New Paradigm in Toxicity Testing: Integrated Discrete Multiple Organ Co-cultures (IdMOC) for the Evaluation of Xenobiotic Toxicity

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Summary

Humans are exposed, intentionally or unintentionally, to a number of novel chemicals due to industrial activities. There is an urgent need to evaluate the biological effects of these novel chemicals efficiently and accurately. The Tox21 and ToxCast™ programs initiated by the National Institutes of Health (NIH) and the EPA recommend the use of innovative chemical testing methods to meet this unprecedented demand. The critical paths initiative of the U.S. FDA emphasizes experimental approaches that are directly translatable to clinical observations. Application of high-throughput, in vitro assays to delineate pharmacological and toxicological pathways in combination with computational modeling represents one of the critical strategies to predict chemical effects on animals and humans. The Integrated Discrete Multiple Organ Co-culture (IdMOC) is a novel in vitro experimental system that allows the evaluation of biological effects of chemicals, with interactions between multiple cell types including endocrine, paracrine, and metabolic interactions. One specific application of IdMOC is the evaluation of metabolism-dependent chemical properties such as metabolism-dependent toxicity and pharmacology.

Keywords: co-culture, IdMOC, ToxCast, Tox21, in vitro toxicity testing

1 Introduction

Concerns regarding the toxicity of environmental chemicals, consumer products, food additives, pharmaceuticals, and biologics have prompted several US legislative and regulatory initiatives. In 1998, the U.S. Environmental Protection Agency (EPA) initiated the High Production Volume (HPV) program[1] to provide health and environmental data for chemicals that are manufactured or imported in the United States in quantities greater than 1 million pounds annually. This represented 95% of US chemical production and use by volume at the time. The extended HPV program[2] was announced in 2005 to address newer HPV chemicals (574) and broaden the scope of the program to include use and exposure information of all HPV chemicals. The European Union passed the REACH law (Registration, Evaluation, Authorization and restriction of Chemicals), which aimed to regulate all chemicals produced in quantities greater than one ton/year. Such initiatives were directed at making industry responsible for assessing and managing the risks posed by chemicals, as well as to provide appropriate safety information to all its users. Furthermore, such legislation has allowed authorities to take appropriate action and ban highly toxic substances (Knight, 2008; Cohen, 2011; Schoeters, 2010).

In 1998, the EPA announced the Endocrine Disrupting Chemicals program[3] to test dietary and environmental chemicals that could potentially disrupt the hormonal milieu of humans and wildlife. In 2009, the EPA proposed a set of 73 chemicals for initial screening, followed by another set in 2010 consisting of 134 chemicals (Borgert, et al., 2011). In addition to the unprecedented demands of REACH and the EPA, the National Toxicology Program (NTP), based at the National Institutes of Health and Environmental Sciences (NIEHS), has actively solicited nominations from the public and scientific community for toxicological evaluation of chemicals[4]. Such nominations are reviewed based on supporting information and priorities of the agency and, when considered appropriate, are evaluated at the laboratories of the NIEHS. The U.S. Food and Drug Administration (FDA) has routinely regulated pharmaceuticals, biologics (vaccines, blood products), devices, food additives, veterinary products, and cosmetics for safety. Thus, there is a clear need for increased testing and early screening of thousands of compounds to eliminate dangerous substances and develop safe products with marketable potential.

[1] http://www.epa.gov/chemrtk/
[2] http://www.americanchemistry.com/Policy/Chemical-Safety/High-Production-Volume
[3] http://www.epa.gov/endo/
[4] http://ntp.niehs.nih.gov/?objectid=25BC6AF8-BDB7-CEBA-F18554656CC4FCD9

2 The challenge

The scientific basis and corresponding methodologies for hazard identification and risk assessment must be modified such that an increasing number of chemicals can be evaluated in a short period of time. This has been recognized recently by U.S. federal and regulatory agencies. In 2008, a partnership was announced between the EPA, NIEHS/NTP, NIH Chemical Genomics Center, and the Food and Drug Administration (FDA) to meet the future toxicity testing and risk assessment needs of the country. This initiative, termed Tox21, recommended implementation of the National Research Council Committee's plan, *Toxicity Testing in the 21st Century: A Vision and a Strategy* (NRC, 2007). The plan proposed the development of innovative chemical testing methods that characterize chemically induced toxicity pathways. The program aimed to identify chemically induced biological mechanisms, prioritize chemicals that require further toxicological evaluation, and develop models that effectively predict how chemicals will affect such biological responses. It recommended exploration of high-throughput screening assays, tests using phylogenetically lower animal species (e.g., fish, worms, etc.), and high-throughput whole genome analytical methods to evaluate biological mechanisms. Hence, these tests are designed to predict toxicological endpoints (e.g., toxic dose) from a cell-based response using pharmacokinetic and computational modeling (Firestone et al., 2010; Kavlock et al., 2009; Locke and Bruce Myers, 2010). While such methodologies are indeed rapid and promising, they rely ultimately on computerized predictions (Kavlock and Dix, 2010). The predictive analysis of new chemicals based on information from previously examined chemicals, however, is dependent upon similarity to the reference chemicals used. If the effect of a reference chemical is unanticipated, e.g., bioactivation of the parent compound into a more toxic metabolite, then similar effects of the test material are likely to remain undiscovered (Schmidt, 2009).

In 2007, the EPA independently launched the ToxCast™ program to develop methods to predict potential toxicity and, based on such predictions, to prioritize the thousands of chemicals that need toxicity testing (Dix et al., 2007). Since then, the EPA has profiled approximately 300 chemicals, primarily pesticides, using high-throughput *in vitro* assays. Dose response relationships evaluating specific endpoints such as gene expression and receptor binding ability have been reported (Knudsen et al., 2011; Rotroff et al., 2010). The EPA also has catalogued traditional toxicity data obtained from animal studies for many of these chemicals (ToxRefDB[5]). The challenge here is to validate the predictive signatures and data so that this endeavor can be translated into a program to successfully screen new compounds for determination of toxicity.

3 Single cell type *in vitro* toxicity and metabolism assays

Two major considerations or research gaps identified in the development of toxicity assays and the identification of pathways include the incorporation of metabolism (hepatic and non-hepatic) into *in vitro* assays and the means to study cell-cell interactions (Schmidt, 2009; Judson et al., 2010; Memorandum of Understanding on High Throughput Screening[6]). Currently, 2-dimensional monolayers of mammalian cells provide the most suitable *in vitro* model. This is due largely to the fact that such models are conducive to repetitive, systematic, and quantitative evaluation of the biological response to a chemical insult. *In vitro* assays also are more cost-effective, less time consuming, and can assess a large number of compounds and experimental parameters compared to *in vivo* assays (Knight, 2008).

3.1 Hepatic metabolism assays

The liver is the central organ for xenobiotic metabolism. All ingested drugs and chemicals undergo a first pass through the liver after intestinal absorption. The hepatocytes in the liver contain various drug metabolizing enzymes, including phase I oxidative enzymes (e.g., CYP 450 dependent monooxygenases) and phase II conjugating enzymes (e.g., UDP-glucuronosyltransferases, Glutathione S-transferases), as well as drug transporters that contribute to the metabolism and disposition of xenobiotics. Thus, it is not surprising that primary hepatocyte cultures generally are regarded as the gold standard for assessing xenobiotic metabolism. Primary hepatocytes derived from cadaver or transplantable human livers provide the most physiologically relevant experimental system, since they are capable of human xenobiotic metabolism. As such, they provide human-specific information for the prediction of xenobiotic toxicity (Li, 2007a). Primary human hepatocytes can be used for studying various drug properties, including metabolic fate, drug-drug interaction potential, and hepatotoxicity. The development of advanced cryopreservation techniques to prevent membrane damage and subsequent lysis during thawing, along with the development of specific hepatocyte media, has facilitated widespread use of these cells in toxicity assays. Monolayers of hepatocytes at 80-95% confluency are routinely cultured after immediate recovery from cryopreservation and can be used to test drugs and other chemicals effectively. Treatment periods of 24-48 hours are well suited to the development of rapid toxicity tests and can be used in the early assessment of hepatotoxicity and xenobiotic metabolism (Li, 2007a; Hewitt et al., 2007; Li et al., 1999a).

The cellular microenvironment is the key to the physiological response to drugs, as it can affect the cell's properties, behavior, and function. Interaction of a cell with the extracellular matrix (ECM) or adhesion molecules can trigger a cascade of molecular

[5] http://www.epa.gov/ncct/toxrefdb/18
[6] http://www.niehs.nih.gov/news/releases/2010/docs/mou-contract.pdf

events within the cell that culminate in specific cellular responses such as proliferation, differentiation, metabolism, or morphogenesis (Elliott and Yuan, 2011). To model appropriate cellular responses, monolayers of hepatocytes often are sandwiched between two layers of the ECM, namely collagen at the bottom and matrigel or collagen on the top. Using this method, Kern et al. showed that metabolic activity in response to the inducer rifampin could be maintained for 9 days and was comparable to that observed *in vivo* (Kern et al., 1997). Kienhuis et al. compared sandwich cultures of rat hepatocytes in media with or without low doses of the P450 inducers-phenobarbital, dexamethasone, and β-naphthoflavone and found that coumarin-induced cytotoxicity, metabolism, and gene expression was more similar to the *in vivo* situation using modified media than to the control media (Kienhuis et al., 2006). The presence of soluble factors such as growth and survival factors, differentiating agents, and inflammatory mediators, also can affect the competency of a cell. Hence, specific factors such as insulin and dexamethasone often are added to increase survival and maintain the differentiated functions of hepatocytes in culture.

Human hepatocytes represent a valuable experimental system for drug metabolism studies. Hepatocytes can be used in screening assays for metabolic stability and metabolite identification, and demonstration of key metabolizing pathways, as well as evaluation of P450 enzyme activity. Unlike cell-free systems such as microsomes, intact hepatocytes contain all the drug metabolizing enzymes and cofactors at physiological levels. Microsomes also contain only phase I oxidizing enzymes, so unless Phase I oxidation is the most important metabolizing pathway of the test material, intact hepatocytes provide a more physiologically relevant model (Li, 2007a). Metabolite profiling of the drug ethynyl estradiol in the presence of microsomes revealed 2-hydroxylated derivative as the predominant metabolite, whereas profiling in hepatocytes revealed glucoronide and sulfate conjugates similar to those seen in *in vivo* studies (Li et al., 1999b).

3.2 Non-hepatic organ-specific metabolism assays

Other cell types besides hepatocytes also have metabolizing abilities. For example, astrocytes from the central nervous system express monoamine oxidase B that can metabolize the drug MPTP in the brain (Brooks et al., 1989; Gerlach et al., 1991). In addition, several cell types contain CYP enzymes that may be induced by certain drugs. CYP1A1 is inducible in the epidermis in response to TCDD (tetrachlorodibenzo-p-dioxin), PCBs (polychlorinated biphenyls) and crude coal tar (Walsh et al., 1992). CYP1B, CYP4A, and CYP2E1 are induced while CYP2B1 and CYP2C11 are inhibited by doxorubicin in the kidney (Zordoky et al., 2011). Hence, metabolism assays using organ-specific target cells capable of metabolizing chemicals are vital in the evaluation of cytotoxicity.

In some organ-specific tissues such as the lung, several P450 enzymes including CYP1A1, CYP1B1, CYP2A6, CYP2B6, CYP2E1, CYP3A5, and Phase II enzymes such as epoxide hydrolase, UGT1A (glucuronyl transferase), as well as GST-P1 (glutathione S-transferase), have been detected (Castell et al., 2005; Mace et al., 1998). However, the key question is whether cells derived from such tissues have the capacity to activate or detoxify chemicals. Since metabolic activity typically diminishes on plating or culture of cells, it becomes imperative to rigorously test all primary cultures and cell lines for metabolic competence. Genetic manipulation of organ-derived cells to re-express key biotransformation enzymes has been suggested as a means to improve their functionality and metabolic performance (Castell et al., 2005).

Hepatic metabolism can lead to detoxification of compounds or biotransformation into highly reactive and toxic metabolites. Bioactivation of pro-carcinogens, i.e., polycyclic aromatic hydrocarbons or N-nitrosamines into reactive intermediates that can form DNA adducts has been shown to occur in the lung (Uppstad et al., 2010; Lao et al., 2007). Current evidence suggests that ifosfamide metabolites, particularly chloroacetaldehyde, produced within the kidney, contribute to nephrotoxicity (Springate and Taub, 2007). While the choice of the metabolically active cell is critical, the target cell may be distinct from the metabolically competent cell type. For example, astrocytes in the substantia nigra of the brain are capable of metabolizing MPTP into the active metabolite MPP^+. MPP^+ has been shown to cause loss of dopaminergic neurons (Brooks et al., 1989), suggesting induced neurotoxicity in the presence of metabolically competent astrocytes. Similarly, certain organophosphates such as isofenphos can be converted into potent toxic oxon metabolites by hepatocytes. These, in turn, can affect neuronal acetylcholinesterase inhibition (Bruinink and Maier, 2007), suggesting a role for hepatocytes in potentiating neurotoxicity.

4 Multiple cell type metabolism and toxicity assays

The use of single cell-type cultures for evaluation of organ-specific toxicity is limiting since such systems ignore the critical interactions between different cell types within an organ or between multiple organs. Co-cultures of rat hepatocytes with stellate cells in spheroidal aggregates produce higher enzymatic activities than monocultures of hepatocytes. This was evidenced by a significant increase in P450-mediated metabolism of testosterone in co-culture compared to monoculture. These studies highlight the importance of parenchymal-stromal cell interactions in retaining metabolic function (Thomas et al., 2006).

Isolated organ cultures, including liver spheroids and artificial liver constructs, mimic some of the histological features of the liver as well as permit interactions between different cell types normally present in the liver. Liver slices, consisting of multiple differentiated cell types also provide simulated cell-cell and cell-matrix interactions. However cellular viability is difficult to maintain in liver slices and is restricted to organ-specific interactions (Knight, 2008).

Toxic metabolites produced in the liver constitute a significant proportion of all toxins. For most compounds, however, the degree of toxicity resulting from metabolic activation is unknown.

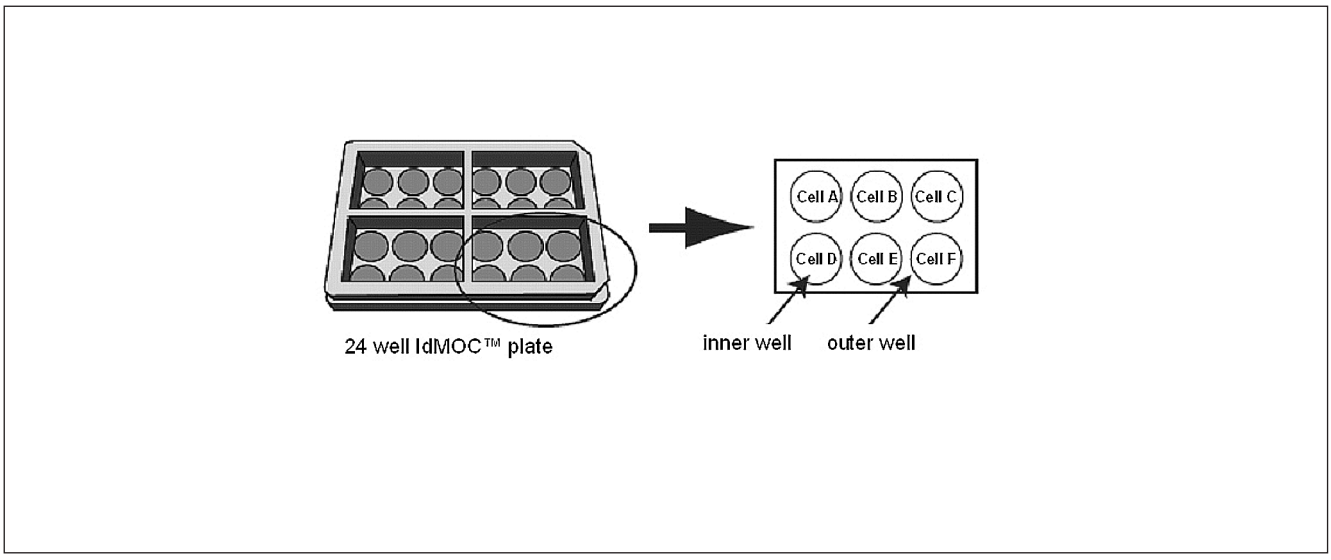

Fig: 1: Diagrammatic representation of a 24 well IdMOC plate
Magnification of a 3x2 array is shown to contain 6 cell types (A-F) in discrete inner wells. The outer well is flooded with a universal medium so that all cell types are in contact with the overlying medium. Test material is added to the universal medium for application to all cell types. Gentle swirling facilitates uniform distribution of test material and metabolites.

Potential toxic metabolites generated in the liver are likely to exert their effects in distal non-hepatic organs; hence, there is a need for co-culturing metabolically competent cells, hepatocytes with non-hepatic target cells from distal organs, followed by assessment of metabolite activity and toxicity (Knight, 2008; Li, 2008). A novel co-culture method, termed "Integrated Discrete Multi Organ Co-culture" provides a means to study cell-cell interactions and to assess toxicity in the presence of multiple cell types, including metabolically competent cells (Li, et al., 2004, 2012; Li, 2004, 2007b, 2009).

5 Integrated discrete Multi-Organ Co-culture (IdMOC)

Developed for the evaluation of xenobiotic toxicity, this model uses a wells-in-a-well concept (Li, 2008). The IdMOC system consists of a unique cell culture plate that contains multiple inner wells embedded in an outer, larger chamber (see Fig. 1). Multiple cell types are first individually cultured in the inner wells using media optimized for each cell type. An advantage of this system is that individual cell types, such as hepatocytes, can be cultured in a sandwich configuration with selective media for optimal expression and activity of metabolizing enzymes. On the day of experimentation, the individual media are removed and the outer chamber is filled with a proprietary universal medium. This floods the inner wells and allows well-to-well communication via the overlying medium. The test material or toxicant is added to the flooding/overlying media providing simultaneous and uniform distribution to all cell types. After incubation of the test material (typically 24-48 h), the overlying medium can be analyzed for overall metabolism of the test material, and individual cell types can be processed for the quantification of associated test material and specific biologic responses such as proliferation, apoptosis, cell cycle arrest, gene expression, and cytokine production.

The IdMOC model is a simple experimental system structured in a 6, 24, or 96-well format and hence is conducive to high throughput techniques. It can be integrated with most common assay platforms that use fluorescence, luminescence, and histochemical analyses, and do not require any specialized equipment (Li, 2008). Applications of this system are highlighted below.

5.1 Detection of organ-selective toxicity with IdMOC

Proof-of principle was established in 2004 when Li et al. simultaneously added different concentrations of tamoxifen to IdMOC plates containing cultures of MCF-7, a human mammary cancer cell line, and five normal primary human cells, namely hepatocytes, aortic endothelial cells, astrocytes, small airway epithelial cells, and renal proximal tubule cells (Li et al., 2004). It was shown that at concentrations of 100 μM the estrogen receptor (ER) antagonist tamoxifen was cytotoxic to ER-positive MCF-7 cells with reduced effects on normal cells. This is in keeping with *in vivo* (Jordan et al., 1980) and clinical data (Hackshaw et al., 2011) that supports the use of tamoxifen as anti-breast cancer therapy. Interestingly, hepatocytes were the most resistant to tamoxifen toxicity in this work (Li et al., 2004).), suggesting detoxification due to metabolic activity.

A similar study monitoring the cytotoxic effects of bisphenol A is presented in Figure 2. Bisphenol A (BPA) is a high production chemical used primarily in the production of polycarbonate plastic and epoxy resins. Polycarbonate plastic is used in reusable food

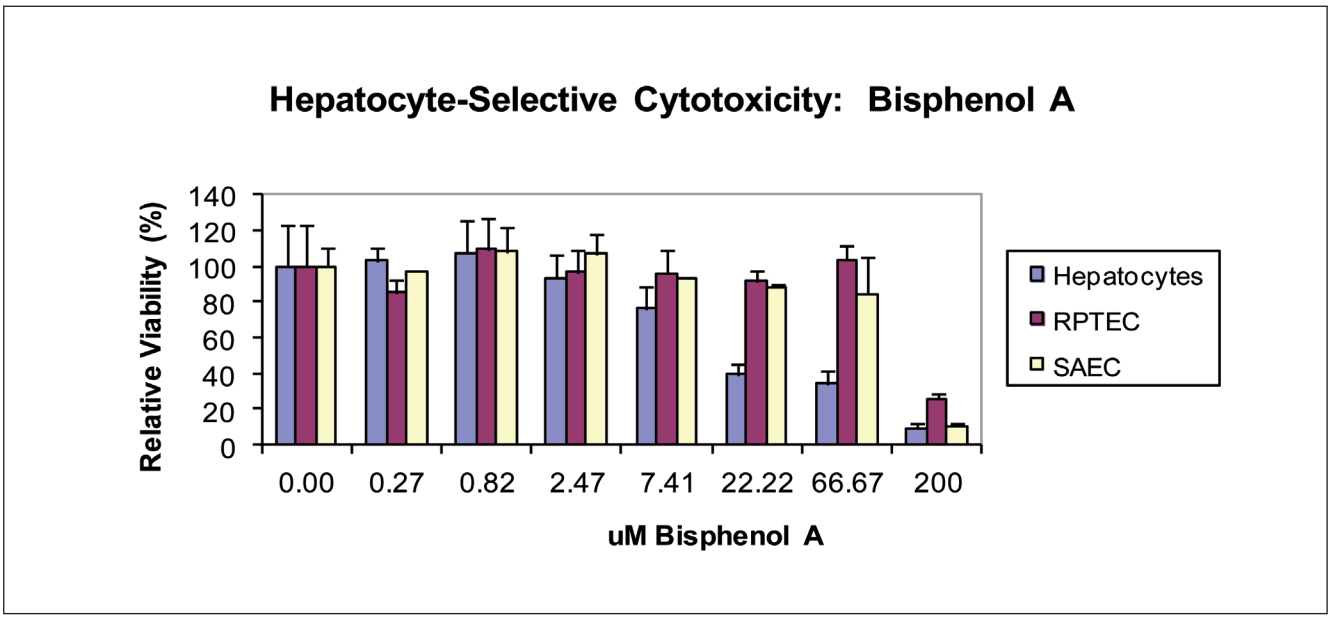

Fig. 2: Bisphenol A cytotoxicity towards human hepatocytes, renal proximal tubule epithelial cells (RPTEC) and small airway epithelial cells (SAEC)

Varying concentrations of Bisphenol A were added to the outer chamber containing 2 wells of each cell type in a 3x2 array and treated for 48 h. Cellular ATP content was estimated as per manufacturer's recommendations (Promega, Inc. Madison, WI) and used as a measure of viability. Relative viability is expressed as percent of DMSO control. Results shown are mean values ±SD.

and drink containers while epoxy resins are used as protective liners in metal cans. Hence, exposure to BPA is likely to occur by consumption of foods and beverages that have contacted these materials (Le et al., 2008). In this study, human hepatocytes, renal proximal tubule cells, and small airway epithelial cells were treated simultaneously with varying degrees of BPA. As shown in Figure 2, BPA was toxic to all three cell types at high concentrations of 200 μM. However, at lower concentrations of 22.2 and 66.7 μM selective toxicity towards hepatocytes was observed. Hepatotoxicity of BPA has been reported both *in vivo* and *in vitro* using microsomal activation systems and has been attributed to the formation of DNA adducts in the liver. This effect was attenuated by known inhibitors of CYP450 enzymes, indicating bioactivation by hepatic metabolizing enzymes (Atkinson and Roy, 1995). Other possible mechanisms of toxicity include generation of reactive oxygen species and oxidative stress (Kovacic, 2010), as well as pronounced effects on the estrogen receptor (Li, 2008; Kurosawa et al., 2002).

IdMOC, in addition to being a multi-organ model, can serve also as a single organ model (Kurosawa et al., 2002). Co-culture of three human pulmonary cell types was established using normal bronchial epithelial cells (NHBE), small airway epithelial cells (SAEC), and human microvascular endothelial cells (HMVEC), and it was treated with increasing doses of different cigarette smoke condensates. Flue-cured tobacco was found to be the most cytotoxic compared to commercial "light" and "full flavored" cigarettes, Burley tobacco, or commercial "filter"-containing cigarettes. These results were comparable to those observed in assays using human lymphoblastoid (TK-6), Balb-c3T3 or Chinese Hamster Ovary (CHO) cells. Increased proliferation, however, was detected at low concentrations of flue-cured tobacco and "light" cigarette blends only in the IdMOC model (Richter et al., 2010). In light of the fact that cigarette smoking has been associated with lung cancer (Wynder and Hoffmann, 1976), these results are physiologically relevant. It is also apparent from these studies that the use of multiple cell types in a single experiment increases the likelihood of detecting cell-type specific toxicity, and overcoming the limitation of choosing only one cell type for an experiment.

5.2 Detection of metabolic activation in IdMOC

IdMOC can be used to detect bioactivation of a parent compound to stable, diffusible, toxic metabolites by co-culturing a metabolically competent cell type (e.g., hepatocytes) with a metabolically incompetent culture (e.g., CHO cells; mouse 3T3 fibroblasts). Cyclophosphamide, a compound that requires metabolic activation to exert its cytotoxic effects, is found to cause toxicity to the metabolically incompetent cells only when co-cultured with hepatocytes (Li, 2008; Li et al., 2012). The studies confirm a major application of the IdMOC system, namely, the evaluation of the effects of hepatic metabolism on the toxicity of a xenobiotic towards nonhepatic cells.

Richter et al. incubated TK-6 cells with phenobarbital and β-naphthoflavone induced rat liver S-9 fractions for metabolic activation of cyclophosphamide and tested several smoke cell condensates in this assay. They found that not only cyclophosphamide but also flue-cured and burley tobacco, along with filter containing cigarette condensates, were biotransformed

into toxic metabolites causing a significant decrease in viability (Richter et al., 2010). Another approach is the transfer of supernatant from a chemically treated metabolically competent cell onto the target (metabolically incompetent) cell (Bruinink and Maier, 2007). While this approach has been used successfully, dilution of the toxic metabolite may prevent detection of the biological response in some cases.

5.3 Pharmacokinetic analysis in IdMOC

Cultivation of multiple cell types in the form of a cellular array in IdMOC plates opens up the possibility of performing drug metabolism studies in a more physiologically relevant manner. Drug stability and metabolite formation can potentially be evaluated after metabolism by cells from multiple organs. Drug distribution also can be estimated in multiple cell types after single or repeated exposure to the xenobiotic. Evaluation of the pharmacological responses to the test material in cells from both target and non-target organs can be used to predict drug efficacy and off-target effects (Li, 2007a).

Further technological advances such as availability of luminogenic substrates of CYP450 enzymes (Promega, Inc.) allows one to monitor enzyme activity in cells while siRNAs or dominant negative cDNAs can be used specifically to inhibit CYP pathways or other metabolizing enzymes and transporters within cells and, hence, validate their role in metabolism. Hepatocytes from commercially available knockout animals such as HRN^{TM} or PXR mice can be used to study the role of P450 metabolism or the Pregnane X receptor, respectively, in drug metabolism. Targeted disruption of specific transporters such as MDR1a (Multi-Drug Resistance gene-1a), OCT1 and 2 (Organic Cation Transporter genes Slc22a1 and Slc22a2), and MRP1 (Multiple Drug Resistance Protein 1), also are useful in validating the contribution of such proteins in drug metabolism and excretion.

5.4 Limitations of IdMOC

One drawback of the IdMOC model is that there is no actual directed flow of the overlying medium from one organ-specific cell type to another. Hence, this system cannot model certain events such as delivery of a bolus dose to the liver or differential exposure due to organ-specific blood flow. With the advent of microfluidics in biological applications, however, one can envision a closed IdMOC system where the inner wells are connected by integrated capillaries and the flow of the interconnecting medium is controlled by a pressure-driven pump. Potentially, such a development will mimic vascular blood flow and enable high-throughput *in vitro* screening alternatives for the study of xenobiotic metabolism and toxicity.

6 Conclusions

In keeping with the evolving regulatory needs of testing thousands of chemicals in a short period of time, innovative and high throughput assays for chemical testing are being developed. Replacement of animal testing with *in vitro* cellular assays, along with the use of intact human cells, has been recommended. Incorporation of metabolism into *in vitro* testing assays, as well as the use of multi-cellular models, have been largely lacking in the development of this new generation of toxicity assays. Metabolism is a key aspect of chemical toxicity. A chemical can be activated from a non-toxic parent molecule to toxic metabolites or, conversely, can be detoxified via biotransformation into non-toxic metabolites. While metabolite identification studies carried out with hepatocytes or microsomes can identify potentially toxic metabolites, subsequent studies on cellular models are required to evaluate toxicity. The presence of target cells with metabolically competent cells, as observed in the IdMOC system, would provide the means to incorporate metabolism into a toxicity assay. Such a model would be more physiologically relevant than current standards. The cultivation of multiple cell types in IdMOC plates without physical mixing allows evaluation of multiple endpoints such as proliferation, apoptosis, gene expression, and enzyme activity. Moreover, several pharmacokinetic studies such as metabolic stability, profiling, drug distribution, and evaluation of drug-drug interactions, can be studied in the presence of multiple cell types. The IdMOC model mimics interactions between different cell types, either within an organ or between multiple organs, thus providing a physiologically relevant model for evaluation of chemical toxicity.

References

Atkinson, A. and Roy, D. (1995). In vivo DNA adduct formation by bisphenol A. *Environ Mol Mutagen 26*, 60-66.

Borgert, C. J., Mihaich, E. M., Quill, T. F., et al. (2011). Evaluation of EPA's Tier 1 Endocrine Screening Battery and recommendations for improving the interpretation of screening results. *Regul Toxicol Pharmacol 59*, 397-411.

Brooks, W. J., Jarvis, M. F., and Wagner, G. C. (1989). Astrocytes as a primary locus for the conversion MPTP into MPP+. *J Neural Transm 76*, 1-12.

Bruinink, A. and Maier, P. (2007). Identification of neurotoxic chemicals in cell cultures. *ALTEX 24, Spec Issue*, 22-25.

Castell, J. V., Donato, M. T., and Gomez-Lechon, M. J. (2005). Metabolism and bioactivation of toxicants in the lung. The in vitro cellular approach. *Exp Toxicol Pathol 57, Suppl 1*, 189-204.

Cohen, A. K. (2011). The implementation of REACH: initial perspectives from government, industry, and civil society. *Int J Occup Environ Health 17*, 57-62.

Dix, D. J., Houck, K. A., Martin, M. T., et al. (2007). The ToxCast program for prioritizing toxicity testing of environmental chemicals. *Toxicol Sci 95*, 5-12.

Elliott, N. T. and Yuan, F. (2011). A review of three-dimensional in vitro tissue models for drug discovery and transport studies. *J Pharm Sci 100*, 59-74.

Firestone, M., Kavlock, R., Zenick, H., and Kramer, M. (2010). The U.S. Environmental Protection Agency strategic plan

for evaluating the toxicity of chemicals. *J Toxicol Environ Health B Crit Rev 13*, 139-162.

Gerlach, M., Riederer, P., Przuntek, H., and Youdim, M. B. (1991). MPTP mechanisms of neurotoxicity and their implications for Parkinson's disease. *Eur J Pharmacol 208*, 273-286.

Hackshaw, A., Roughton, M., Forsyth, S., et al. (2011). Long-term benefits of 5 years of tamoxifen: 10-year follow-up of a large randomized trial in women at least 50 years of age with early breast cancer. *J Clin Oncol 29*, 1657-1663.

Hewitt, N. J., Lechon, M. J., Houston, J. B., et al. (2007). 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 39*, 159-234.

Jordan, V. C., Allen, K. E., and Dix, C. J. (1980). Pharmacology of tamoxifen in laboratory animals. *Cancer Treat Rep 64*, 745-759.

Judson, R. S., Houck, K. A., Kavlock, R. J., et al. (2010). In vitro screening of environmental chemicals for targeted testing prioritization: the ToxCast project. *Environ Health Perspect 118*, 485-492.

Kavlock, R. J., Austin, C. P., and Tice, R. R. (2009). Toxicity testing in the 21st century: implications for human health risk assessment. *Risk Anal 29*, 485-487, discussion 492-487.

Kavlock, R. and Dix, D. (2010). Computational toxicology as implemented by the U.S. EPA: providing high throughput decision support tools for screening and assessing chemical exposure, hazard and risk. *J Toxicol Environ Health B Crit Rev 13*, 197-217.

Kern, A., Bader, A., Pichlmayr, R., and Sewing, K. F. (1997). Drug metabolism in hepatocyte sandwich cultures of rats and humans. *Biochem Pharmacol 54*, 761-772.

Kienhuis, A. S., Wortelboer, H. M., Hoflack, J. C., et al. (2006). Comparison of coumarin-induced toxicity between sandwich-cultured primary rat hepatocytes and rats in vivo: a toxicogenomics approach. *Drug Metab Dispos 34*, 2083-2090.

Knight, A. (2008). Non-animal methodologies within biomedical research and toxicity testing. *ALTEX 25*, 213-231.

Knudsen, T. B., Houck, K. A., Sipes, N. S., et al. (2011). Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets. *Toxicology 282*, 1-15.

Kovacic, P. (2010). How safe is bisphenol A? Fundamentals of toxicity: metabolism, electron transfer and oxidative stress. *Med Hypotheses 75*, 1-4.

Kurosawa, T., Hiroi, H., Tsutsumi, O., et al. (2002). The activity of bisphenol A depends on both the estrogen receptor subtype and the cell type. *Endocr J 49*, 465-471.

Lao, Y., Yu, N., Kassie, F., et al. (2007). Analysis of pyridyloxobutyl DNA adducts in F344 rats chronically treated with (R)- and (S)-N'-nitrosonornicotine. *Chem Res Toxicol 20*, 246-256.

Le, H. H., Carlson, E. M., Chua, J. P., and Belcher, S. M. (2008). Bisphenol A is released from polycarbonate drinking bottles and mimics the neurotoxic actions of estrogen in developing cerebellar neurons. *Toxicol Lett 176*, 149-156.

Li, A. P., Lu, C., Brent, J. A., et al. (1999a). 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 121*, 17-35.

Li, A. P., Hartman, N. R., Lu, C., et al. (1999b). Effects of cytochrome P450 inducers on 17alpha-ethinyloestradiol (EE2) conjugation by primary human hepatocytes. *Br J Clin Pharmacol 48*, 733-742.

Li, A. P. (2004). In vitro approaches to evaluate ADMET drug properties. *Curr Top Med Chem 4*, 701-706.

Li, A. P., Bode, C., and Sakai, Y. (2004). 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 150*, 129-136.

Li, A. P. (2007a). Human hepatocytes: isolation, cryopreservation and applications in drug development. *Chem Biol Interact 168*, 16-29.

Li, A. P. (2007b). Human-based in vitro experimental systems for the evaluation of human drug safety. *Curr Drug Saf 2*, 193-199.

Li, A. P. (2008). In vitro evaluation of human xenobiotic toxicity: scientific concepts and the novel integrated discrete multiple cell co-culture (IdMOC) technology. *ALTEX 25*, 43-49.

Li, A. P. (2009). The use of the Integrated Discrete Multiple Organ Co-culture (IdMOC) system for the evaluation of multiple organ toxicity. *Altern Lab Anim 37*, 377-385.

Li, A. P., Uzgare, A., and Sakai, Y. (2012). Definition of metabolism-dependent xenobiotic toxicity with co-cultures of human hepatocytes and mouse 3T3 fibroblasts in the novel integrated discrete multiple organ co-culture (IdMOC) experimental system: Results with model toxicants aflatoxin B1, cyclophosphamide and tamoxifen. *Chem Biol Interact 199,* 1-8.

Locke, P. A. and Bruce Myers, D., Jr. (2010). Implementing the National Academy's Vision and Strategy for Toxicity Testing: opportunities and challenges under the U.S. Toxic Substances Control Act. *J Toxicol Environ Health B Crit Rev 13*, 376-384.

Mace, K., Bowman, E. D., Vautravers, P., et al. (1998). Characterisation of xenobiotic-metabolising enzyme expression in human bronchial mucosa and peripheral lung tissues. *Eur J Cancer 34*, 914-920.

NRC (2007). *Toxicity Testing in the 21st Century: A Vision and Strategy*. Washington DC, USA: National Academies Press.

Richter, P. A., Li, A. P., Polzin, G., and Roy, S. K. (2010).

Cytotoxicity of eight cigarette smoke condensates in three test systems: comparisons between assays and condensates. *Regul Toxicol Pharmacol 58*, 428-436.

Rotroff, D. M., Beam, A. L., Dix, D. J., et al. (2010). Xenobiotic-metabolizing enzyme and transporter gene expression in primary cultures of human hepatocytes modulated by ToxCast chemicals. *J Toxicol Environ Health B Crit Rev 13*, 329-346.

Schmidt, C. W. (2009). TOX 21: new dimensions of toxicity testing. *Environ Health Perspect 117*, A348-353.

Schoeters, G. (2010). The REACH perspective: toward a new concept of toxicity testing. *J Toxicol Environ Health B Crit Rev 13*, 232-241.

Springate, J. and Taub, M. (2007). Ifosfamide toxicity in cultured proximal renal tubule cells. *Pediatr Nephrol 22*, 358-365.

Thomas, R. J., Bennett, A., Thomson, B., and Shakesheff, K. M. (2006). Hepatic stellate cells on poly(DL-lactic acid) surfaces control the formation of 3D hepatocyte co-culture aggregates in vitro. *Eur Cell Mater 11*, 16-26, discussion 26.

Uppstad, H., Ovrebo, S., Haugen, A., and Mollerup, S. (2010). Importance of CYP1A1 and CYP1B1 in bioactivation of benzo[a]pyrene in human lung cell lines. *Toxicol Lett 192*, 221-228.

Walsh, A. A., Hsieh, D. P., and Rice, R. H. (1992). Aflatoxin toxicity in cultured human epidermal cells: stimulation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Carcinogenesis 13*, 2029-2033.

Wynder, E. L. and Hoffmann, D. (1976). Tobacco and tobacco smoke. *Semin Oncol 3*, 5-15.

Zordoky, B. N., Anwar-Mohamed, A., Aboutabl, M. E., and El-Kadi, A. O. (2011). Acute doxorubicin toxicity differentially alters cytochrome P450 expression and arachidonic acid metabolism in rat kidney and liver. *Drug Metab Dispos 39*, 1440-1450.

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