used as positive con-

trols for the assay are shown in Table 5. Typical experimental results are shown in Figure 5.

b. Hepatocyte P450 Induction Assay (evaluation of inductive drug-drug interaction potential):

- Add 500 µl of Hepatocyte Plating Medium (HPM) containing 0.35 million plateable cryopreserved human hepatocytes or freshly isolated human hepatocytes into each well of a collagen-coated 24-well plate.
- Culture the hepatocytes for 2 days with daily change of medium to Hepatocyte Incubation Medium (HIM).
- On day three, change medium to HIM containing the desired concentration of the drug to be evaluated for enzyme induction potential.
- On days four, five, and six, change medium daily to HIM containing the drugs to be evaluated to allow a total of 72-hours of treatment.
- On day seven, remove treatment medium and replace with 0.5 ml of HMM containing specific drug metabolizing enzyme substrates and incubate for an additional 30 minutes.
- Add 1 ml of ACN to terminate reaction.
- LC/MS or HPLC quantification of drug metabolizing enzyme metabolism of the substrate.

The U. S. FDA recommends the evaluation of CYP1A2 and CYP3A4 for induction studies, using the substrates specified in Table 5. The argument is that CYP2B6, CYP2C9, and CYP2C19 inducers are found to be CYP3A4 inducers. The U. S. FDA also recommends enzyme induction studies with human hepatocytes from three individual donors from either plateable cryopreserved human hepatocytes or freshly isolated human hepatocytes.

Induction results are usually expressed as % of negative (solvent) control:

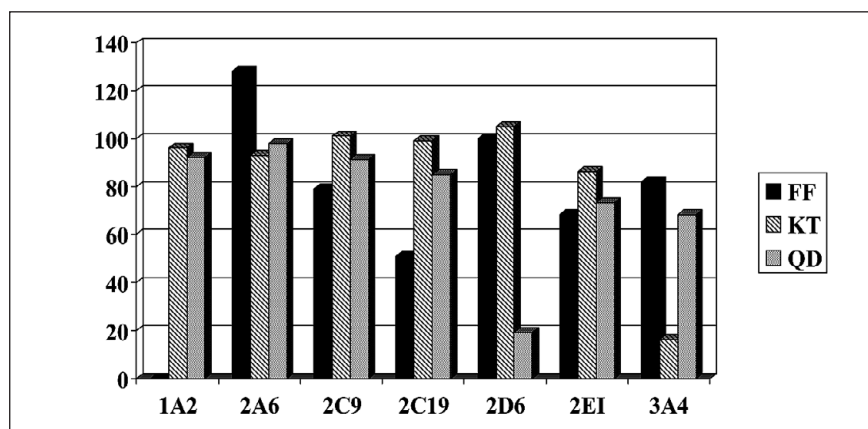
Induction (%) = Activity (treatment)/Activity (solvent control) x 100%

Results with the positive controls, omeprazole (for CYP1A2 induction) and rifampin (for CYP3A4 induction) in the Hepatocyte P450 Induction Assay using cryopreserved human hepatocytes from three donors are shown in Figure 6.

**Tab. 5: Substrates and corresponding metabolites for the evaluation of drug metabolizing enzyme activities for the evaluation of enzyme inhibition and enzyme induction potential**

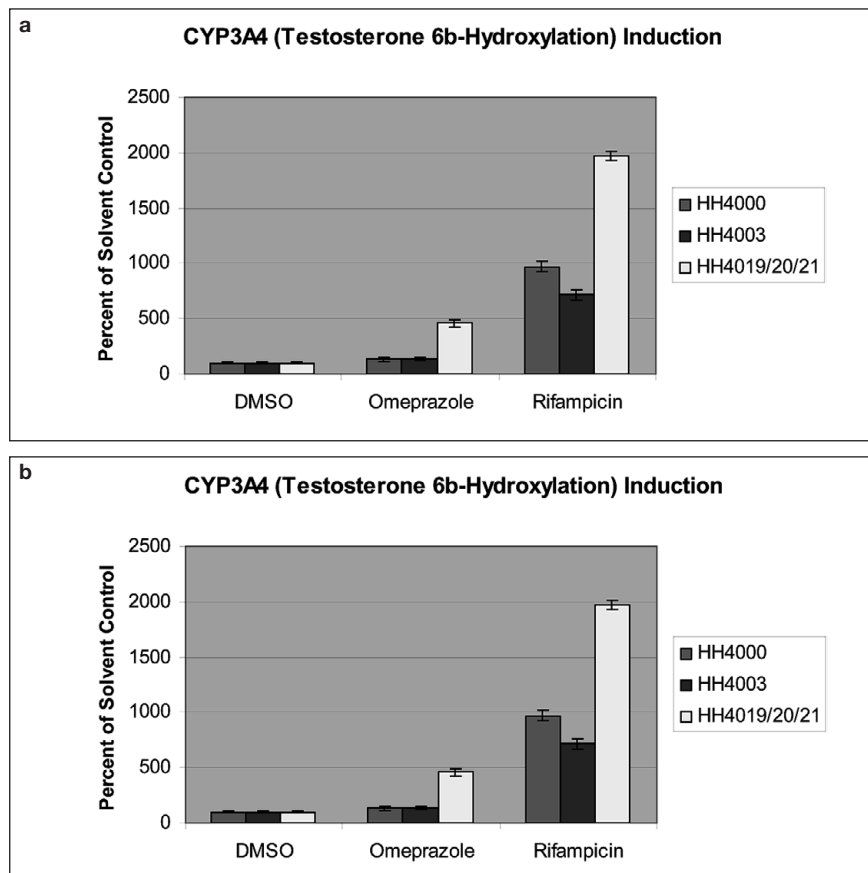
The drug metabolizing enzymes are the various P450 isoforms (CYP), UDP-dependent glucuronyl transferase (UGT), and sulfotransferase (ST).

Drug Metabolizing Enzyme	Substrate Conc.	Substrates	Metabolites
CYP1A2	50 µM	Phenacetin	Acetaminophen
CYP2A6	50 µM	Coumarin	7-OH Coumarin
CYP2B6	50 µM	7-ethoxy-4-trifluoromethyl coumarin	7-OH-4-trifluoromethyl coumarin
CYP2C8	50 µM	Taxol	6-OH Taxol
CYP2C9	75 µM	Tolbutamide	4-OH Tolbutamide
CYP2C19	50 µM	S-mephenytoin	4'-OH mephenytoin
CYP2E1	50 µM	Chloroxazone	6-OH Chloroxazone
CYP3A4	125 µM	Testosterone	6β-OH Testosterone
UGT	12.5 µM	7-OH Coumarin	Coumarin-7-glucuronide
ST	12.5 µM	7-OH Coumarin	Coumarin-7-sulfate



**Fig. 5: Hepatocyte P450 inhibition assay for the evaluation of inhibitory drug-drug interaction potential**

The effects of three known P450 inhibitors, furafylline (FF), ketoconazole (KT) and quinidine (QD) are shown. FF, KT, QD are known inhibitors of CYP1A2, CYP3A4, and CYP2D6, respectively, and are shown to inhibit the activities of these isoforms in the assay.



**Fig. 6: Hepatocyte P450 induction assay for the evaluation of inductive drug-drug interaction potential**

Results of two known P450 inducers which are commonly used as positive controls, namely, omeprazole (CYP1A2 inducer) (a), and rifampin (CYP3A4) (b) are shown. CYP1A2 activity was measured as 7-ethoxyresorufin deethylase activity, and CYP3A4 was measured as testosterone 6 $\beta$ -hydroxylation activity. Results using plateable cryopreserved human hepatocytes from three donors are shown. The U. S. FDA recommends the use of hepatocytes from three individual donors for the evaluation of enzyme induction potential. The use of cryopreserved human hepatocytes allows hepatocytes from three donors to be evaluated in the same experiment as shown here.

#### 4. Hepatotoxicity screening

Hepatotoxicity is a major manifestation of drug toxicity, the reason being that the liver usually would receive the highest, bolus concentration of an ingested drug. Further, the hepatocytes, being the cells responsible for drug metabolism, are the first cells to be affected by reactive or toxic metabolites.

Isolated hepatocytes therefore represent a physiologically relevant experimental model for the evaluation of hepatotoxicity. *In vitro* hepatocyte cytotoxicity measurements have been found to be effective in the delineation of hepatotoxic and less hepatotoxic structures (Li, 2007).

Hepatocyte cytotoxicity assays can be performed using cryopreserved human hepatocytes in suspension or as plated cells. We recommend the use of plated cells to allow a prolonged treatment period (at least 24-hours). The procedures are as follows:

- i. Add 100  $\mu$ l of Hepatocyte Plating Medium containing 35,000 hepatocytes into each well of a collagen-coated 96-well plate.
- ii. Incubate for 24-hours to allow attachment and the formation of a monolayer culture.
- iii. Change medium to Hepatocyte Incubation Medium containing the desired concentration of the drug to be evaluated for hepatotoxic potential.
- iv. Incubate for 24-hours at 37°C.
- v. Assay for cytotoxicity using a desired cytotoxicity endpoint (e.g. for the quantification of cellular ATP content, add 50  $\mu$ l of lysis buffer followed by 50  $\mu$ l of luciferin-luciferase reagent followed by quantification of luminescence using a multiwell plate reader).

The results of the human hepatocyte cytotoxicity assay in the evaluation of several toxic/less toxic structural analogs: Acetaminophen/gentamicin; tamoxifen/estradiol; troglitazone/rosiglitazone are shown in Figure 7. The results show that the less toxic structure can be readily distinguished from the more toxic structure, illustrating the application of this assay in early drug development for the selection of the drug candidates with the most appropriate safety profile for further development.

#### 4 Discussion

An ideal drug candidate is one that is readily absorbed, has an acceptable plasma



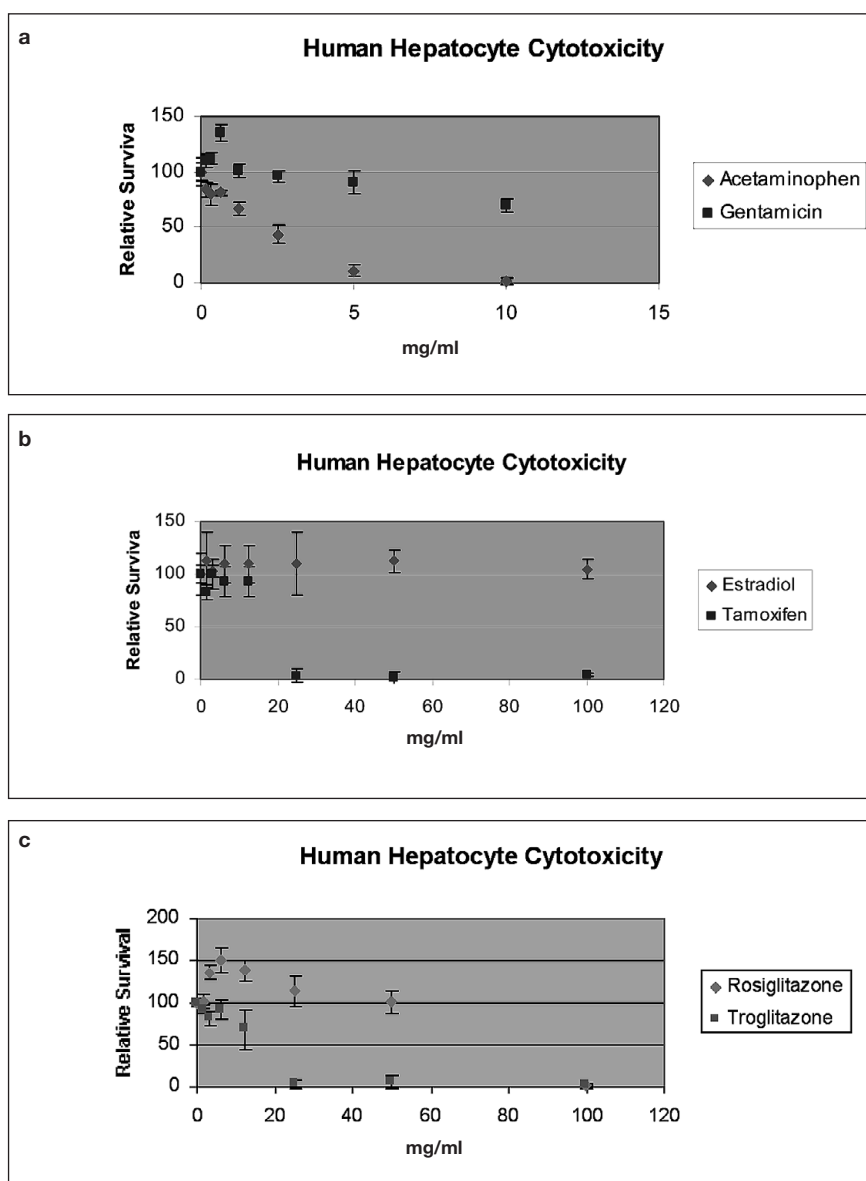
half-life to accommodate a convenient drug administration schedule, high efficacy, minimum toxicity, and minimum drug-drug interaction potential. Successful selection of drug candidates with these desired properties would greatly enhance the efficiency of drug development.

The liver, as the major organ of xenobiotic metabolism, is responsible for several of these critical drug properties, namely, metabolic stability, drug-drug interaction potential, and toxic potential. An orally administered drug, upon intestinal absorption, is delivered firstly via portal circulation to the liver where it is metabolized, with the metabolites either excreted via the bile (which may be reabsorbed via enterohepatic recirculation) or distributed into the systemic circulation. The hepatic metabolites can be more toxic (metabolic activation), or less toxic (metabolic inactivation) than the parent drug. The drug may have metabolic interaction (metabolic drug-drug interactions) via the inhibition or induction of specific drug metabolizing enzyme pathways such as P450 isoform activities.

Because of species-species differences in drug properties, results with laboratory animals are not always predictive of human drug properties. The use of human-based *in vitro* experimental systems during preclinical trials allows the early assessment of human-specific drug properties. The reduction of pharmacokinetics as a contributing factor in clinical trial failures of drug candidates is attributed to the application of the *in vitro* hepatic system in the definition of human drug metabolism.

The human-based *in vitro* liver-based system is successful in achieving the objectives of reduction, refinement, and replacement of the use of animals in drug development research:

1. Reduction: Early screening using *in vitro* systems for drug properties, for instance metabolic stability and hepatotoxicity, greatly reduces the number of NCE that would require *in vivo* studies in laboratory animals.
2. Refinement: The use of human *in vitro* results aid the selection of relevant animal species, i.e. animal species with drug metabolism activities similar to those of humans, for *in vivo* experimentation, leading to the generation of higher quality data for the prediction of human drug properties.



**Fig. 7: Hepatocyte cytotoxicity assay**

Known hepatotoxicants and their respective less hepatotoxic analogs were evaluated in cryopreserved human hepatocytes. Plateable human hepatocytes were used. The hepatocytes were treated for 24 hours followed by viability determination based on cellular ATP content. The toxic/less hepatotoxic pairs were: acetaminophen/gentamicin (a); tamoxifen/estradiol (b); and troglitazone/rosiglitazone (c). The results of the assay illustrate that this assay can be used in early drug development to aid the selection of compounds with the most appropriate safety profile for further development.

3. Replacement: The U. S. FDA requires only human-based *in vitro* systems for the evaluation of drug-drug interaction potential, with no requirement for evaluation in laboratory animals.

The successes of the hepatic *in vitro* systems underscore the importance of a science-based approach to the development

and application of alternative methods. The scientific understanding of the basis of species-species differences in drug metabolizing enzymes has argued strongly for the deficiency of nonhuman laboratory animal models and the relevance of human-based metabolically competent experimental systems. The persistence of the pioneers of the field in the painstaking



characterization of the *in vitro* systems and the generation of supportive data has led to the endorsement of the U. S. FDA, which ultimately led to the universal acceptance of the use of *in vitro* approaches to define human drug metabolism and drug-drug interaction potential. The positive experience with *in vitro* hepatic systems has paved the way for an important goal of alternative experimental systems, namely, the reduction, refinement, and replacement of the use of nonhuman laboratory animals in the assessment of human drug toxicity.

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