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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

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Abstract

In vitro assays involving primary cells are used routinely to evaluate organ-specific toxic effects, for instance, the use of primary hepatocytes to evaluate hepatotoxicity. A major drawback of an in vitro system is the lack of multiple organ interactions as observed in a whole organism. A novel cell culture system, the integrated discrete multiorgan cell culture system (IdMOC), is described here. The IdMOC is based on the "wells within a well" concept, consisting of a cell culture plate with larger, containing wells, within each of which are multiple smaller wells. Cells from multiple organs can be cultured initially in the small wells (one organ per well, each in its specialized medium). On the day of toxicity testing, a volume of drug-containing medium is added to the containing well to flood all inner wells, thereby interconnecting all the small wells. After testing, the overlying medium is removed and each cell type is evaluated for toxicity using appropriate endpoints. We report here the application of IdMOC in the evaluation of the cytotoxicity of tamoxifen, an anticancer agent with known human toxicity, on primary cells from multiple human organs: liver (hepatocytes), kidney (kidney cortical cells), lung (small airway epithelial cells), central nervous system (astrocytes), blood vessels (aortic endothelial cells) as well as the MCF-7 human breast adenocarcinoma cells. IdMOC produced results that can be used for the quantitative evaluation of its anticancer effects (i.e., cytotoxicity towards MCF-7 cells) versus its toxicity toward normal organs (i.e., liver, kidney, lung, CNS, blood vessels).

Keywords: In vitro toxicity assay; Anticancer agents; Tamoxifen; Hepatocytes; Renal tubule epithelial cells; Astrocytes; Small airway epithelial cells; Endothelial cells; Hepatotoxicity; Neurotoxicity; Pulmonary toxicity; Vascular toxicity; Renal toxicity; Co-culturing

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1. Introduction

Technologies for culturing primary cells from human and animal organs have been developed and have long been applied toward the evaluation of drug toxicity. Human primary cells are especially important as experimental systems for the prediction of human-specific drug properties [1–4]. Of the various human primary cell systems, human hepatocytes may be the most useful, as the cells retain human-specific metabolism pathways [4–6] and transporters [7]. Human hepatocytes are now used routinely for the evaluation of human drug properties including drug metabolism [1–6], drug–drug interactions [7–12], and drug toxicity [13–16].

A major drawback of in vitro systems is that each cell type, representing a single organ, is evaluated in isolation with no interaction with cells from other organs. In vivo, multiple organ interaction are important aspects of drug toxicity, for example, metabolites produced in one organ (e.g., liver) may cause damage to a different organ (e.g., heart).

An in vitro system allowing multiple organ interactions would be a desirable system, providing a more relevant model for the prediction of in vivo drug toxicity. This manuscript represents the first description of a novel cell culture system that allows multiple organ interactions—the integrated discrete multiple organ culture (IdMOC) system (patent pending [17]). We report here the comparative cytotoxicity (using ATP content as endpoint) of an anticancer agent, tamoxifen, toward normal human primary cells, hepatocytes; kidney proximal tubule cells; small airway epithelial cells; aortic endothelial cells and astrocytes; and a human tumor cell line, the MCF-7 human breast adenocarcinoma cells.

2. Materials and methods

2.1. IdMOC

The IdMOC plates were manufactured by AP-Sciences Inc., Baltimore, Maryland. The plates were modified from six-well tissue culture plates (well diameter of 35 mm; Corning Inc., New York), with seven wells of a diameter of 8 mm and a height of 0.8 mm inside each well. The inner wells have a capacity of approximately 100 uL. The concept of IdMOC is presented in Fig. 1.

2.2. Human cells and culturing media

Cryopreserved human hepatocytes (lot HH227) were obtained from Tissue Transformation Technologies Inc. (Edison, New Jersey). This lot of hepatocytes was chosen because of its high attachment efficiency (approximately 80%). Human renal proximal tubule cells (RPTC), human small airway epithelial cells (SAEC), human aortic endothelial cells (HAEC), and normal human astrocytes were obtained from Cambrex, Walkersville, Maryland. MCF-7 cells were obtained from Sitek Laboratories (Rockville, Maryland). The media used for each cell type were: Li's Universal Medium (APSciences, Baltimore, Maryland) for hepatocytes; and the following media from Cambrex:

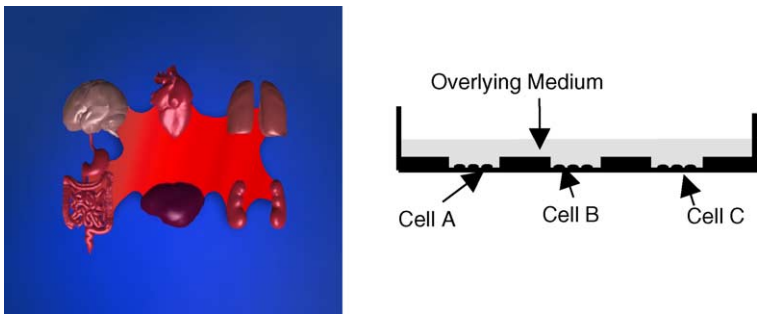

Fig. 1. The human body is conceptualized here as multiple organs connected by blood, the principle of the IdMOC system (left panel). A cross-section (right panel) of the IdMOC plate is shown demonstrating the culturing of multiple cell types (cells A–C) as physically separated cultures in different wells but interconnected by a common medium. The IdMOC thereby mimics an animal in vivo, with multiple organs as physically separated entities connected by a common fluid (blood in vivo; the overlaying medium in the IdMOC).

REGM (renal proximal tubule cells), SAGM (small airway epithelial cells), EGM-2 (aortic endothelial cells), AGM (astrocytes).

2.3. Cell culture and drug treatment

The primary cells from Cambrex were supplied to our laboratory as dividing cultures in 25-cm^2 flasks. The cells were subcultured into 75-cm^2 flasks after approximately 4 days (75% confluent). The cells were cultured for another 7 days before they were trypsinized and plated for cytotoxicity studies. The cells were plated into IdMOC plates or 96-well plates 1 day before drug treatment. For the IdMOC plates, 10,000 cells were plated into each inner well, using 80 uL of medium specific for each cell type as described earlier. Human hepatocytes were thawed (viability approximately 82% based on trypan blue) and plated at 100,000 cells per well in 80 uL of medium. The wells receiving human hepatocytes were coated with Vitrogen (Cohesion Inc., Palo Alto, California). The cells were similarly plated into 96-well plates (Corning Inc., New York). The cells were cultured in an incubator kept at 37 °C, and a highly humidified atmosphere of 95% air and 5% carbon dioxide for approximately 24 h before treatment.

2.4. Drug treatment

Tamoxifen (Sigma-Aldrich Inc., St. Louis, Missouri) was initially dissolved in methanol as a 100× stock solution. On the day of treatment, media were removed from all inner wells of the IdMOC and replaced with 2.5 mL of Eagle's minimal essential medium (MEM; Invitrogen, Carlsbad, California) containing various concentrations of tamoxifen. Treatment was performed in triplicate for an incubation period of approximately 24 h. After the treatment period, the cells in individual wells were processed for viability determination.

2.5. Viability determination

Cellular ATP content was measured as an index of viability, using ATPlite®-M luminescence assay system (Perkin-Elmer Inc., Boston, Massachussets). The treatment media were removed. Each of the individual wells (inner wells)was washed twice with isotonic phosphate buffered saline (pH 7.4), followed by the addition of 50 ul of mammalian cell lysis solution (Perkin-Elmer Inc.). The cell lysate was combined with the substrate solutions of the ATPlite® system and analyzed for chemiluminescence using a Wallac Victor 1420 Multilabel Counter (Wallac, Turku, Finland). Results are expressed as relative viability as follows:

$$\text{relative viability} = \frac{\text{ATP(treatment)}}{\text{ATP(control)}} \times 100\%$$

2.6. Data analysis

Mean and standard error of the mean were determined using Microsoft Office 2000 Excel software (Microsoft Inc., Redman, Washington). Data plotting and curve fitting were performed using the Kaleidagraph 3.6 software (Synergy Software, Reading, PA). The concentrations of tamoxifen to cause 50%, 90% and 99% decreases in survival (EC50, EC90, and EC99, respectively) are calculated based on the following equation:

$$\log S = a + b(X);$$

where S, relative viability; X, tamoxifen concentration; and a and b represent Y intercept and slope of the fitted curve, respectively.

Therapeutic index values are calculated by dividing the EC values of each normal cell type by that for MCF-7 cells as follows:

$$\text{TI(EC50)} = \frac{\text{EC50(normal cells)}}{\text{EC50(MCF7)}}$$

$$\text{TI(EC90)} = \frac{\text{EC90(normal cells)}}{\text{EC90(MCF7)}}$$

$$\text{TI(EC99)} = \frac{\text{EC99(normal cells)}}{\text{EC99(MCF7)}}$$

3. Results

3.1. Cell morphology

The morphology of the cells was evaluated using phase contrast microscopy. The cells cultured in IdMOC exhibited cell morphology similar to that observed in mass cultures of individual cell types. There was no apparent alteration in cell morphology after the

24-h co-culturing period in the solvent control-treated IdMOC cultures. Morphologically, dose-dependent cytotoxicity of tamoxifen could be discerned after the 24-h treatment period with tamoxifen, with the appearance of rounded cells at the toxic concentrations (data not shown).

3.2. Cytotoxicity

Tamoxifen caused dose-dependent cytotoxicity, as indicated by a dose-dependent decrease in cellular ATP content, in all six cell types evaluated. The survival curves after drug treatment were plotted as survival versus drug concentration (Fig. 2), and as log(survival)

Table 1
Calculated concentrations (in uM) of tamoxifen causing a reduction in viability of each cell type by 50% (EC50), 90% (EC90), and 99% (EC99)

	Hepatocytes	HAEC	Astrocytes	RPTC	SAEC	MCF-7
EC50	58.4	24.1	31.9	34.9	11.4	19.9
EC90	204.2	137.9	105.9	115.5	121.8	62.5
EC99	412.9	300.6	211.8	230.9	279.7	123.5

The cells co-cultured in the IdMOC were human hepatocytes (hepatocytes), human aortic endothelial cells (HAEC), human astrocytes (astrocytes), human renal proximal tubule epithelial cells (RPTC), human small airway epithelial cells (SAEC), and human breast adenocarcinoma cells (MCF-7).

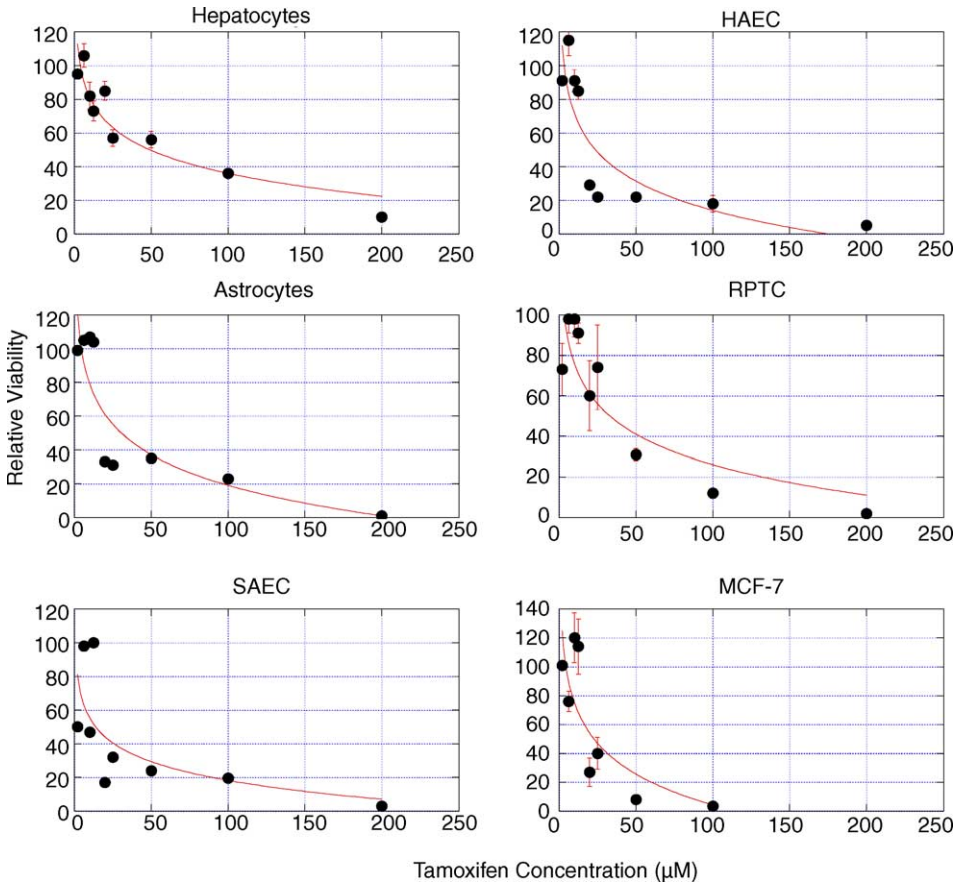

Fig. 2. Relative viability (*Y*-axis; percentage of control) vs. tamoxifen concentration (*X*-axis; uM) for the multiple cell types. The error bars shown represent standard errors of triplicate treatments. The curves were fit using a logarithmic curve fitting function. The cells co-cultured in the IdMOC were human hepatocytes (hepatocytes), human aortic endothelial cells (HAEC), human astrocytes (astrocytes), human renal proximal tubule epithelial cells (RPTC), human small airway epithelial cells (SAEC), and human breast adenocarcinoma cells (MCF-7). {Question: I know the standard error is smaller than the standard deviation; but in this case, is it statistically correct to show the standard errors?}

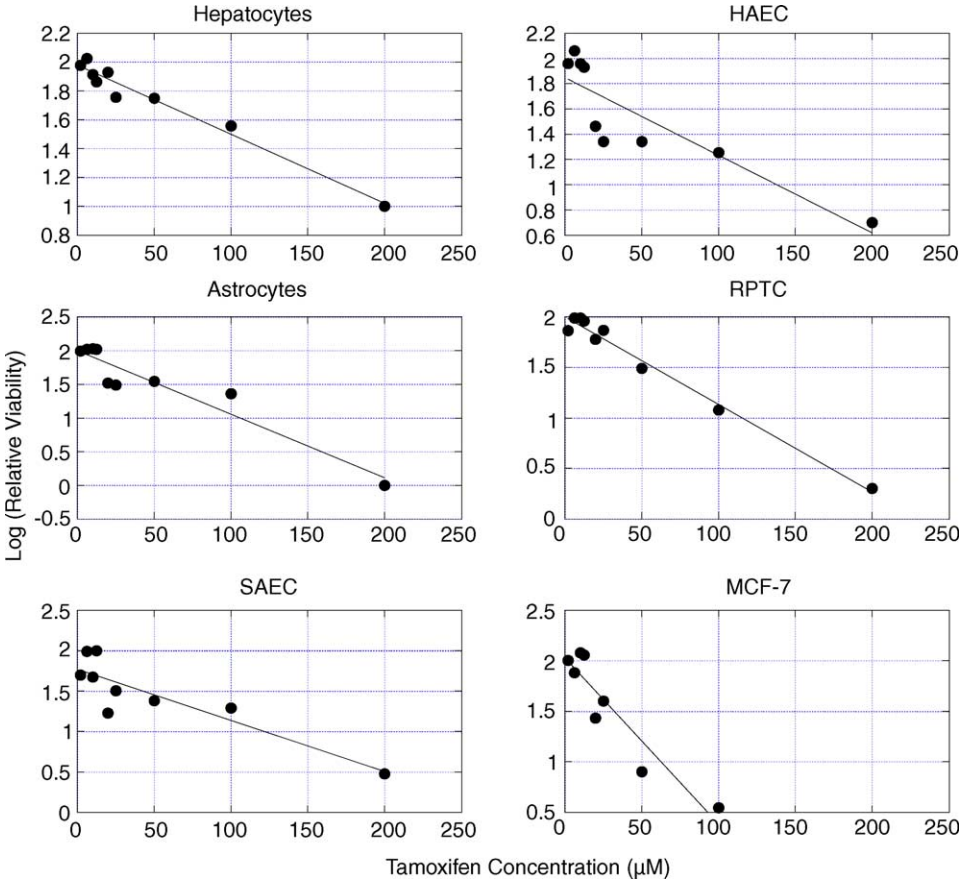

Fig. 3. Logarithm of relative viability (*Y*-axis; log(relative viability)) vs. tamoxifen concentration (*X*-axis; uM). The linearity of the plots demonstrate the ability of IdMOC to generate survival curves as expected from the known relationship between cell survival and toxicant concentration. The cells co-cultured in the IdMOC were human hepatocytes (hepatocytes), human aortic endothelial cells (HAEC), human astrocytes (astrocytes), human renal proximal tubule epithelial cells (RPTC), human small airway epithelial cells (SAEC), and human breast adenocarcinoma cells (MCF-7).

Table 2
Calculated therapeutic index values of tamoxifen for the treatment of the MCF-7 adenocarcinoma cells vs. the multiple normal cell types based on EC50, EC90, and EC99 values

	Hepatocytes	HAEC	Astrocytes	RPTC	SAEC
TI (EC50)	2.93	1.21	1.60	1.75	0.57
TI (EC90)	3.27	2.21	1.69	1.85	1.95
TI (EC99)	3.34	2.43	1.72	1.87	2.27

The cells co-cultured in the IdMOC were human hepatocytes (hepatocytes), human aortic endothelial cells (HAEC), human astrocytes (astrocytes), human renal proximal tubule epithelial cells (RPTC), human small airway epithelial cells (SAEC), and human breast adenocarcinoma cells (MCF-7).

versus drug concentration (Fig. 3). The calculated EC50, EC90, and EC99 values are shown in Table 1. The resulting therapeutic index values are shown in Table 2.

4. Discussion

Human cell-based in vitro experimental systems represent important tools for the evaluation of mechanisms of human drug toxicity and the prediction of human toxicity of new drug entities. Human hepatocytes, for instance, are now routinely used in the evaluation of drug metabolism, drug–drug interactions, and drug

toxicity [1–15]. Other differentiated cell systems that have been applied towards the evaluation of drug toxicity include renal proximal tubule epithelial cells for the evaluation of nephrotoxic agents (e.g., [18–20]); endothelial cell cultures for the evaluation of vascular toxins [21–23]; astrocytes for the evaluation of neurotoxins [24–26]; and small airway epithelial cells for the evaluation of pulmonary toxicants [27–29]. This report represents the first description of a novel experimental system, the IdMOC, which allows the treatment of multiple cell types with one single overlying medium (integrated) while each cell type is cultured physically separated (discrete) from each other. The human differentiated cells used were primary cultures of human hepatocytes, aortic endothelial cells, astrocytes, renal proximal tubule epithelial cells, and small airway epithelial cells, representing the liver, vascular vessels, central nervous system, kidney, and lung, respectively. As the toxicant studied, tamoxifen, is used in the treatment of breast cancer, we also include the human breast adenocarcinoma cell line, MCF-7, in the study.

The interconnection of the multiple cell types in the IdMOC by a common medium has two advantages: (1) it ensures that all cell types are treated under virtually identical conditions, thereby avoiding differences in response due to experimental artefacts; (2) it allows metabolites from one cell type to interact with a different cell type. Metabolites generated by hepatocytes, for instance, can diffuse and interact with nonhepatic cells. The physical separation of the cell types (each in an individual well) mimics the in vivo condition, where organs are physically separated from each other. The IdMOC, therefore, represents an in vitro system resembling a whole organism with multiple organs, with the organs physically separated from each other but connected by a common medium (the overlying medium in the IdMOC versus blood in vivo). The IdMOC, therefore, represents a new generation of in vitro experimental system, taking into account the complexity of multiple organ interactions.

The results show that the IdMOC allows the quantitative evaluation of the cytotoxicity of a chemical towards multiple cell types. Dose-dependent cytotoxicity, as indicated by cellular ATP content, was observed for all cell types used. The linear plots of log viability versus tamoxifen drug concentration (Fig. 3) for all six cell types co-culture in the IdMOC indicate that results obtained are consistent with the theorectical relationship between dose and effects, thereby suggesting that this novel experimental systemis appropriate for the evaluation of cytotoxicity.

The results illustrate an important application of IdMOC: evaluation of the comparative toxicity of an agent toward multiple cell types. As expected, the MCF-7 cells were the cell type most sensitive to the cytotoxicity of tamoxifen. The EC50 values for hepatocytes (58.4 uM), HAEC (24.1 uM), astrocytes (31.9 uM), and RPTC (34.9 uM) were higher than that of MCF-7 (19.9 uM). SAEC had an EC50 (11.4 uM) lower than that for MCF-7. The EC90 values for all normal cell types (from 105.9 uM [astrocytes] to 204.2 uM [hepatocytes]) were higher than that for MCF-7 (62.5 uM). The EC99 values also higher for the normal cells (from 211.8 uM [astrocytes] to 412.9 uM [hepatocytes]) than that for MCF-7 (123.5 uM). It is to be noted that the highest concentration used was 200 uM; calculated EC values over 200 uM are shown only for illustrative purposes. The limited solubility of tamoxifen precludes experimentation at dose levels above 200 uM.

The results show that IdMOC can be used to evaluate the toxicity of a drug towards multiple cell types. The rank ordering of the multiple cell types towards tamoxifen cytotoxicity, from the most sensitive to the most resistant, based on the different EC values are as follows:

EC50: SAEC > MCF-7 > HAEC > astrocytes > RPTC > hepatocytes
EC90: MCF-7 > astrocytes > RPTC > SAEC > HAEC > hepatocytes
EC99: MCF7 > astrocytes > RPTC > SAEC > HAEC > hepatocytes

As our study was performed using relatively low cell density (subconfluence), the results probably represent an accurate depiction of the relative cytotoxicity of the multiple cell types under conditions that allow cell division. It is commonly known that cytotoxic anticancer agents in general are cytotoxic to most dividing cells. The relative resistance of hepatocytes towards tamoxifen relative to the other cell types is probably a result of its high metabolic capacity as well as it being a nondividing cell type. Furthermore, as the treatment period used was 24 h, the results obtained probably are most applicable to the acute toxicity of tamoxifen.

Our in vitro results with IdMOC appear to be consistent with the known toxicity of tamoxifen, both in terms of the multiplicity of target organs and their relative sensitivities. The toxicity of tamoxifen towards multiple organs and tissues is a well-established phenomenon, and has been attributed to estrogen receptor-independent effects [30,31]. It is to be noted that low dose tamoxifen treatment has been associated with neurotoxicity, especially optic neuritis [32], which is consistent with our observation of the high sensitivity of astrocytes to its cytotoxicity.

The results illustrate the utility of the novel cell culture system, IdMOC, for the evaluation of drug toxicity, especially for studying the comparative cytotoxicity of an agent toward multiple cell type. It is to be emphasized in the IdMOC, the multiple co-cultured cell types are exposed to the toxicant under virtually identical conditions, thereby producing results most likely to reflect differences in sensitivity of the cell types to the toxic effects of the drug being tested. Another advantage of the IdMOC which is not addressed in this study is the multiple organ metabolism and the effects of metabolites on multiple cell types. In our laboratories, an extensive research program is underway to further characterize the IdMOC system for its application in drug development, with emphasis on drug metabolism, drug distribution, and drug toxicity.

We believe that the IdMOC represents a new generation of in vitro system for cell biology, pharmacology, and toxicology investigation. The presence of multiple organ interaction and the possibility of discrete evaluation of organ-specific effects in the IdMOC should yield information more relevant to the prediction of human in vivo drug properties.

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