In Vitro Evaluation of Human Xenobiotic Toxicity: Scientific Concepts and the Novel Integrated Discrete Multiple Cell Co-Culture (IdMOC) Technology

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Summary

In vitro human-based experimental systems represent a relevant preclinical tool for the definition of human xenobiotic properties as human-specific xenobiotic properties, by definition, cannot be detected with nonhuman laboratory animals. For the evaluation of human-specific xenobiotic toxicity, the experimental system should have human xenobiotic metabolism as well as cell populations representing the target cells in vivo. Based on these requirements, we have developed a novel cell culture technology, the Integrated Discrete Multiple Organ Co-culture (IdMOC) system, as an alternative experimental model for the evaluation of xenobiotic toxicity. The IdMOC system employs a wells-in-a-well concept for the co-culturing of cells from different organs as physically separated (discrete) entities in the inner wells, but interconnected by an overlying medium (integrated) in the outer well. The IdMOC system models a multi-organ animal/human in vivo with organs that are physically separated but interconnected by the systemic circulation. The IdMOC allows multi-organ interactions that are absent in the conventional single-cell type cultures. Applications of IdMOC include the evaluation of multiple organ metabolism as well as organ-specific drug distribution and toxicity. A particularly interesting application of IdMOC is the co-culturing of cancer cells and cells representing major organs for the selection of anticancer agents with minimal organ toxicity.

Zusammenfassung: *In vitro* Evaluierung xenobiotischer Toxizität für den Menschen: wissenschaftliche Konzepte und die neuartige integrierte diskrete multiple Zell-Kokultur (IdMOC) Technologie
Human-basierte in vitro Testsysteme stellen relevante, präklinische Werkzeuge zur Definition von xenobiotischen Wirkungen auf den Menschen dar, denn human-spezifische xenobiotische Wirkungen können per Definition nicht mittels nicht-humaner Labortiere nachgewiesen werden. Um eine Evaluierung human-spezifischer xenobiotischer Toxizität zu ermöglichen, muss das System sowohl über humanen xenobiotischen Metabolismus als auch über Zellpopulationen, welche die Zielzellen in vivo repräsentieren, verfügen. Um diese Bedingungen zu erfüllen, haben wir eine neuartige Zellkulturtechnologie entwickelt: Das integrierte diskrete multiple Zell-Kokultur (IdMOC) System, ein alternatives experimentelles Modell für die Evaluierung xenobiotischer Toxizität. Das IdMOC System verwendet ein „Well in Well" Konzept für die Kokultur von Zellen verschiedener Organe als physisch getrennte (diskrete) Einheiten in den inneren Vertiefungen, die durch das überliegende Medium (integriert) in der äußeren Vertiefung der Zellkulturschale miteinander verbunden sind. Das IdMOC System ist ein Modell eines Multiorgantieres/-menschen in vivo mit Organen, die physisch von einander getrennt, aber über den Kreislauf miteinander verbunden sind. Das IdMOC erlaubt Multi-Organ-Interaktionen, die in konventionellen Einzelzellkulturen nicht vorkommen können. Anwendungen des IdMOC schliessen die Evaluierung von Metabolismus in multiplen Organen wie auch organspezifische Verteilung und Toxizität von Substanzen ein. Eine besonders interessante Anwendung des IdMOC ist die Kokultur von Tumorzellen mit Zellen, die unterschiedliche Organe repräsentieren, zur Selektion von Chemotherapeutika mit minimaler Organtoxizität.

1 Introduction

While *in vitro* toxicity assays with primary cells and cell lines have been used for decades for toxicity evaluation, they are generally viewed as inadequate as definitive tools for the assessment of *in vivo* toxicity. A major criticism is the lack of key *in vivo* variables that are required for the manifestation of toxicity. Multiple organ interactions represent one of these key variables. An example of multiple organ interactions is the generation of toxic metabolites by the major organ for xenobiotic metabolism, the liver, with the toxic metabolites causing toxicity in distal, non-hepatic organs.

The mission of our laboratory is to develop *in vitro* technologies for xenobiotic evaluation to improve upon the rou-

tinely used approaches. We have previously developed *in vitro* hepatic systems for the evaluation of drug metabolism and drug-drug interactions which are now universally applied in drug development (Li, 2001; 2004a). We now are focusing our efforts on the development and application of human-based *in vitro* experimental systems for the evaluation of human xenobiotic toxicity. We consider the following properties as critical to the success of such *in vitro* systems:

1. Human-specific xenobiotic metabolism
2. Human target cells
3. Multiple organ interactions
4. Relevant, predictive endpoints
5. High throughput applications

The scientific concepts behind the need to apply *in vitro* technologies to evaluate human-specific xenobiotic effects are reviewed here. A patented novel technology, the Integrated Discrete Multiple-organ Co-culture (IdMOC) system (Li et al., 2004; Li, 2005) is described as one of the promising *in vitro* experimental systems for the evaluation of xenobiotic toxicity.

2 Species-species differences in xenobiotic toxicity

Species difference in xenobiotic toxicity is a well-documented phenomenon. A well-established example of this phenomenon is the lethality of the environmental pollutant TCDD-dioxin, which displays a more than 1000-fold (100,000%) difference in LD_{50} value in guinea pig (approximately 1-2 μg/kg) compared to hamster (>3000 μg/kg) (Hengstler et al., 1999). It has been suggested that human-laboratory animal difference in toxicity is the major reason for clinical trial failure of drug candidates that have been found to be safe based on preclinical laboratory animal results (DiMasi et al., 2003; Kola and Landis, 2004; Li, 2004b).

This occurrence of species-species differences in xenobiotic toxicity illustrates the major deficiency of the routine practice of xenobiotic safety evaluation, namely, human-specific effects cannot be detected with nonhuman animals.

There are two major contributing factors to species-differences in xenobiotic toxicity:

1. Species-differences in drug metabolism.
2. Species-differences in target organ sensitivity.

2.1 Species-species differences in xenobiotic metabolism

Xenobiotic metabolism is in general a protective mechanism against toxic substances that can enter an organism. In general, toxic substances are modified by xenobiotic metabolism to neutralize their toxicity and to facilitate their excretion. Variations in drug metabolizing enzymes evolved among the various animal species, presumably due to the specific exposure of each species towards environmental toxicants.

Species-species differences in xenobiotic metabolizing enzymes can be illustrated with the occurrence of species-specific isoforms for the major family of xenobiotic metabolizing enzymes: the P450-dependent monooxygenases (CYP) (Guengerich, 2006). The major human hepatic isoforms are: CYP1A1/2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1 and CYP3A4. Of these isoforms, only CYP1A2 and CYP2E1 are found in nonhuman animals, including mouse, rat, dog, and monkey, routinely used in safety evaluation. For instance, in the rat, the major corresponding isoforms are: CYP1A1/2, CYP2A5, CYP2B1, CYP2C7, CYP2C9, CYP2C11, CYP2D1, CYP2E1 and CYP3A1/2. The species-species differences in P450 isoforms can lead to differences in the fate of a xenobiotic upon metabolism, including differences in the rate of metabolism and the structure of the metabolites (Lewis et al., 1998). Species-difference in metabolite formation is believed to be one of the major contributors to species-difference in xenobiotic toxicity for the following reasons:

1. A toxic metabolite that is formed in humans but not in nonhuman animals would lead to underestimation of the toxicity of the parent xenobiotic.
2. A toxic metabolite that is formed in nonhuman animals but not in humans would lead to the overestimation of the toxicity of the parent xenobiotic.

There are numerous reports on the human-animal differences in xenobiotic metabolism. A clear example is the formation of 7-OH-coumarin in humans but not in rodents (Easterbrook et al., 2001).

2.2 Species-species differences in target cell sensitivity towards toxicants

Besides differences in metabolism, species-differences in xenobiotic toxicity can also be due to differences in the sensitivity of the cells in the target organs towards the toxicants in question. The following are well-defined examples of this phenomenon.

1. Peroxisome proliferation receptor ligands: Antilipidemic agents such as the fibrates are agonists of PPARα, leading to the upregulation of lipid metabolism pathways. These agents have been found be nongenotoxic hepatocarcinogens in rodents but are generally believed to be noncarcinogenic in primates, including humans. In a recent report by Yang et al. (2007) using mice with humanized PPARα the peroxisome proliferator fenofibrate induced hepatomegaly and hepatocyte proliferation in wild type mice (with mouse PPARα) but not in the mice with human PPARα. The species-difference in peroxisome proliferator carcinogenicity therefore could be a result of species-differences in the function of the PPARα.
2. Ah-receptor ligands: The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates many of the biological and toxic effects of halogenated aromatic hydrocarbons (HAHs), polycyclic aromatic hydrocarbons (PAHs), and other structurally diverse ligands. Species differences in the toxicity of these ligands, for instance, TCDD-dioxin and polybrominated biphenol (PBB), are well-established (Hengstler et al., 1999). A hallmark of AhR binding, CYP1A induction, has been used to demonstrate the species differences in AhR affinity of carcinogenic halogenated hydrocarbons (Merrill et al., 1995; Xu et al., 2000; Silkworth et al., 2005).
3. PXR-ligands: Pregnane X receptor (PXR) is an orphan nuclear receptor

that regulates the expression of genes encoding drug-metabolizing enzymes and transporters (Stanley et al., 2006). Activation of PXR leads to up-regulation of drug metabolizing enzymes including cytochrome P450 isoforms and the transporter Pgp (Handschin and Meyer, 2003). Although the consequence is mainly in drug-drug interactions, there is an apparent correlation between enzyme induction potential and hepatotoxicity for drugs that are known to cause idiosyncratic toxicity (Li, 2002). Human-rodent differences in PXR activation are well-established. For instance, rifampin has a higher affinity for the human than for rodent PXR ligand, while pregnenolone 16alpha-carbonitrile (PCN) has a higher affinity for rodent PXR than human PXR, with CYP3A induction potential reflecting these differences (Lu and Li, 2001; Ma et al., 2007).

4. Anticancer agents: Species-species differences in target cell sensitivity can be illustrated with directly acting cytotoxicants evaluated *in vitro* using metabolically incompetent cells. Erickson-Miller et al. (1997) evaluated the toxicity of ercamptothecin (CAM), topotecan (TPT) and 9-aminocamptothecin (9-AC) on human, canine, and murine myeloid progenitors (CFU-GM) in vitro and found that the murine IC_{90} (concentration of the toxicant causing 90% cytotoxicity) values were 2.6-, 2.3-, 10-, 21-, 5.9-, and 11-fold higher than human values for CAM lactone (NSC-94600) and sodium salt (NSC-100880), TPT (NSC-609699), and racemic (NSC-629971), semisynthetic and synthetic preparations (NSC-603071) of 9-AC, respectively. In contrast, the canine IC_{90} values were the same as, or lower than, the human IC_{90} values for all six compounds.

3 *In vitro* toxicity assays as an approach to define human-specific xenobiotic toxicity

Human-specific xenobiotic toxicity, whether as a result of drug metabolism or target cell sensitivity, by definition cannot be accurately evaluated with nonhuman animal models. Short of experimentation in humans *in vivo*, toxicity testing *in vitro* using experimental systems with relevant human-specific properties represents the only practical preclinical approach to derive human-specific information for the accurate prediction of human xenobiotic toxicity.

It is important to note that the *in vitro* experimental systems used should have the following components that are critical to human-specific xenobiotic toxicity as discussed earlier:
1. Human xenobiotic metabolism
2. Human target cells

The use of dedifferentiated cell lines such as transformed or immortalized mouse or human fibroblasts would not be useful, as neither of the critical properties mentioned above are present. The use of human organ-derived primary cells as monogenic culture (single cell type cultures) will only allow the evaluation of the effect of xenobiotics on a specific cell type which may or may not possess significant human xenobiotic metabolism pathways.

One cell system that represents the target cells and has human metabolism capacity is the human hepatocyte system. This cell system is used routinely for the evaluation of human-specific xenobiotic metabolism (Li, 2004a; 2007) and is used routinely for the early assessment of hepatotoxic potential (Li et al., 1999; Dambach et al., 2005). This cell system has the following advantages:
1. Human xenobiotic metabolism: Freshly isolated or cryopreserved fresh isolates of human hepatocytes are known to contain most, if not all, of the *in vivo* hepatic xenobiotic metabolism capacity (Li et al., 1999).
2. Human target cells: The hepatocytes are the cells in the human liver that are damaged by hepatotoxicants, leading ultimately to liver failure (Abboud and Kaplowitz, 2007).

A commonly used cell line, the HepG2 cell line, does not have these properties, and therefore would not represent a relevant *in vitro* model for the investigation of hepatotoxicity:
1. Human xenobiotic metabolism: HepG2 cells only retain a minimal capacity

(estimated to be less than 1%) of the normal hepatic xenobiotic metabolism (Rodríguez-Antona et al., 2002; Wilkening et al., 2003; Westerink and Schoonen 2007).
2. Human target cells: HepG2 cells are derived from a human adenocarcinoma of the liver (Schwartz et al., 1981), not from parenchymal cells which are the *in vivo* target of hepatotoxicants.

It must be emphasized that the use of cell lines without human metabolism and not representing *in vivo* target cells will not have any advantage over the use of laboratory animals in the evaluation of xenobiotic toxicity.

Besides hepatocytes, primary cells cultured from various organs such as kidney (Dietrich et al., 2001; Li et al., 2006; Lock et al., 2007), nervous system (Stair et al., 2005; Guizzetti et al., 2005), heart (Hasinoff et al., 2007), skeletal muscle (Mazno et al., 2003), vascular endothelium (Harlan et al., 1983; Kim et al., 2007), lung (Li, 1986; Li and Myers, 1988; Bakand et al., 2007; Han et al., 2007; Sayes et al., 2007), and blood/bone marrow cells (Deldar and Stevens, 1993; Rich and Hall, 2005) have also been applied towards the evaluation of xenobiotic toxicity. The various primary cell systems used may be representative of the appropriate *in vivo* target cells for their respective organs. However, the use of these cells alone would lack an important *in vivo* determinant of xenobiotic toxicity, namely, hepatic metabolism.

In our laboratory, efforts are focused on the development of an *in vitro* experimental model to incorporate hepatic metabolism and both hepatic and nonhepatic target cells to allow a comprehensive evaluation of xenobiotic toxicity.

4 Integrated Discrete Multiple Organ Co-culture (IdMOC)

The IdMOC is a novel technology developed in our laboratory as an *in vitro* experimental system for the evaluation of human xenobiotic metabolism, distribution, and toxicity (Li et al., 2004; Li, 2007). It is based on the concept that in the human body, there are multiple

organs that are physically separated but are interconnected by the systemic circulation. The systemic circulation allows multiple organ interactions. An example of multiple organ interaction is the metabolism of a toxicant by the liver, with the resulting metabolites entering the systemic circulation, leading to the exposure of distal, nonhepatic organs to these metabolites, resulting in toxicity in these organs.

The schematic representation of the scientific concept and configuration of IdMOC is presented in Figure 1, with a photograph of an IdMOC plate shown as Figure 2. The IdMOC uses a wells-in a well concept. Cells from individual organs are cultured separately in each of the inner wells. The inner wells are interconnected by filling the outer well with an overlying medium to cover all inner wells. A xenobiotic introduced into the overlying medium will interact with the multiple cell types in each of the inner wells, and these will be exposed to the metabolites collectively generated by the cells. The IdMOC system therefore satisfies our requirements for human metabolism (via the use of human hepatocytes as one of the cell types to provide hepatic xenobiotic metabolism), and human target cells (via the use of cell types from different organs to allow evaluation of organ-specific toxicity). An advantage of the use of IdMOC over the conventional mixed-cell type cultures is that after treatment, the cells from each well can be evaluated for cytotoxicity, thereby allowing the evaluation of cell-type specific effects after co-culturing that is extremely difficult with mixed-cell type co-cultures.

Two proof-of-concept experiments were performed to demonstrate the application of IdMOC:

1. Quantification of differential toxicity: We evaluated tamoxifen toxicity towards the mammary cancer cell MCF-7 and cells from 5 major organs – liver (hepatocytes), kidney (proximal tubule epithelial cells), nervous system (astrocytes), vascular endothelium (human aortic endothelial cells), and lung (airway epithelial cells) (Li et al., 2004). The results show that tamoxifen, while toxic to all six cell types, was selectively toxic to MCF-7 cells. Of the multiple cell types from the various organs, the hepatocytes were the most resistant to tamoxifen toxicity, which probably is a result of the high detoxifying metabolic capacity of these cells. The astrocytes were the
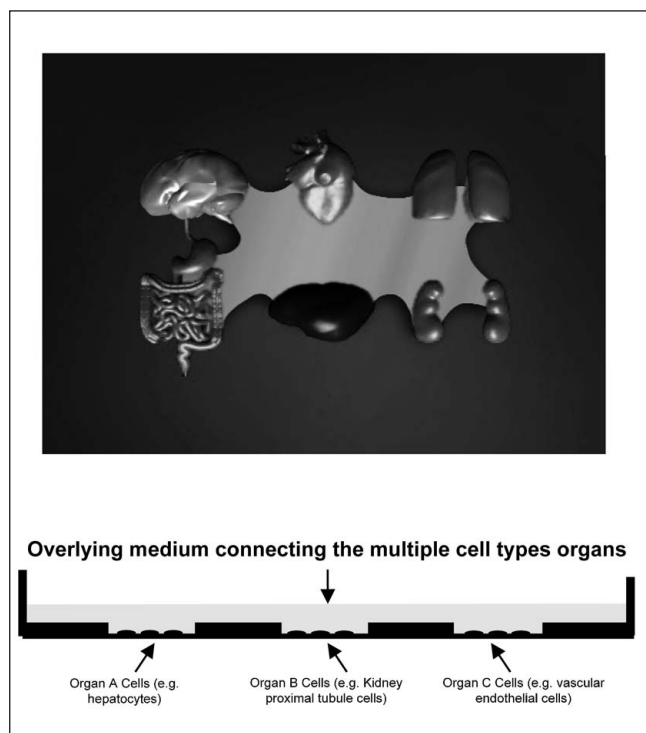

Fig. 1: Schematic presentation of the principles of the Integrated Discrete Multiple Organ Co-Culture (IdMOC) experimental system.
The human body is envisioned as consisting of multiple organs, which are physically discrete but are interconnected by blood (top figure). This concept was applied in the development of IdMOC (bottom), consisting of multiple inner wells, with cells from each organ cultured individually in the inner wells, and an overlying medium overfilling the inner wells. The IdMOC therefore has multiple organs that are physically discrete but interconnected by an overlying medium akin to a human *in vivo*.
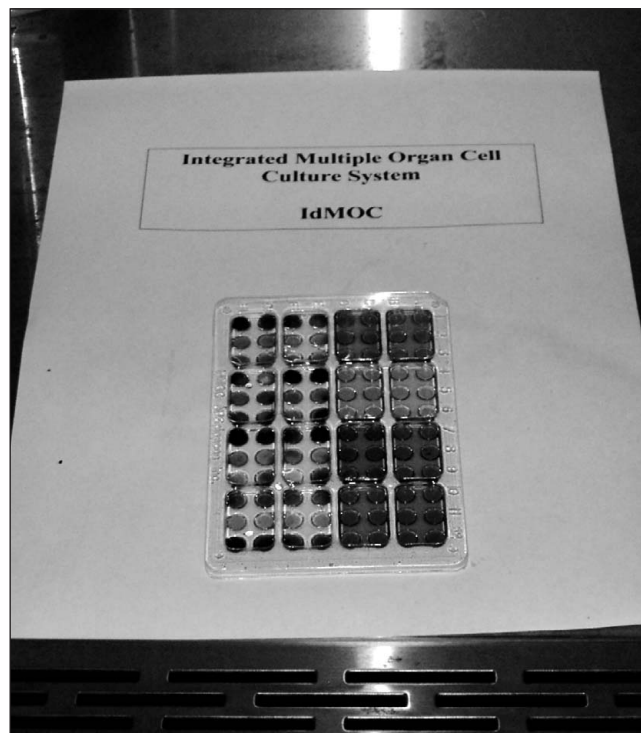
Overlying medium connecting the multiple cell types organs

Organ A Cells (e.g. hepatocytes)   Organ B Cells (e.g. Kidney proximal tubule cells)   Organ C Cells (e.g. vascular endothelial cells)

Fig. 2: A photograph of an IdMOC plate with 6 inner wells per outer well, and with 16 outer wells per plate.
The separation of the inner wells from each other is illustrated by the colored fluids on the left half of the IdMOC plate. The use of an overlying medium to integrate the inner wells in each outer well is illustrated on the right half of the plate. This IdMOC plate therefore has 16 independent units for experimentation.

most sensitive (besides the MCF-7 cells). It is to be noted that tamoxifen is used at hormonal levels as an anti-estrogen for the treatment of estrogen-dependent breast cancer, therefore clinical toxicity is not a complicating factor. Our results on the cytotoxicity of tamoxifen are consistent with its known *in vivo* toxicity when used at relatively high levels, which include hepatotoxicity (Liu et al., 2006) and renal toxicity (McClay et al., 2001). Results of this study are shown in Figure 3.

2. Metabolic cooperation: To demonstrate IdMOC can be used to evaluate the effects of metabolites generated by one cell type on a different target cell type, we evaluated the cytotoxicity of an anticancer agent that requires metabolic activation, i.e. cyclophosphamide, in IdMOC with co-cultures of a metabolically-competent cell type (male Sprague-Dawley rat hepatocytes) and a metabolically non-competent cell type (Chinese hamster ovary (CHO) cells), using an IdMOC plate with only CHO cells without co-culturing with rat hepatocytes as a control. Cyclophosphamide was found to be cytotoxic to CHO cells in the IdMOC with the rat hepatocytes, and noncytotoxic to the CHO cells in the IdMOC without the rat hepatocytes. The results therefore demonstrate the metabolic cooperation of the multiple cell types, with cyclophosphamide metabolized by the hepatocytes, generating toxic metabolites which in turn cause cytotoxicity in the CHO cells (Fig. 4).

Discussion

Demonstration of advantages over conventional approaches using laboratory animals is key to the acceptance and universal application of alternative methods. A major advantage of the use of *in vitro* methods is that human-specific information can be obtained with human-based *in vitro* experimental systems. The same information, by definition, cannot be obtained with nonhuman laboratory animals. This advantage, namely, that *in vitro* systems can provide human-specific

information, has led to the present universal acceptance of human-based *in vitro* hepatic experimental systems such as human hepatocytes and human liver microsomes in the evaluation of drug

metabolism and drug-drug interactions. It is to be noted that the U. S. FDA now accepts data from human *in vitro* hepatic systems alone, and considers *in vivo* animal tests as inappropriate, for the defini-
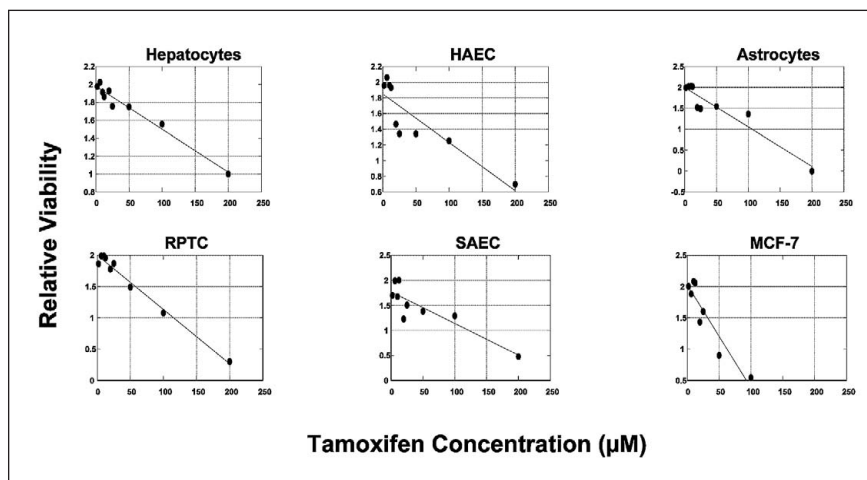

Fig. 3: Evaluation of multiple organ cytotoxicity of tamoxifen, an anticancer agent used to treat estrogen-dependent mammary cancer, in IdMOC with cells from 5 major organs and estrogen-dependent mammary carcinoma cells.
The co-cultured cells and their respective organs were: hepatocytes (liver), human aortic endothelial cells (HAEC; vascular endothelium), human astrocytes (nervous system), human renal proximal tubule cells (RPTC; kidney), human small airway epithelial cells (SAEC; lung) and MCF-7 adenocarcinoma cells (cancer). Cellular ATP was used as the viability endpoint in this study. The results illustrate the use of IdMOC as an *in vitro* "tumor-bearing-man" for the selection of anticancer agents with the most appropriate therapeutic index: agents that are highly cytotoxic towards the intended target, the cancer cells, but noncytotoxic towards the cells representing the major organs. Data from Li et al., 2004.
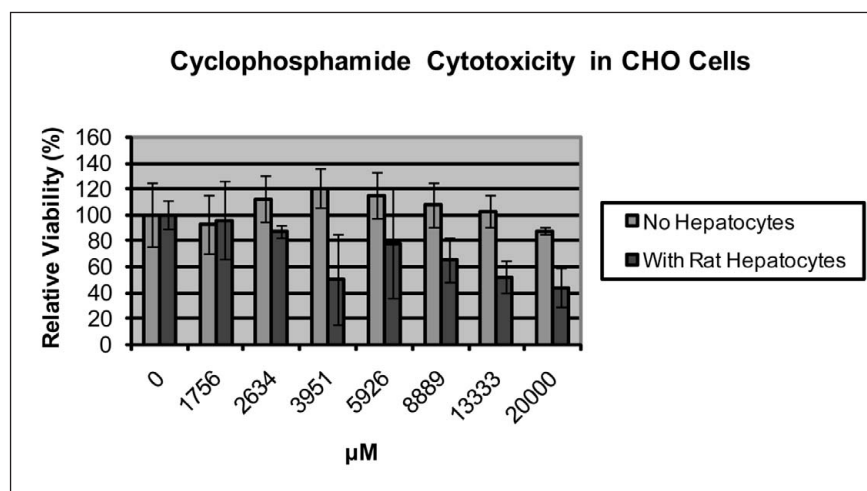

Fig. 4: Cyclophosphamide cytotoxicity towards Chinese hamster ovary (CHO) cells co-cultured with and without hepatocytes in IdMOC.
Male Sprague-Dawley rat hepatocytes were used in this study. The results showed that cyclophosphamide was more cytotoxic to the metabolically-noncompetent CHO cells when hepatocytes were present in the IdMOC, demonstrating the metabolic cooperation between the two cell types, with hepatocytes metabolizing cyclophosphamide to toxic metabolites which in turn caused cytotoxicity in the CHO cells.

tion of drug-drug interaction potential of human pharmaceuticals (FDA, 2006).

In vitro toxicity has always been highly criticized, mainly due to the lack of *in vivo* factors that are believed to be crucial to the manifestation of *in vivo* toxicity. We therefore embarked on the development of *in vitro* approaches incorporating these key *in vivo* factors. Our research has led to the development of the IdMOC technology. The IdMOC system has the simplicity and practicality of an *in vitro* system, but with the incorporation of the following key components that are crucial to the manifestation of *in vivo* toxicity:

1. Hepatic metabolism via the use of hepatocytes.
2. Hepatic and nonhepatic target cells via the co-culturing of hepatocytes with key cell types from major organs susceptible to toxicant effects.
3. Multiple organ interactions via an overlying medium.
4. Discrete cultures allowing evaluation of effects of a toxicant on a specific cell type.

The IdMOC is a simple experimental system that can be used without the need for specialized laboratory equipment. Virtually all assays developed for cells in culture can be applied to the IdMOC, including higher-throughput approaches. IdMOC has the potential to be a valuable, universally applicable *in vitro* system for the evaluation of xenobiotic properties including metabolism, distribution, and toxicity.

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