THE USE OF IMMORTALIZED HEPATOCYTES IN INDUCTION STUDIES

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INTRODUCTION

Primary cultures of human hepatocytes are widely used to evaluate the cytochrome P450 (CYP) enzyme-inducing potential and/or toxicity of drug candidates. However, the availability of human hepatocytes for this purpose is limited and erratic, and there are large inter-individual differences in the magnitude of induction (due to differences in both "control" and "induced" CYP activities). Immortalized human hepatocytes, Fa2N-4, developed by Multicell Technologies, (Warwick, RI) have the potential to overcome these limitations. The cells, immortalized through transformation of human hepatocytes with SV40 T antigen, display *in vitro* cell morphology that closely resembles primary human hepatocytes (Figure 1). The Fa2N-4 cells retain many of the characteristics of primary hepatocytes, including the inducibility of multiple CYP mRNAs and enzyme activities (Mills *et al*., 2002; Morris *et al*., 2003; Czerwinski *et al*., 2003). In this study, we further characterized the ability of these cells to respond to enzyme-inducing xenobiotics. Additionally, we examined the toxicity profile of multiple enzyme inducers in primary hepatocytes and Fa2N-4 cells.

MATERIALS AND METHODS

The Fa2N-4 cells were propagated in MFE media (Multicell Technologies) on plasticware coated with Vitrogen (Cohesion Technologies, Palo Alto, CA) and maintained at 37°C, 5% CO_2, 95% humidity. Immortalized hepatocytes were grown to confluency in 24 or 96-well plates and treated with enzyme inducers for up to 6 consecutive days with daily changes of medium. All enzyme inducers were dissolved in DMSO (final concentration of solvent 0.1%, v/v). Primary human hepatocytes were isolated and cultured as described (LeCluyse *et al*., 2000, Madan *et al*., 2003). Enzymatic activities were determined by incubating the cells with CYP-specific substrates. Metabolite formation was measured by LC-MS as summarized in Table 1.

Leakage of lactate dehydrogenase (LDH) into the medium, an indicator of cellular toxicity, was measured with the Cytotoxicity Detection Kit (LDH) (Roche Diagnostics Corp., Indianapolis, IN). Another marker of toxicity, mitochondrial respiration, was determined by fluorescent monitoring of the reduction of resazurin (Alamar Blue) to resorufin.

Figure 1: Fa2N-4 cells

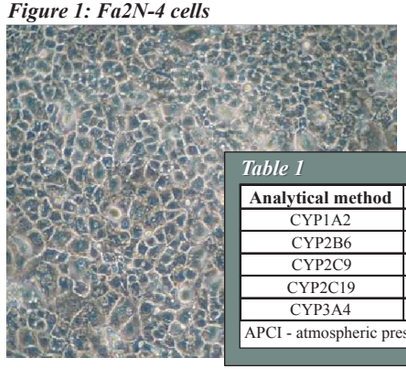


Table 1

Analytical method	Incubation time	Substrate	Metabolite	Ionizaton Mode
CYP1A2	6 hour	Phenacetin	Acetaminophen	APCI+
CYP2B6	6 hour	Bupropion	Hydroxybupropion	ESI+
CYP2C9	6 hour	Diclofenac	4′-Hydroxydiclofenac	ESI-
CYP2C19	6 hour	*S*-mephenytoin	4′-Hydroxymephenytoin	ESI-
CYP3A4	1 hour	Midazolam	1′-Hydroxymidazolam	ESI+

APCI - atmospheric pressure chemical ionization, ESI - electrospray ionization

RESULTS

1. Fa2N-4 cells metabolize CYP-specific substrates. In Fa2N-4 cells, activity of phenacetin *O*-dealkylase (CYP1A2) increased 11 fold in response to three days' treatment with 100 µM omeprazole. In response to a three-day treatment with 20 µM rifampin, the rates of midazolam 1′-hydroxylation (CYP3A4), bupropion hydroxylation (CYP2B6), and diclofenac 4′-hydroxylation (CYP2C9) increased by 4.9, 2.2, and 4.0 fold, respectively. The activity of *S*-mephenytoin hydroxylase (CYP2C19) was not induced following the treatment with 20 µM rifampin (fold induction < 2) (Figure 2).

2. A time-dependent increase in midazolam 1′-hydroxylase activity was seen in Fa2N-4 cells over the course of a six day-treatment with 20 µM rifampin, but not in the control (DMSO-treated) cells (Figure 3). On the last day of treatment CYP3A4 activity was induced 8.3 fold. A time-dependent increase in phenacetin *O*-dealkylase activity was seen in Fa2N-4 cells over the course of six day-treatment with 100 µM omeprazole (and to a much lower extent, in the control cultures). On day 5 of treatment, induction of CYP1A2 activity reached 8.6 fold, based on amount of acetaminophen formed/incubation (data not shown).

3. Fa2N-4 cells plated in 96-well plates correctly differentiated compounds that induce CYP3A4 (*e.g.* efavirenz, hyperforin) in primary hepatocytes from those that do not (*e.g.* probenecid) (Figure 4). Both toxicity markers used in this study, LDH release and reduction of resazurin, identified toxic concentration of selected xenobiotics. Fa2N-4 cells plated in 24-well plates correctly identified those compounds that induce CYP1A2 or CYP3A4 in primary hepatocytes (Figure 5).

4. Cultures of both primary and immortalized hepatocytes were exposed to several known toxicants and their effects were monitored by LDH release into the medium and by changes in mitochondrial respiration (based on resazurin reduction). The toxicity profiles of Fa2N-4 and primary hepatocytes were similar for most compounds (Table 2). Few quantitative differences in the response of the two cell types were observed. The Fa2N-4 cells were more sensitive than primary hepatocytes to hepatotoxins such as troglitazone, hyperforin, and benzo[a]pyrene, but less sensitive than the primary hepatocytes to a mitochondrial respiration inhibitor, menadione.

5. Cultures of Fa2N-4 immortalized hepatocytes, representing cell passages 32 - 47, were treated with 100 µM omeprazole or 20 µM rifampin and analyzed for induction of CYP1A2 and CYP3A4, respectively. Regardless of passage number, the cells responded consistently to the inducers by increasing the activity of both enzymes to the extent that has been observed in extensive series of primary hepatocyte cultures (Figure 6, Madan *et al*., 2003).

Figure 2: CYP induction in Fa2N-4 cells

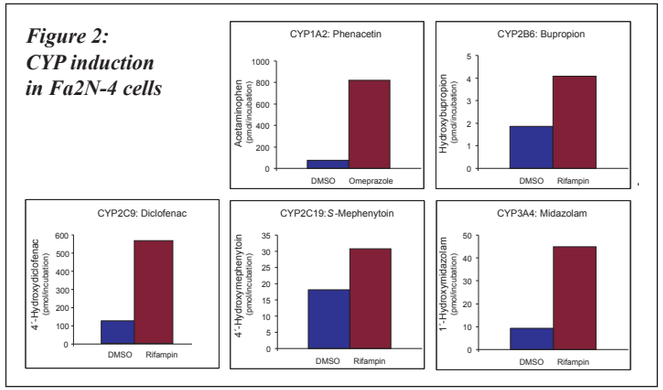
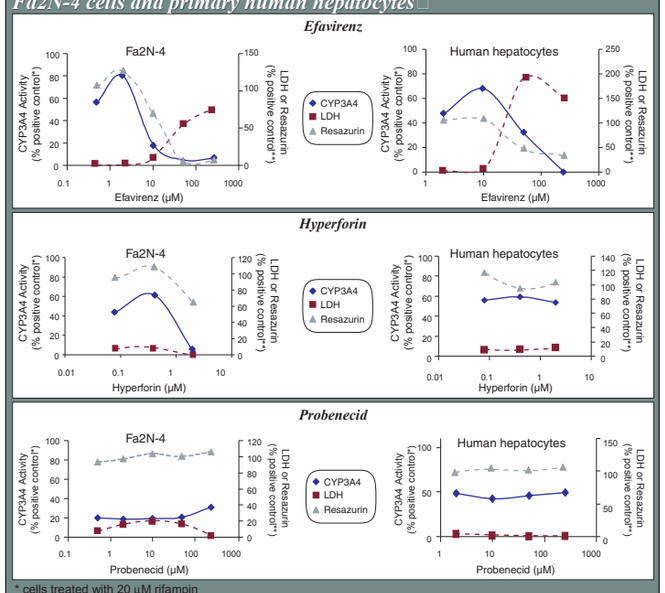


Figure 4: Effects of efavirenz, hyperforin and probenecid on Fa2N-4 cells and primary human hepatocytes



* cells treated with 20 µM rifampin
**cells treated with 0.1% v/v DMSO

Figure 6: Impact of cell passage on CYP1A2 and CYP3A4 induction in Fa2N-4 cells

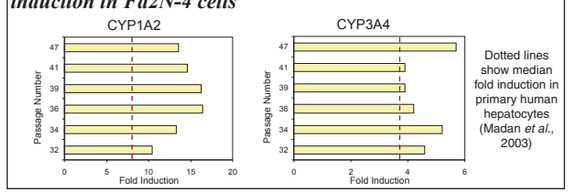


Figure 3: Time course of CYP3A4 induction in Fa2N-4 cells

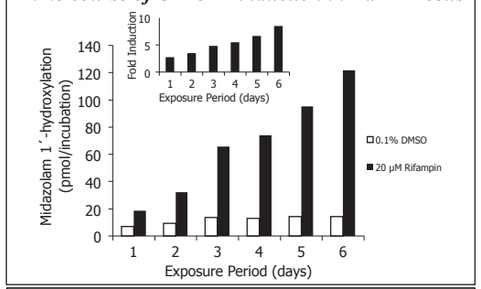
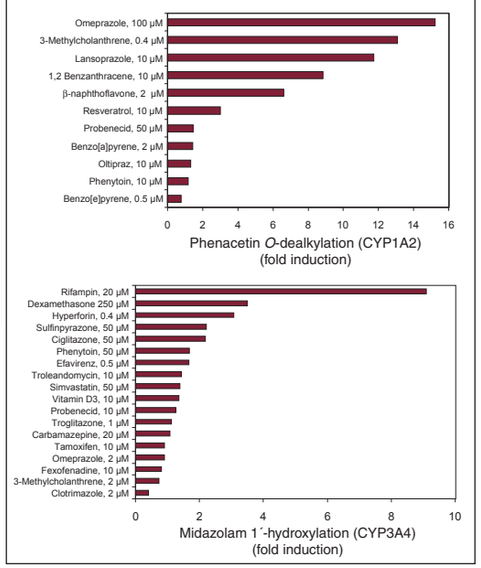


Table 2: Cellular response of Fa2N-4 cells and primary hepatocytes

Cellular response:	Compound: Non-toxic	Non-toxic	Toxic
Same	Rifampin, Phenobarbital, Phenytoin, Carbamazepine, Troleandomycin, Lansoprazole, Omeprazole	Probenecid, Felbamate, Acetaminophen, Ciglitazone, Sulfinpyrazone, Simvastatin, Fexofenadine	3-Methylcholanthrene, Methotrexate, Rotenone, Efavirenz
Different			Troglitazone (A), Benzo[a]pyrene (A), Hyperforin (A), Menadione (B)

(A) = Fa2N-4 cells more sensitive than human hepatocytes
(B) = Fa2N-4 cells less sensitive than human hepatocytes

Figure 5: Effect of enzyme inducers on CYP1A2 and CYP3A4 activity in Fa2N-4 cells



CONCLUSIONS

1. The Fa2N-4 cells respond like primary cultures of human hepatocytes to a wide range of xenobiotics. The Fa2N-4 cells respond to prototypical PXR and AhR agonists in a manner that closely resembles the magnitude of induction observed in freshly plated hepatocytes but is largely free of the variability associated with the use of primary hepatocytes.

2. Fa2N-4 cells and human hepatocytes respond similarly to several drugs and other xenobiotics, although some differences are noted. For example, Fa2N-4 cells are more sensitive than human hepatocytes to the toxic effects of troglitazone, and benzo[a]pyrene, whereas they are less sensitive than human hepatocytes to menadione.

3. The Fa2N-4 hepatocyte-based analysis of enzyme induction and cellular toxicity has been carried out in 6-, 12-, 24- and 96-well plates. Data from the different plating formats is highly reproducible. The use of 96-well plates with immortalized hepatocytes bodes well for the development of the higher throughput screening of CYP induction and toxicity in a reproducible human-based cell system.

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