

# We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

186,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index  
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?  
Contact [book.department@intechopen.com](mailto:book.department@intechopen.com)

Numbers displayed above are based on latest data collected.  
For more information visit [www.intechopen.com](http://www.intechopen.com)



# Critical Human Hepatocyte-Based *In Vitro* Assays for the Evaluation of Adverse Drug Effects

Albert P. Li

*In Vitro* ADMET Laboratories LLC and  
Advanced Pharmaceutical Sciences Inc., Columbia, MD  
USA

## 1. Introduction

A major challenge in drug development is to accurately estimate human adverse drug effects to allow the selection and advancement of drug candidates with the best safety profile for further development. Due to species differences, safety data obtained with the routine *in vivo* studies with nonhuman laboratory animals do not always correctly predict human outcome. Human liver-derived systems, especially human hepatocytes, represent physiologically-relevant experimental systems for the evaluation of human adverse drug effects. The assays developed with human-based *in vitro* experimental systems for the assessment of two major adverse drug effects: drug-drug interactions and drug toxicity can be used routinely during drug development to select and optimize drug candidates to enhance the probability of clinical success.

## 2. Current challenges in drug development

Efficacy and safety are two co-dependent requirements for successful drug development – clinical failure will result if the drug candidate possesses only one of these two properties. For the past 50 years, drug candidates are evaluated for pharmacological and safety properties using *in vivo* animal models. It is now known that this paradigm, namely, prediction of human drug properties with animals *in vivo*, is no longer valid. DiMassi et al (2003)<sup>1</sup> has estimated that for R&D initiated in 2001 with approval 12 years later (based on the average time required for approval), the out-of-pocket cost for a single approved drug is estimated to be U. S. \$ 970 million, equivalent to a capitalized cost of U. S. \$ 1.9 billion. Frequent clinical trial failure, with lack of efficacy and the occurrence of unexpected adverse drug effects as major reasons, accounts for astronomical time and costs involved in the development of a successful drug. The most recent published estimation of the clinical approval success rate for investigational drugs is 16%<sup>2</sup>. Furthermore, marketed drugs are frequently withdrawn or have their use limited due to adverse effects, with dire consequences to the welfare of the patients and the financial status of the drug manufacturers<sup>3</sup>.

### 3. Overcoming species-species differences

True advancement in the efficiency of drug development can only be made if one accepts that, due to species differences, data from nonhuman laboratory animals do not always predict human drug properties. As *in vivo* experimentation with humans *in vivo* during preclinical phases is neither practical nor ethical, surrogates for humans *in vivo* need to be applied. Experimental models with human tissues and human cells represent practical and relevant surrogates.

A major breakthrough in the acceptance of the reliability of *in vitro* human-based system in the prediction of human drug properties is the advancement of human-based drug metabolism systems. Human liver fractions (e.g. human liver microsomes), human hepatocytes, and cDNA-derived human drug metabolizing enzymes have been found to provide useful information for the prediction of human metabolism *in vivo*. These systems are now used routinely for the evaluation of drug metabolism and drug-drug interaction potential of drug candidate in various phases of drug development <sup>4, 5</sup>, with the approaches fully endorsed by U. S. FDA <sup>6</sup>. It is interesting to note that the application of *in vitro* drug metabolism technologies using human-based experimental systems has been attributed to the removal of pharmacokinetics as a major reason for clinical trial failure.

The success in the application of *in vitro* drug metabolism systems, in combination with data from relevant *in vivo* animal models, in the prediction of human metabolism suggest that the same approach will also be successful for safety evaluation <sup>7, 8</sup>. Based on the premise that the inability to accurately predict human drug toxicity is due to species-species differences, i. e., there are human-specific drug properties that cannot be revealed by nonhuman animal studies, a safety evaluation strategy is proposed here for the preclinical evaluation of human drug toxicity:

1. Application of **human**-based *in vitro* systems to provide human-specific toxicity data;
2. Select a relevant animal species to develop **in vivo** parameters;
3. Predict **human in vivo** drug toxicity via a combination of human-specific information obtained *in vitro*, and *in vivo* parameters obtained from nonhuman animals *in vivo*.

Success of this In Vitro-In Vivo Strategy (IVIVS) requires the development of *in vitro* experimental systems with human-specific properties to cover the key adverse drug effects in humans, and a vigorous set of parameters defining the relevant nonhuman animal species.

### 4. Human hepatocytes as a key *in vitro* experimental system for the evaluation of human-specific drug properties

The liver is a key determinant of drug properties. It is a major organ for drug metabolism, and is often a target for drug toxicity <sup>9,10</sup>. Hepatocytes or liver parenchymal cells are the cells in the liver responsible for drug metabolism and are the target cells for hepatotoxic drugs. Isolated hepatocytes therefore represent the most physiologically-relevant experimental system for the evaluation of hepatic drug metabolism and hepatotoxicity <sup>11-13</sup> for the following reasons:

1. Human xenobiotic metabolism: Fresh isolates or cryopreserved fresh isolates of human hepatocytes are known to contain most, if not all, of the *in vivo* hepatic xenobiotic metabolism capacity <sup>12</sup>.

2. Human target cells: The hepatocytes are the cells in the human liver that are damaged by hepatotoxicants, leading ultimately to liver failure <sup>14,15</sup>.
3. Endpoints: Myriad of toxicological endpoints allowing measurements of necrosis, apoptosis, nuclear receptor interactions, P450 functions, transporter functions etc. have been developed in hepatocytes for the evaluation of adverse drug properties<sup>15,16</sup>.

In the past, the use of human hepatocytes has been severely limited by their availability. This limitation has been overcome in the past decade due to advances in the procurement of human livers for research, and the commercial availability of isolated human hepatocytes. The application of human hepatocytes in drug metabolism studies is greatly aided by the successful cryopreservation of human hepatocytes to retain drug metabolism activities <sup>12, 13, 17</sup>. Recently, the usefulness of cryopreserved human hepatocytes is further extended through the development of technologies to cryopreserve human hepatocytes to retain their ability to be cultured as attached cultures (plateable cryopreserved hepatocytes) which can be used for longer term studies such as enzyme induction studies <sup>12</sup>.

Cryopreserved human hepatocytes have several advantages over the use of freshly isolated cells:

1. Experimentation can be readily scheduled;
2. There are little or no deleterious effects of cryopreservation on key hepatocyte properties;
3. Repeat of experimentation can be performed at different times or by different laboratories with cells from the same donor;
4. The hepatocytes can be pre-characterized for properties relevant to a specific study before they are used for experimentation;
5. Hepatocytes from multiple donor can be used in the same study.

## 5. Critical assays for the evaluation of adverse drug effects

Two adverse drug effects are responsible for clinical failures and drug withdrawal: drug-drug interactions and drug toxicity. Below are the critical assays for these adverse drug effects. In this chapter, the overall scientific concepts behind these assays and the general approaches used in the assays are described.

## 6. Critical assays for drug-drug interactions

Metabolic drug-drug interaction results from the alteration of the metabolic clearance of one drug by a co-administered drug. There are two major pathways of metabolic drug-drug interactions:

*Inhibitory drug-drug interaction:* When one drug inhibits the drug metabolism enzyme responsible for the metabolism of a co-administered drug, the result is a decreased metabolic clearance of the affected drug, resulting in a higher than desired systemic burden. For drugs with a narrow therapeutic index, this may lead to serious toxicological concerns. Most fatal drug-drug interactions are due to inhibitory drug-drug interactions.

*Inductive drug-drug interactions:* Drug-drug interactions can also be a result of the acceleration of the metabolism of a drug by a co-administered drug. Acceleration of metabolism is usually due to the induction of the gene expression, leading to higher rates of protein synthesis and therefore higher cellular content of the induced drug-metabolizing enzyme and a higher rate of metabolism of the substrates of the induced enzyme. Inductive

drug-drug interactions can lead to a higher metabolic clearance of the affected drug, leading to a decrease in plasma concentration and loss of efficacy. Inductive drug-drug interactions can also lead to a higher systemic burden of metabolites, which, if toxic, may lead to safety concerns.

Due to the realization that it is physically impossible to evaluate empirically the possible interaction between one drug and all marketed drugs, and that most drug-metabolizing enzyme pathways are well-defined, a mechanism-based approach is used for the evaluation of drug-drug interaction potential of a new drug or drug candidate<sup>18-20</sup>. This mechanistic-based approach is endorsed and required by the U. S. FDA ([www.fda.gov/cber/gdlns/interactstud.htm](http://www.fda.gov/cber/gdlns/interactstud.htm)) for new drug applications. The approach consists of the following major studies:

1. Metabolic phenotyping: The major enzymes involved in the biotransformation of the drug candidate are identified. The major emphasis in the past has been on phase 1 oxidation pathways and on P450 isoforms. Elucidation of enzyme pathways involved in the biotransformation of a drug candidate will allow the identification of potential drug-drug interactions with drugs that are known modifiers (inhibitors and inducers) of the pathways.
  - a. Metabolite identification: Structural identification of the metabolites allow one to deduce the major pathways of metabolism. Identification of
    - i. Experimental systems: Human liver homogenate 9000 x g supernatant (S9); human liver microsomes (HLM); hepatocytes
    - ii. General incubation conditions:
      1. S9 or HLM: 0.25 to 1.0 mg protein/mL in 0.1 M phosphate buffer at pH 7.4 containing NADPH or NADPH regenerating system (phase 1 oxidation); uridine 5'-diphospho-glucuronic acid (UDPGA; cofactor for glucuronidation) and 3'-phosphoadenosine 5'- phosphosulfate (PAPS; cofactor for sulfation).
      2. Hepatocytes: 0.5 to 1.0 million cells/mL in Isotonic buffer (e.g. Krebs-Hensleit Buffer) maintained at pH 7.2.
      3. Temperature: 37 deg. C
      4. Compound concentration: Generally 10 uM
      5. Time: Multiple time points up to 30 minutes (HLM); 2 hrs (hepatocytes in suspension); 24 hrs. (hepatocytes in monolayer culture)
    - iii. Metabolite identification: HPLC-MS/MS is the most commonly used approach for the initial identification of the metabolites. NMR is used for definitive structural identification.
  - b. Major pathway identification: Chemical inhibitors are used to identify of the major oxidative pathways involved in the formation of the metabolites. Inhibition of metabolism of the parent compound, as indicated by metabolic stability or decreased formation of metabolites, would suggest that the participation of the pathway in the metabolism of the compound. Examples of inhibitors for the major pathways are as follows:
    - i. P450 inhibition: 1-aminobenzotriazole (S9; HLM; hepatocytes)
    - ii. MAO inhibitors: pargyline (S9)
    - iii. FMO inhibitors: 45 deg. C inactivation (S9; HLM).
  - c. P450 isoform identification:



- i. Experimental system: HLM or cDNA-P450 isoforms
- ii. Incubation with HLM in the presence of isoform-selective inhibitors or individual cDNA-P450 isoforms to determine pathway responsible for metabolism. Inhibition of metabolism by an inhibitor of a specific isoform (Table 1) with corroborative data using the identified cDNA-P450 isoform would allow the identification of the isoform for the metabolism of the compound in question.
- d. Evaluation of inhibitory potential for drug-metabolizing enzymes: The drug candidate will be evaluated for its ability to inhibit known drug metabolizing enzymes, with emphasis on the P450 isoforms. The incubation conditions are similar to that described above for metabolite identification, using substrates that are selective for the pathways in question (Table 1).
- e. Evaluation of induction potential for drug metabolizing enzymes: The drug candidate will be evaluated for its ability to induce known drug metabolizing enzymes. The inducible P450 isoforms: CYP1A, 2B and 3A are the ones required by U. S. FDA. Human hepatocytes are considered the “gold standard” for induction studies, with cryopreserved hepatocytes that can be cultured after thawing and have been characterized to be responsive to prototypical inducers as the preferred system. As of this writing, virtually all known inducers of P450 isoforms *in vivo* are inducers in human hepatocytes *in vitro* (Table 1)<sup>12</sup>. Experimental evaluation of enzyme induction involves the treatment of human hepatocytes for several days with the test article followed by evaluation of enzyme activities using P450 isoform-specific substrates<sup>20</sup>.

The general experimental conditions are as follows:

- i. Experimental system: Primary cultured human hepatocytes
- ii. Culturing condition: Matrigel-collagen sandwich (requirement: >80% confluent cultures).
- iii. Treatment regiment: Culturing of hepatocytes for 2 days followed by 3 days of treatment
- iv. Endpoints: Quantification of CYP1A2, 2B6 and 3A4 gene expression by RT-PCR as well as activities using isoform-specific substrates (Table 1).

## 7. Higher throughput human hepatocyte-based drug-drug interaction studies

Of the multiple P450 isoforms, CYP3A4 is the most abundant of the isoforms in the human liver. CYP3A4 has been found to be responsible for the metabolism of a large variety of exogenous and endogenous substrates<sup>21, 22</sup>. In drug development, there is a need to evaluate the inhibitory and inductive potential of drug candidates towards CYP3A4 to estimate their drug-drug interaction potential with the myriad drugs that are substrates of this important P450 isoform<sup>23-25</sup>. In our laboratory, we have developed cost- and time-effective higher throughput screening assays for the evaluation of drug-drug interaction potential of drug candidates involving CYP3A4. The assays are as follows:

1. 384-well CYP3A4 inhibition assay<sup>26</sup>;
2. 96-well time-dependent CYP3A4 inhibition assay<sup>27</sup>;
3. 96-well CYP3A4 induction assay<sup>26</sup>.

The throughput of the assays are increased via the use of the following technologies:

1. Cryopreserved human hepatocytes cultured in micro-well cell culture plates: The properties and advantages of cryopreserved human hepatocytes have been discussed earlier. The use of micr-owell (96 and 384 well plates) allows the ease of sample organization, decreased cost of cells and reagents, and allows the use of automation.
2. Luciferin-IPA as CYP3A4 substrate: Luciferin-IPA is metabolized to luciferin specifically by CYP3A4. The use of this substrate allows CYP3A4 activity to be quantified using a plate-reader, thereby eliminating the need for the time-consuming and costly LC/MS assays that are used with conventional substrates.

P450 Isoforms	Substrates	Inhibitors	Inducers
CYP1A2	7-ethoxyresorufin dealkylation; Phenacetin-O-deethylation	Furafylline; a-naphthoflavone	3-methylcholanthrene; omeprazole
CYP2A6	Courmarin 7-hydroxylation	Tranlycypromine; methoxsalen	Dexamethasone
CYP2B6	Bupropion hydroxylation	Ticlopidine; clopidogrel	Phenobarbital; phenytoin
CYP2C8	Taxol 6-hydroxylation	Quercetin	Rifampin
CYP2C9	Tolbutamide methyl-hydroxylation	Sulphenazole	Rifampin
CYP2C19	S-mephenytoin 4'-hydroxylation	Omeprazole	Rifampin
CYP2D6	Dextromethorphan O-demethylation	Quinidine	none
CYP2E1	Chloroxazone 6-hydroxylation	Diethyldithiocarbamide	none
CYP3A4/5	Midazolam 1-hydroxylation; testosterone 6b-hydroxylation; luciferin-IPA dealkylation	Ketoconazole; itraconazole; troleandomycin; verapamil	Rifampin; phenobarbital; phenytoin; troglitazone

Table 1. Model P450 isoform-selective substrates, inhibitors, and inducers. These compounds can be used for pathway identification (inhibitors); evaluation of isoform-selective inhibition (substrates); and as positive controls for the evaluation of P450 induction (inducers).

8. 384 well CYP3A inhibition assay with intact human hepatocytes

Evaluation of P450 inhibition is traditionally performed with liver microsomes and recombinant CYP enzymes <sup>28, 29</sup>. Intact hepatocytes represent an additional experimental system that may provide useful information to improve the accuracy of the prediction of in vivo effects. A chemical, for instance, may be metabolized by non-CYP pathways to a metabolite that is a potent P450 inhibitor and therefore would be inhibitory in hepatocytes but not in microsomes or recombinant CYP enzymes. Gemfibrozil, for instance, requires

glucuronidation for its CYP2C8 inhibitory effects and is found to be a potent CYP2C8 inhibitor in hepatocytes but not in liver microsomes nor recombinant CYP2C8<sup>30</sup>. Hepatocytes can also be used for the modeling of differential inhibitor distribution between plasma and intracellular compartments. Lu et al. reported the use of hepatocytes suspended in 100% human plasma to accurately predict CYP3A4 inhibitory effects of several CYP3A inhibitors *in vivo*<sup>31</sup>. The presence of active transporters in human hepatocytes, including cryopreserved hepatocytes, also suggests that an inhibitor may be actively accumulated inside the cells, leading to substantially higher concentration and a correspondingly higher inhibitory effect which would not be observed using cell free systems<sup>32,33</sup>.

We have previously introduced the use of human hepatocytes in P450 inhibition studies<sup>20,34,35</sup>. In the HTS human hepatocyte CYP3A4 inhibition assay described here, 384-well plates were used to reduce the quantity of hepatocytes, reagents, as well as the chemical to be evaluated<sup>26</sup>. The use of LIPA as CYP3A4 substrate substantially enhances the efficiency of the assay, as its metabolism can be quantified based of luminescence using a plate reader<sup>35</sup>, thereby eliminating the need for HPLC and mass spectrometry that are routinely required with conventional substrates such as testosterone and midazolam. The use of robotics allowed rapid and accurate delivery of relatively small volumes of reagents into the 384 well plates. The accuracy of the assay is demonstrated by the relatively low coefficient of variation (standard deviations <10% of mean values) of the results.

A homogenous (addition assay) has been developed in our laboratory using cryopreserved human hepatocytes cultured in 384 well plates. An automated workstation is used for the performance of the assay. The workstation is programmed to perform serial dilutions of the model inhibitors and for the initiation of the assay. White opaque 384-well plates are used. The workstation is programmed to add into each of the wells of the 384-well plates 10 uL of hepatocytes (containing 10,000 cells) and 10 uL of Hepatocyte Metabolism Medium containing either solvent (0.1% v/v of acetonitrile) or P450 inhibitors at the designated concentrations (at 3X of the designated concentrations). The assay is initiated by the addition of 10 uL of 3 uM LIPA (final concentration 1 uM). The plates are returned to a cell culture incubator maintained at 37 deg. C, in a highly humidified atmosphere of 95% air and 5% carbon dioxide. After an incubation period of 120 minutes, the plates are returned to the workstation for the addition of 10 uL of Luciferin Detection Reagent. Luminescence is quantified using a multichannel plate reader.

Representative results of the application of this HTS assay to evaluate CYP3A4 inhibitory potential of drug substances, using model CYP3A4 inhibitors, are shown in Fig. 1.

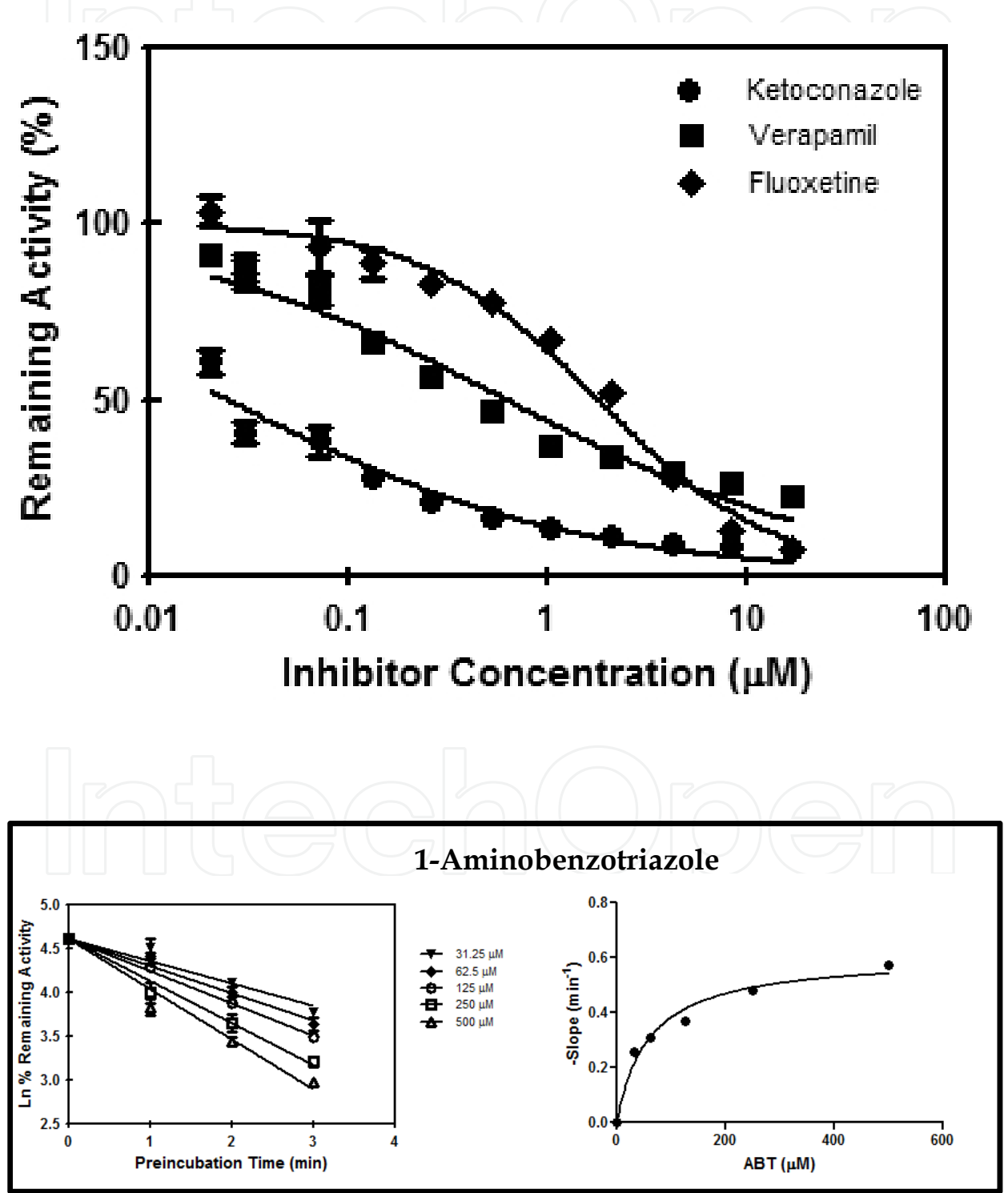
## 9. 96-well time-dependent inhibition assay for CYP3A4 in human hepatocytes

In terms of P450 inhibition, time-dependent inhibition (TDI) or mechanism-based P450 inhibition is of particular concern. In TDI, the inactivated P450 needs to be replaced by newly synthesized proteins to return to its normal activity. After cessation of administration with the TDI inhibitor, the patient would continue to have decreased drug metabolizing capacity before the inactivated enzymes are fully replaced<sup>23,36</sup>.

While TDI is generally studied using liver microsomes or recombinant CYP<sup>37,38</sup>, there are substantial efforts in the evaluation of this important mechanism of drug-drug interaction in human hepatocytes<sup>39,40</sup>. Human hepatocytes, because of the intact plasma membrane, complete and uninterrupted drug metabolism enzymes and cofactors, represent a desirable *in vitro* experimental system for the evaluation of human drug properties.



Traditionally, TDI studies with hepatocytes utilize suspension cultures <sup>40</sup>. The use of hepatocytes in suspension culture is a common practice with cryopreserved cells as most preparations of cryopreserved hepatocytes would have compromised ability to be cultured as monolayer cultures. Due to our success in cryopreservation of human hepatocytes to retain their ability to be cultured, a convenient and quantitative approach for the evaluation of TDI using monolayer cultures of plateable cryopreserved human hepatocytes has been developed in our laboratory<sup>41</sup>.



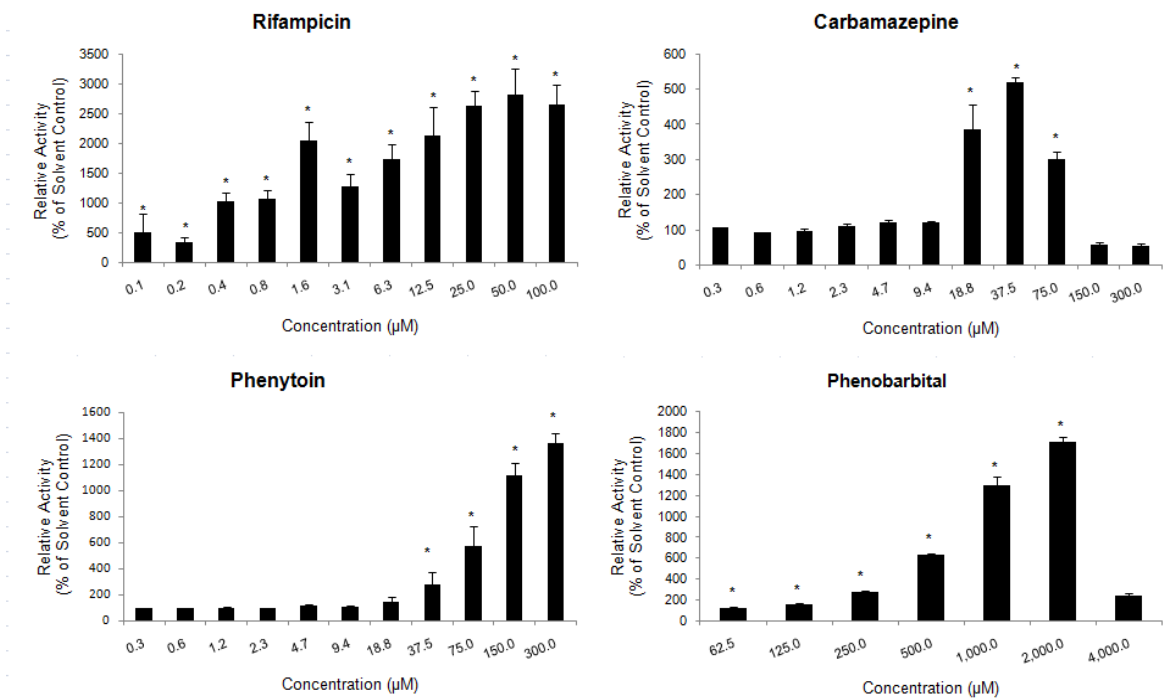


Fig. 1. Examples of the application of the higher throughput hepatocyte assays in the evaluation of CYP3A4 inhibition (top panel), time-dependent CYP3A4 inhibition (middle panel), and CYP3A4 induction. For the CYP3A4 inhibition assay, dose-dependent inhibition was observed for the three model inhibitors, ketoconazole, verapamil, and fluoxetine (top panel). The model time-dependent inhibitor, 1-aminobenzotriazole, yielded time-dependent and dose-dependent inhibition (left figure, middle panel). A plot of the slope of the time-dependent decrease in activity versus inhibitor concentration yielded the classical saturation curve (right figure, middle panel) which can be used to calculate the time-dependent inhibition enzyme kinetic constants  $k_{inact}$  and  $K_I$ . The model CYP3A4 inducers rifampin, carbamazepine, phenytoin and phenobarbital yielded dose dependent induction of CYP3A4 activity (bottom panel). From Li<sup>35</sup>; Doshi and Li<sup>26</sup>; and Li and Doshi<sup>27</sup>.

In this assay, the cryopreserved human hepatocytes are thawed from cryopreservation using Cryopreserved Hepatocytes Recovery Medium and plated at 50,000 cells per well in 96-well collagen coated plates in Cryopreserved Hepatocytes Plating Medium at a volume of 100  $\mu$ L per well. The cells are cultured for 4 hours in a cell culture incubator maintained at 37 deg. C with a highly humidified atmosphere of 5% carbon dioxide and 95% air. The cells on the day of plating (4 hour cultures) are used for the evaluation of TDI. The plating medium is removed and the cells are washed 3 times with Hepatocyte Metabolism Medium (HMM), followed by the addition of 50  $\mu$ L of HMM per well. At designated times 50  $\mu$ L of treatment media consisting of HMM containing 2X concentrated solutions of the inhibitors or medium control is added. At designated periods after treatment media are removed by quickly inverting the 96-well plates on absorbent paper. The cells are washed 5 times with 100  $\mu$ L of HMM to remove the inhibitors. The cells are incubated at 37 deg. C with 100  $\mu$ L per well of HMM for a 60 min “washout” period to allow removal of intracellular inhibitors by diffusion to minimize competitive inhibition with CYP3A4 substrate. After the washout period, medium is replaced with that containing 3  $\mu$ M of the CYP3A4-specific substrate LIPA. After an incubation period of 30 min, 50  $\mu$ L of the incubated substrate solution from each well is removed and placed into

a white 96-well plate. After all the solutions are collected from the various time points, 50  $\mu$ L of Luciferin Detection Reagent is added to each well containing incubated substrate solution followed by quantification of luminescence using a Wallac Victor-3 plate reader. Luminescence signals are converted to pmoles of luciferin based on a standard curve generated from luciferin. Viability of the hepatocytes after treatment is determined after CYP3A4 activity quantification using cellular ATP as an endpoint using a commercially available ATP kit consisting of lysis buffer and ATP detection reagent.

Results are expressed as % remaining activity, which is calculated as a ratio of the activity in the presence of inhibitors to that of the solvent control using the following equation:

$$\% \text{ Remaining Activity (\%)} = [\text{Normalized Activity (Treatment)} / \text{Normalized Activity (Solvent Control)}] \times 100;$$

whereas activity represents luciferin generated in each well quantified by luminescence normalized by relative activity based on ATP content using the following equations:

$$\text{Normalized Activity} = \text{CYP3A4 Activity} / \text{Relative Viability}$$

$$\text{Relative Viability (\%)} = \text{ATP Content (Treatment)} / \text{ATP Content (Solvent Control)}$$

Enzyme kinetic parameters for TDI are derived as follows: The observed rate of enzyme inactivation ( $k_{obs}$ ) is determined as the initial slope of the linear regression line of a semi-logarithmic plot of the natural logarithm of remaining activity versus preincubation time.  $k_{inact}$  and  $K_I$  values are determined based on the double reciprocal Lineweaver-Burk plot ( $1/k_{obs}$  versus  $1/[I]$ , whereas  $[I]$  represents inhibitor concentration), where  $k_{inact}$  is estimated as the reciprocal of the Y-intercept and  $K_I$  as the negative reciprocal of the x-intercept.

Representative results of the application of this HTS assay to evaluate time-dependent CYP3A4 inhibitory potential of drug substances, using the model time-dependent CYP3A4 inhibitor, 1-aminobenzotriazole, are shown in Fig. 1.

## 10. 96-well CYP3A4 induction assay with human hepatocytes

Enzyme induction is a major mechanism for drug-drug interactions. Induction of a drug metabolizing enzyme by one drug would lead to the enhanced metabolism of co-administered drugs that are substrates of the induced enzyme.

As freshly isolated hepatocytes possess endogenous activities which may be the result of inducers present in the donor's systemic circulation, the isolated hepatocytes are cultured for 2 to 3 days to allow the P450 enzyme activities to return to a basal level. Testing for induction potential is that initiated by treatment of the cultured hepatocytes for 2 to 3 days to allow full expression of the induced enzyme. Induction is generally evaluated by measuring enzyme activity as activity represents the most relevant endpoint for drug-drug interaction. Both freshly isolated and plateable cryopreserved human hepatocytes can be used for the induction study.

In our laboratory, we have developed a higher-throughput P450 induction assay using 96 well plates<sup>26</sup>. The procedures are as follows:

1. Day 0: Plate human hepatocytes (freshly isolated or plateable cryopreserved human hepatocytes) with 50  $\mu$ L of cell suspension per well, at a cell density of 1 million cells/mL thereby delivering 50,000 cells per well.

2. Day 1: Change medium to cold (4 to 10 deg. C) medium containing 0.25 mg/mL of Matrigel®.
3. Day 2: Change medium to treatment medium containing test articles at the desired concentrations.
4. Day 3, 4, 5: Continue treatment. Medium change is not necessary unless the test article is known to be unstable under the culturing conditions.
5. Day 6: Measure activity (in situ incubation with LIPA) or extraction of RNA for the evaluation of gene expression.

Representative results of the application of this HTS assay to evaluate CYP3A4 induction potential of drug substances, using model CYP3A4 inducers, are shown in Fig. 1.

## 11. In vitro evaluation of drug toxicity

The current success in the application of human-based in vitro experimental models in the evaluation of drug metabolism and drug-drug interactions paths the way for a similar approach to evaluate drug toxicity, especially human-specific toxic events that cannot be observed in laboratory animals. In vitro toxicity assays are can be applied in various during phases of drug development:

1. **Early screening of intrinsic toxicity:** Cell-based systems are used for rapid screening of drug candidates, especially structural analogs, to allow the selection of less toxic structures for further development. The screening assay can allow logical evaluation of structures responsible for toxicity (toxicophore) which, hopefully, can be separated from structures for pharmacological activity (pharmacophore). Toxicity screening with in vitro systems require only limited amount of test articles, and is rapid and quantitative. Toxicity is most effective when one has an indication for in vivo toxicity (e.g. hepatotoxicity or nephrotoxicity) for a lead molecule, therefore allowing the selection of the most appropriate in vitro system for screening (e.g. hepatocytes for hepatotoxicity and renal proximal tubule cells for nephrotoxicity).
2. **Mechanistic evaluations:** Mechanistic understanding is critical to drug development. It allows a better understanding of human health risks, defines potential risk factors, and evaluates the relationship between efficacy and adverse effects. Mechanistic studies may be performed after adverse effects are observed in nonhuman animals to aid the prediction of human toxicity as well as the development of approaches for a more acceptable replacement. The defined experimental conditions and the availability of reagents and approaches for multiple endpoints of in vitro experimental systems allow one to define the key pathways involved in a toxicology phenomenon.

The preferred human in vitro systems for the evaluation of drug toxicity are primary cells derived from human organs, used within a period that the cells would retain differentiated functions, thereby serving as surrogates of the similar cells in vivo.

Primary cell culture systems, including stem-cell derived differentiated cells representative of the key cell types in each organ, are currently available and the respective organ-specific toxicity:

- 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)

## 12. Overcoming the major deficiencies of in vitro system

An argument routinely raised against the application of in vitro systems in safety evaluation is that toxicity is a complex phenomenon and therefore cannot be adequately modeled by simple in vitro systems such as cell culture assays.

The major deficiencies of in vitro experimental systems can be defined as follows:

1. **Lack of systemic effects.** In vitro experimental systems in general consist of single cell types. Toxic effects are evaluated in the absence of influences from systemic effects that may be critical to drug toxicity. An example is the participation of the immune system in organ toxicity. One hypothesis for idiosyncratic hepatotoxicity, for instance, is the hapten-hypothesis which postulates that liver failure arises from the cytotoxicity of antibodies towards antigens developed between the idiosyncratic drug (or its metabolites) on the plasma membrane of the hepatocytes.
2. **Absence of chronic dosing.** It is generally believed that drug toxicity due to acute cytotoxic events can be studied effectively with in vitro systems. However, toxic effects due to chronic, low-dose treatments may require multiple events that may or may not be obtained with in vitro studies, with cells treated for a relatively short time period (e.g. 24-hours). Long-term treatments (e.g. months to years) of cells in culture is theoretically possible but in practice near impossible. Further, it is extremely difficult to maintain primary cells, the preferred cell system, in a differentiated state for a long time period.

For in vitro systems to be useful, one needs to develop experimental approaches to overcome these deficiencies.

## 13. In vitro experimental model for multiple organ interactions: Integrated discrete multiple organ co-culture ( IdMOC)

One major drawback of in vitro system is that each cell type is studied in isolation. In the human body, multiple organ interactions may be critical to drug toxicity. An example of multiple organ interactions is a drug which is firstly metabolized by one organ (e.g. liver) to form metabolites which may enter the general systemic circulation to cause toxicity in a distant organ (e.g. heart).

The multiple organ interaction is not covered by the TACIT approach<sup>8</sup> using a single cell type, as the initiating events may include effects of a toxicant on a nontarget cell. To overcome this deficiency, we have developed the IdMOC (Independent Discrete Multiple Organ Co-culture) system (<sup>42-44</sup>). The IdMOC allows the co-culturing of cells from different organs as physically separated cultures that are interconnected by an overlying medium, akin to the blood circulation connecting the multiple organs in the human body (Fig. 1). The IdMOC models the multiple organ interaction in the whole organism in vivo, allowing the evaluation of organ-specific effects a drug and its metabolites. The IdMOC represents an improved in vitro experimental system for routine screening of ADMET drug properties.

The IdMOC involves the “wells-in-a-well” concept. The typical IdMOC plate consists of a chamber within which are several wells (Fig. 2). 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. 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 IdMOC system can be used for 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. Aflatoxin B1, a known hepatotoxicant in humans *in vivo*, is shown to have selectively higher cytotoxicity in hepatocytes in the IdMOC co-culture of hepatocytes, renal proximal tubule cells, and small airway epithelial cells.
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. Multiple organ metabolism: Evaluate the ultimate metabolic fate of a substance upon metabolism by cells representing multiple organs with metabolic functions (e.g. liver, kidney, lung). This application allows the development of metabolite profiling of drugs which are subjected to both hepatic and extrahepatic metabolism.

Evaluation of organ-specific toxicity is illustrated by the treatment of IdMOC with a known hepatotoxicant, aflatoxin B1, in IdMOC with three human primary cell types: hepatocytes, renal proximal tubule epithelial cells, and pulmonary (small airway) epithelial cells. Aflatoxin B1 was found to be significantly more cytotoxic towards human hepatocytes, presumably due to the higher P450 activities of the cells versus the other two cell types (Fig. 3), as it is known that aflatoxin requires P450 metabolism to toxic metabolites to exert its toxicity.

## 14. Conclusion

Accurate prediction of human adverse drug effects represents a major challenge for drug development. The high rate of clinical failure of drug candidates that have been carefully selected from preclinical studies illustrates clearly that the routine, "classical" approach of preclinical safety evaluation is inadequate. It is argued here that species-species differences in drug toxicity is a major reason - human-specific toxicity, by definition, cannot be predicted with nonhuman laboratory animals. It is proposed here that human *in vivo* drug toxicity can be predicted using a combination of human-based *in vitro* experimental systems and appropriate *in vivo* laboratory animals - the In Vitro-In Vivo Strategy (IVIVS). The success of IVIVS will depend on the selection of appropriate *in vitro* models. Human-specific drug metabolism, appropriate target cell populations, and relevant endpoints are three key parameters for the selection of an appropriate *in vitro* model. Human hepatocytes and human liver fractions represent useful appropriate experimental models to evaluate liver specific events such as hepatic metabolism, drug-drug interactions, and hepatotoxicity. Higher throughput screening assays have been developed to allow early screening of human-specific adverse drug effects. IdMOC allows the co-culturing of multiple cell types modeling *in vivo* multiple organ interactions and thereby represent a more complete *in vitro* experimental system for the prediction of *in vivo* drug properties.

It is to be noted that recent research findings have demonstrated that in addition to drug metabolizing enzyme activities, uptake and efflux transporters also play critical roles in the manifestation of adverse drug effects<sup>10, 45</sup>. Human hepatocyte assays for the evaluation of uptake and efflux transporters have been established and are being applied towards drug development<sup>46-50</sup>. These transporter assays, when applied in conjunction with the assays described in this chapter, should aid the selection of the most appropriate drug candidates for further drug development.

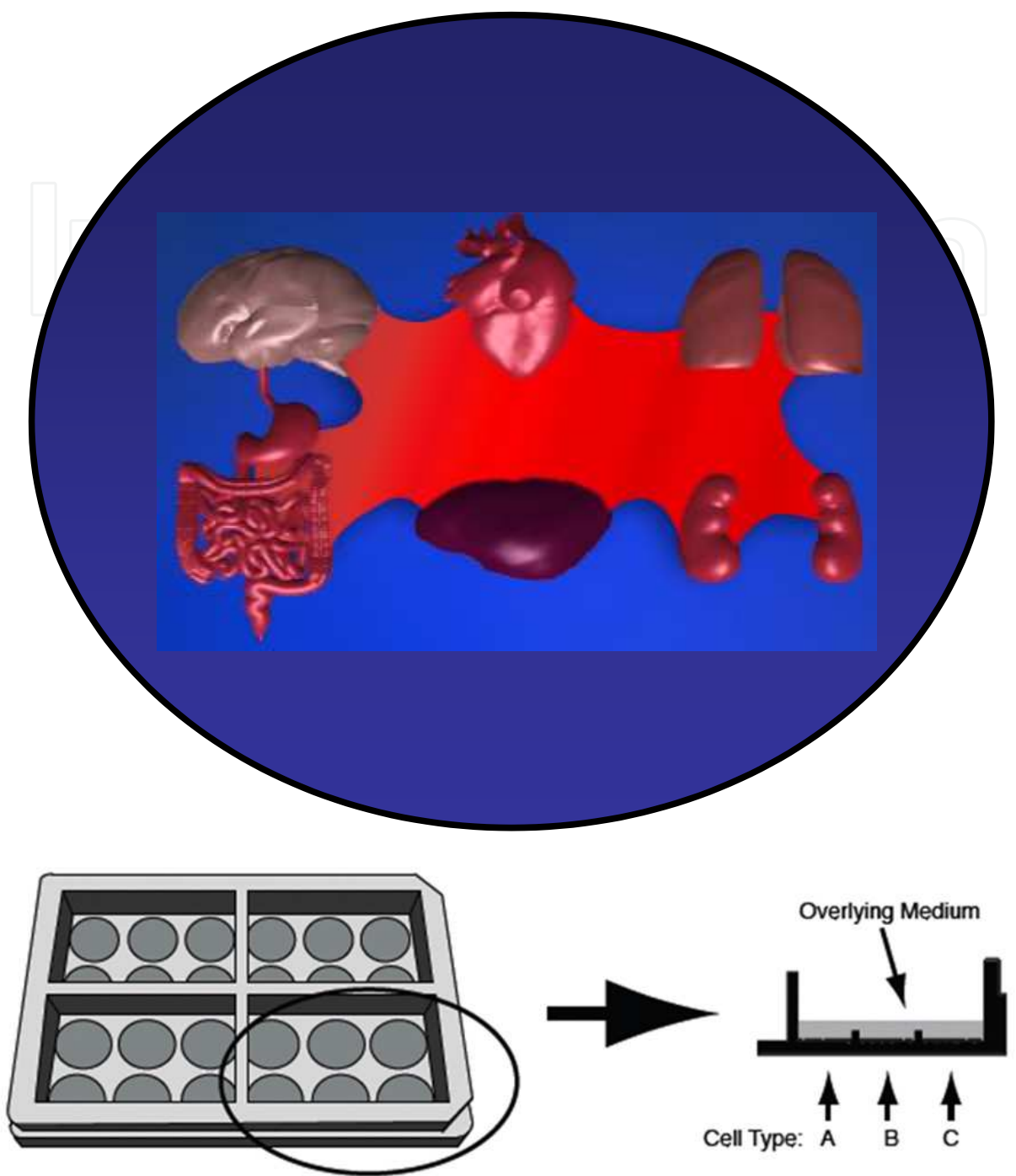


Fig. 2. The Integrated Discrete Multiple Organ Co-culture (IdMOC) experiment system is based on the concept that in the human body consists of multiple organs interacting via the systemic circulation (Top figure). A toxicant may be metabolized by one or more of the organs, and the resulting metabolites may interact with one or more organs via the systemic circulation. This concept is reduced to practice as an IdMOC plate (Lower Figure), with multiple wells within a chamber. Cells from individual organs are cultured physically separated in the wells, with the cells of the multiple organs interconnected via an overlying medium. From Li <sup>43</sup>

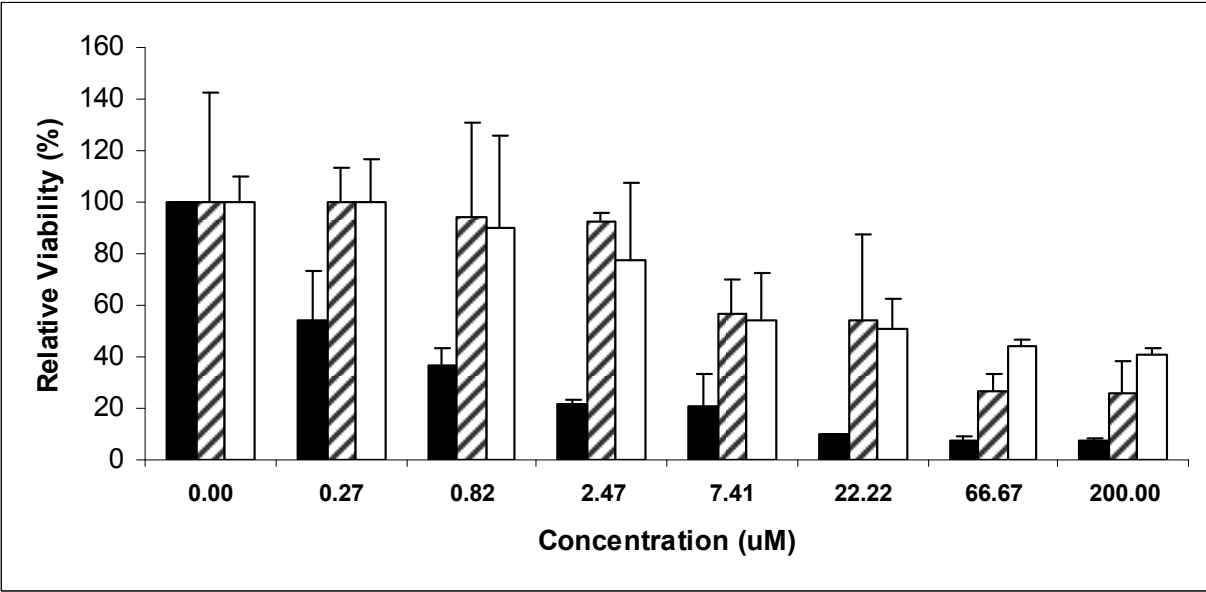


Fig. 3. Application of the Integrated Discrete Multiple Organ Co-culture (IdMOC) experiment system in the evaluation of organ specific toxicity. IdMOC with co-cultures of human hepatocytes (solid bars), renal proximal tubule cells (shaded bars), and small airway epithelial cells (open bars) was used to evaluate the cytotoxicity of the known hepatotoxic agent, aflatoxin B1. While dose-dependent cytotoxicity was observed for all cell types, aflatoxin B1 was significantly more cytotoxic towards human hepatocytes. The results illustrate the application of IdMOC in the evaluation of organ-selective toxicity of drug substances. Other applications of IdMOC include organ-selective drug distribution and integrated multiple organ metabolism.

15. References

[1] DiMasi JA, Hansen RW, Grabowski HG: The price of innovation: new estimates of drug development costs, *J Health Econ* 2003, 22:151-185

[2] DiMasi JA, Feldman L, Seckler A, Wilson A: Trends in risks associated with new drug development: success rates for investigational drugs, *Clin Pharmacol Ther* 2010, 87:272-277

[3] Wysowski DK, Swartz L: Adverse drug event surveillance and drug withdrawals in the United States, 1969-2002: the importance of reporting suspected reactions, *Arch Intern Med* 2005, 165:1363-1369

[4] Li AP, Jurima-Romet M: Overview: pharmacokinetic drug-drug interactions, *Adv Pharmacol* 1997, 43:1-6

[5] Davit B, Reynolds K, Yuan R, Ajayi F, Conner D, Fadiran E, Gillespie B, Sahajwalla C, Huang SM, Lesko LJ: FDA evaluations using in vitro metabolism to predict and interpret in vivo metabolic drug-drug interactions: impact on labeling, *J Clin Pharmacol* 1999, 39:899-910

[6] Zhang L, Zhang YD, Zhao P, Huang SM: Predicting drug-drug interactions: an FDA perspective, *AAPS J* 2009, 11:300-306

[7] MacGregor JT, Collins JM, Sugiyama Y, Tyson CA, Dean J, Smith L, Andersen M, Curren RD, Houston JB, Kadlubar FF, Kedderis GL, Krishnan K, Li AP, Parchment RE,

- Thummel K, Tomaszewski JE, Ulrich R, Vickers AE, Wrighton SA: In vitro human tissue models in risk assessment: report of a consensus-building workshop, *Toxicol Sci* 2001, 59:17-36
- [8] Li AP: Human-based in vitro experimental systems for the evaluation of human drug safety, *Curr Drug Saf* 2007, 2:193-199
- [9] Ikeda T: Drug-induced idiosyncratic hepatotoxicity: prevention strategy developed after the troglitazone case, *Drug Metab Pharmacokinet* 2011, 26:60-70
- [10] Assis DN, Navarro VJ: Human drug hepatotoxicity: a contemporary clinical perspective, *Expert Opin Drug Metab Toxicol* 2009, 5:463-473
- [11] Hewitt NJ, Lechon MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM, Hengstler JG: Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies, *Drug Metab Rev* 2007, 39:159-234
- [12] Li AP: Human hepatocytes: isolation, cryopreservation and applications in drug development, *Chem Biol Interact* 2007, 168:16-29
- [13] Li AP: Human hepatocytes as an effective alternative experimental system for the evaluation of human drug properties: general concepts and assay procedures, *ALTEX* 2008, 25:33-42
- [14] Ulrich RG, Rockett JC, Gibson GG, Pettit SD: Overview of an interlaboratory collaboration on evaluating the effects of model hepatotoxicants on hepatic gene expression, *Environ Health Perspect* 2004, 112:423-427
- [15] Gomez-Lechon MJ, Castell JV, Donato MT: The use of hepatocytes to investigate drug toxicity, *Methods Mol Biol* 2010, 640:389-415
- [16] O'Brien PJ, Chan K, Silber PM: Human and animal hepatocytes in vitro with extrapolation in vivo, *Chem Biol Interact* 2004, 150:97-114
- [17] Loretz LJ, Li AP, Flye MW, Wilson AG: Optimization of cryopreservation procedures for rat and human hepatocytes, *Xenobiotica* 1989, 19:489-498
- [18] Li AP: Preclinical evaluation of drug-drug interactions using human in vitro experimental systems, *IDrugs* 1998, 1:311-314
- [19] Li AP: Primary hepatocyte cultures as an in vitro experimental model for the evaluation of pharmacokinetic drug-drug interactions, *Adv Pharmacol* 1997, 43:103-130
- [20] Li AP: Evaluation of drug metabolism, drug-drug interactions, and in vitro hepatotoxicity with cryopreserved human hepatocytes, *Methods Mol Biol* 2010, 640:281-294
- [21] Li AP, Kaminski DL, Rasmussen A: Substrates of human hepatic cytochrome P450 3A4, *Toxicology* 1995, 104:1-8
- [22] Guengerich FP: Cytochrome P450: what have we learned and what are the future issues?, *Drug Metab Rev* 2004, 36:159-197
- [23] Zhou SF, Xue CC, Yu XQ, Li C, Wang G: Clinically important drug interactions potentially involving mechanism-based inhibition of cytochrome P450 3A4 and the role of therapeutic drug monitoring, *Ther Drug Monit* 2007, 29:687-710
- [24] Flockhart DA, Oesterheld JR: Cytochrome P450-mediated drug interactions, *Child Adolesc Psychiatr Clin N Am* 2000, 9:43-76

- [25] Fujita K: Food-drug interactions via human cytochrome P450 3A (CYP3A), *Drug Metabol Drug Interact* 2004, 20:195-217
- [26] Doshi U, Li AP: Luciferin IPA-Based Higher Throughput Human Hepatocyte Screening Assays for CYP3A4 Inhibition and Induction, *J Biomol Screen* 2011,
- [27] Li AP, Doshi U: Higher Throughput Human Hepatocyte Assays for the Evaluation of Time-Dependent Inhibition of CYP3A4, *Drug Metab Lett* 2011,
- [28] Chauret N, Tremblay N, Lackman RL, Gauthier JY, Silva JM, Marois J, Yergey JA, Nicoll-Griffith DA: Description of a 96-well plate assay to measure cytochrome P4503A inhibition in human liver microsomes using a selective fluorescent probe, *Anal Biochem* 1999, 276:215-226
- [29] Crespi CL, Penman BW: Use of cDNA-expressed human cytochrome P450 enzymes to study potential drug-drug interactions, *Adv Pharmacol* 1997, 43:171-188
- [30] Ogilvie BW, Zhang D, Li W, Rodrigues AD, Gipson AE, Holsapple J, Toren P, Parkinson A: Glucuronidation converts gemfibrozil to a potent, metabolism-dependent inhibitor of CYP2C8: implications for drug-drug interactions, *Drug Metab Dispos* 2006, 34:191-197
- [31] Lu C, Hatsis P, Berg C, Lee FW, Balani SK: Prediction of pharmacokinetic drug-drug interactions using human hepatocyte suspension in plasma and cytochrome P450 phenotypic data. II. In vitro-in vivo correlation with ketoconazole, *Drug Metab Dispos* 2008, 36:1255-1260
- [32] Shitara Y, Li AP, Kato Y, Lu C, Ito K, Itoh T, Sugiyama Y: Function of uptake transporters for taurocholate and estradiol 17beta-D-glucuronide in cryopreserved human hepatocytes, *Drug Metab Pharmacokinet* 2003, 18:33-41
- [33] Maeda K, Kambara M, Tian Y, Hofmann AF, Sugiyama Y: Uptake of ursodeoxycholate and its conjugates by human hepatocytes: role of Na(+)-taurocholate cotransporting polypeptide (NTCP), organic anion transporting polypeptide (OATP) 1B1 (OATP-C), and oatp1B3 (OATP8), *Mol Pharm* 2006, 3:70-77
- [34] Li AP, Lu C, Brent JA, Pham C, Fackett A, Ruegg CE, Silber PM: Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential, *Chem Biol Interact* 1999, 121:17-35
- [35] Li AP: Evaluation of luciferin-isopropyl acetal as a CYP3A4 substrate for human hepatocytes: effects of organic solvents, cytochrome P450 (P450) inhibitors, and P450 inducers, *Drug Metab Dispos* 2009, 37:1598-1603
- [36] Zhou ZW, Zhou SF: Application of mechanism-based CYP inhibition for predicting drug-drug interactions, *Expert Opin Drug Metab Toxicol* 2009, 5:579-605
- [37] Wang X, Wang Y, Chunsheng Y, Wang L, Han S: Mechanism-based quantitative structure-phytotoxicity relationships comparative inhibition of substituted phenols on root elongation of *Cucumis sativus*, *Arch Environ Contam Toxicol* 2002, 42:29-35
- [38] Mori K, Hashimoto H, Takatsu H, Tsuda-Tsukimoto M, Kume T: Cocktail-substrate assay system for mechanism-based inhibition of CYP2C9, CYP2D6, and CYP3A using human liver microsomes at an early stage of drug development, *Xenobiotica* 2009, 39:415-422
- [39] McGinnity DF, Berry AJ, Kenny JR, Grime K, Riley RJ: Evaluation of time-dependent cytochrome P450 inhibition using cultured human hepatocytes, *Drug Metab Dispos* 2006, 34:1291-1300



- [40] Mao J, Mohutsky MA, Harrelson JP, Wrighton SA, Hall SD: Prediction of CYP3A-Mediated Drug-Drug Interactions Using Human Hepatocytes Suspended in Human Plasma, *Drug Metab Dispos* 2011, 39:591-602
- [41] Li AP, Doshi U: Higher Throughput Human Hepatocyte Assays for the Evaluation of Time-Dependent Inhibition of CYP3A4, *Drug Metab Lett* 2011, 5:183-191
- [42] Li AP, Bode C, Sakai Y: A novel in vitro system, the integrated discrete multiple organ cell culture (IdMOC) system, for the evaluation of human drug toxicity: comparative cytotoxicity of tamoxifen towards normal human cells from five major organs and MCF-7 adenocarcinoma breast cancer cells, *Chem Biol Interact* 2004, 150:129-136
- [43] Li AP: In vitro evaluation of human xenobiotic toxicity: scientific concepts and the novel integrated discrete multiple cell co-culture (IdMOC) technology, *ALTEX* 2008, 25:43-49
- [44] Li AP: The use of the Integrated Discrete Multiple Organ Co-culture (IdMOC) system for the evaluation of multiple organ toxicity, *Altern Lab Anim* 2009, 37:377-385
- [45] Morgan RE, Trauner M, van Staden CJ, Lee PH, Ramachandran B, Eschenberg M, Afshari CA, Qualls CW, Jr., Lightfoot-Dunn R, Hamadeh HK: Interference with bile salt export pump function is a susceptibility factor for human liver injury in drug development, *Toxicol Sci* 2010, 118:485-500
- [46] Diao L, Li N, Brayman TG, Hotz KJ, Lai Y: Regulation of MRP2/ABCC2 and BSEP/ABCB11 expression in sandwich cultured human and rat hepatocytes exposed to inflammatory cytokines TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , *J Biol Chem* 2010, 285:31185-31192
- [47] Maeda K, Sugiyama Y: The use of hepatocytes to investigate drug uptake transporters, *Methods Mol Biol* 2010, 640:327-353
- [48] Rotroff DM, Beam AL, Dix DJ, Farmer A, Freeman KM, Houck KA, Judson RS, LeCluyse EL, Martin MT, Reif DM, Ferguson SS: Xenobiotic-metabolizing enzyme and transporter gene expression in primary cultures of human hepatocytes modulated by ToxCast chemicals, *J Toxicol Environ Health B Crit Rev* 2010, 13:329-346
- [49] Liao M, Raczynski AR, Chen M, Chuang BC, Zhu Q, Shipman R, Morrison J, Lee D, Lee FW, Balani SK, Xia CQ: Inhibition of hepatic organic anion-transporting polypeptide by RNA interference in sandwich-cultured human hepatocytes: an in vitro model to assess transporter-mediated drug-drug interactions, *Drug Metab Dispos* 2010, 38:1612-1622
- [50] Badolo L, Rasmussen LM, Hansen HR, Sveigaard C: Screening of OATP1B1/3 and OCT1 inhibitors in cryopreserved hepatocytes in suspension, *Eur J Pharm Sci* 2010, 40:282-288



## **Drug Discovery and Development - Present and Future**

Edited by Dr. Izet Kapetanović

ISBN 978-953-307-615-7

Hard cover, 528 pages

**Publisher** InTech

**Published online** 16, December, 2011

**Published in print edition** December, 2011

Drug discovery and development process aims to make available medications that are safe and effective in improving the length and quality of life and relieving pain and suffering. However, the process is very complex, time consuming, resource intensive, requiring multi-disciplinary expertise and innovative approaches. There is a growing urgency to identify and develop more effective, efficient, and expedient ways to bring safe and effective products to the market. The drug discovery and development process relies on the utilization of relevant and robust tools, methods, models, and validated biomarkers that are predictive of clinical effects in terms of diagnosis, prevention, therapy, and prognosis. There is a growing emphasis on translational research, a bidirectional bench to the bedside approach, in an effort to improve the process efficiency and the need for further innovations. The authors in the book discuss the current and evolving state of drug discovery and development.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Albert P. Li (2011). Critical Human Hepatocyte-Based In Vitro Assays for the Evaluation of Adverse Drug Effects, Drug Discovery and Development - Present and Future, Dr. Izet Kapetanović (Ed.), ISBN: 978-953-307-615-7, InTech, Available from: <http://www.intechopen.com/books/drug-discovery-and-development-present-and-future/critical-human-hepatocyte-based-in-vitro-assays-for-the-evaluation-of-adverse-drug-effects>

**INTECH**  
open science | open minds

### **InTech Europe**

University Campus STeP Ri  
Slavka Krautzeka 83/A  
51000 Rijeka, Croatia  
Phone: +385 (51) 770 447  
Fax: +385 (51) 686 166  
[www.intechopen.com](http://www.intechopen.com)

### **InTech China**

Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821

© 2011 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the [Creative Commons Attribution 3.0 License](https://creativecommons.org/licenses/by/3.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

IntechOpen

IntechOpen