#367

CYTOCHROME P450 3A4 AND 1A2 INDUCTION IN THE IMMORTALIZED HEPATOCYTE LINE, Fa2N-4, AND COMPARISON WITH PRIMARY CULTURES OF HUMAN HEPATOCYTES.

Kevin C. Lyon, Maciej Czerwinski, Martin Perry, Paul Toren* and Andrew Parkinson*

XenoTech
16825 W 116th Street Lenexa, KS 66219

ABSTRACT

Primary cultures of human hepatocytes are the gold standard for evaluating induction of cytochrome P450 (CYP) enzymes by new molecular entities. Recently, immortalized human hepatocytes, known as Fa2N-4 cells, have been shown to express CYP3A4 and CYP1A2. Additionally, these cells exhibited enzyme induction in response to treatment with agonists of nuclear receptors (Mills *et al.*, 2004). To establish the Fa2N-4 cells as a tool for determining CYP3A4 and CYP1A2 induction, we measured the cells' response to drugs previously evaluated in multiple primary hepatocyte cultures (Luo *et al.*, 2002). Midazolam 1'-hydroxylase and phenacetin *O*-dealkylase activity were monitored using LC/MS/MS methods to determine the effects of the drugs on expression of CYP3A4 and CYP1A2, respectively. Toxic effects of the compounds were monitored with lactate dehydrogenase (LDH) leakage into the culture media. The magnitude of CYP3A4 and CYP1A2 induction in Fa2N-4 cells agrees closely with primary cultures of human hepatocytes. The comparison of the CYP response in the two cell culture systems validates immortalized hepatocytes as a preclinical tool for assessment of CYP3A4 and CYP1A2 induction.

INTRODUCTION

Primary cultures of human hepatocytes are the gold standard for evaluating the induction of cytochrome P450 (CYP) enzymes by new molecular entities (NME). Unfortunately, the availability of human hepatocytes for such studies is sporadic, and their response to prototypical CYP enzyme inducers is highly variable. Alternatively, reporter gene assays are adequate for evaluating the binding of NME-activated receptors to fragments of a specific CYP promoter, which serves to identify those NMEs capable of activating the aryl hydrocarbon receptor (AhR) and pregnane X receptor (PXR). Due to their narrow specificity, the reporter gene assays cannot account for the pleiotypic response characteristic of receptor-mediated. Hepatoma cell lines, such as HepG2 and its derivatives, proposed in the past to be an adequate model to study induction of CYP enzymes, lack many hepatocyte characteristics, for which reason their applicability is limited to the evaluation of induction of the CYP1 family (Silva *et al.*, 2002). Because of the drawbacks (*e.g.* scarce availability, limited representation of in vivo processes) these systems are unsuitable for high throughput screening of the induction of multiple CYPs and other enzymes.

Recently, immortalized human hepatocytes, Fa2N-4 cells, were developed from the stable transformation of a primary culture of human hepatocytes with SV40 T-antigen (Multicell Technologies, Warwick, RI). The cells maintain hepatocyte functions such as the production of liver-specific proteins and expression and inducibility of drug metabolizing enzymes (Mills *et al.*, 2004; Lyon *et al.*, 2004; Morris *et al.*, 2003). This study compared CYP activity of immortalized Fa2N-4 hepatocytes with that of human hepatocytes in primary culture and the hepatocellular carcinoma-derived cell line, HepG2. We observed strong correlation between the induction of CYP1A2 and CYP3A4 enzymes in Fa2N-4 cells and primary cultures of human hepatocytes and demonstrated that the immortalized cells have maintained, post crisis, a stable enzyme induction phenotype over for more than 100 population doublings.

Table 1: Outline of cell-based assays to measure CYP induction

CYP enzyme	Incubation Time	Substrate	Metabolite	Ionization Mode for LC-MS
CYP1A2	6 hours	Phenacetin (100µM)	Acetaminophen	APCI+
CYP3A4	1 hour	Midazolam (100µM)	1´-hydroxymidazolam	ESI+

APCI: Atmospheric Pressure Chemical Ionization ESI: Electrospray Ionization

MATERIALS AND METHODS

For routine passaging, Fa2N-4 cells were cultured in Multi-function Enhancing (MFE™) media (Multicell Technologies, Warwick, RI) on Vitrogen-coated (Cohesion Technologies, Palo Alto, CA) polystyrene T150 cell culture flasks (Corning, Corning, NY). For enzyme induction studies, the cells were grown to confluency in Vitrogen-coated 24-well plates (Nunc, Rochester, NY). Following adaptation to cell culture conditions for 96 hr, the cells were dosed for 72 hours with daily medium changes. HepG2 cells, obtained from American Type Cell Collection (ATCC, Manassas, VA) were cultured in MEM media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, as recommended by the cell bank. Fa2N-4 cells and HepG2 cells were both plated at a concentration of 330,000 cells per well. Enzyme inducers were dissolved in DMSO. Enzymatic activities of CYP1A2 and CYP3A4 were determined by incubating the cells with the CYP-specific substrates phenacetin and midazolam, respectively. Details of the methods of measuring the formation of specific metabolites by LC-MS are summarized in Table 1. The Cytotoxicity Detection Kit (LDH) (Roche Diagnostics Corp., Indianapolis, IN) was used to determine cellular toxicity in accordance with the manufacturer's instructions.

Figure 1: Impact of cell passage on the induction of CYP1A2 and CYP3A4 in Fa2N-4 cells treated with omeprazole or rifampin, respectively

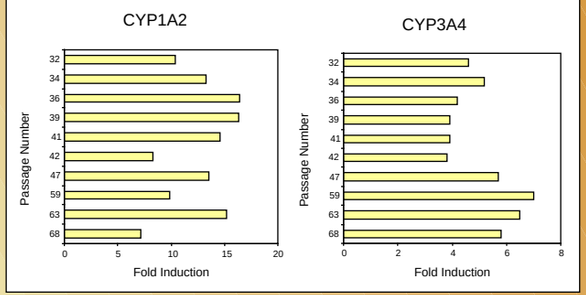


Figure 2: Constitutive CYP1A2 and CYP3A4 activity in control (DMSO-treated) Fa2N-4 cells and primary cultures of human hepatocytes

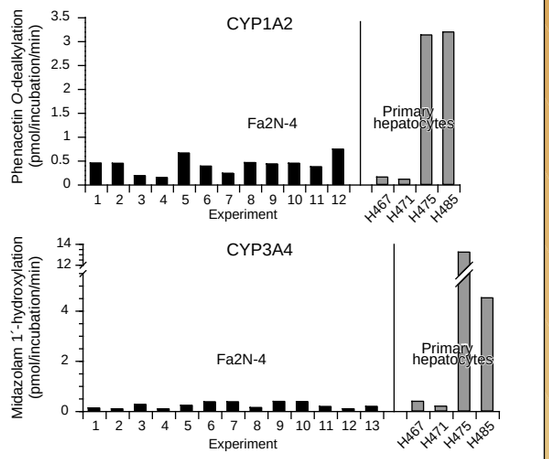


Figure 3: A comparison of constitutive and induced CYP3A4 and CYP1A2 activity in Fa2N-4 cells and HepG2 cells

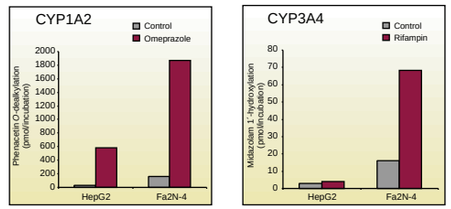
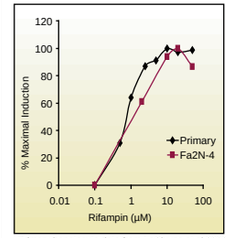


Figure 4: A comparison of the induction of CYP3A4 by rifampin in Fa2N-4 cells and primary cultures of human hepatocytes



Primary hepatocyte data from LeCluyse et al 2000

Figure 5: Comparison of CYP3A4 induction by selected inducers in Fa2N-4 cells and primary cultures of human hepatocytes

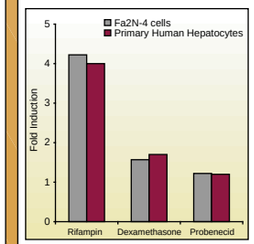


Figure 6: Induction of CYP1A2 in Fa2N-4 cells treated with various prototypical inducers. Precipitate was observed in β-naphthoflavone samples at concentrations of 50 µM or greater. 3-Methylcholanthrene and 1,2-benzanthracene were toxic at the highest concentrations.

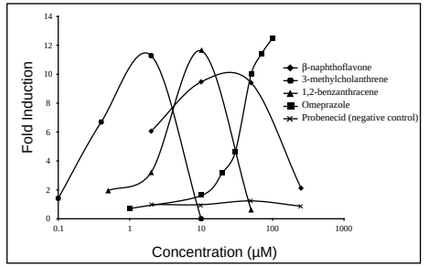
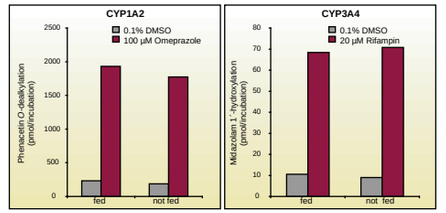


Figure 7: CYP induction Fa2N-4 cells: The MFE medium need not be changed during a weekend adaptation period



RESULTS

1. We have established a cell-based enzymatic assay to evaluate CYP induction in Fa2N-4 cells in order to document their performance over an extended period of continuous culture. The Fa2N-4 cells have been passaged at XenoTech for over 100 population doublings, post crisis, without apparent loss of CYP expression or inducibility. Fold induction of enzymatic activity of CYP1A2 and CYP3A4 averaged 4.8 and 12.0, respectively (Fig. 1).
2. Constitutive CYP1A2 and CYP3A4 activity in Fa2N-4 cells treated with DMSO (0.1%, v/v), ranged from 0.1 - 0.7 pmol of acetaminophen formed per incubation per minute and 0.1 - 0.4 pmol 1´-hydroxymidazolam formed per incubation per minute, respectively. Four randomly selected primary cultures of human hepatocytes assayed under identical conditions exhibited CYP1A2 activity of 0.1 - 3.2 pmol of acetaminophen formed per incubation per minute and CYP3A4 activity of 0.2 - 13 pmol 1´-hydroxymidazolam formed per incubation per minute (Fig. 2). The wide range of CYP activities in these four primary cultures, which is characteristic of the heterogeneity observed in a large number of human hepatocyte cultures (Madan *et al.*, 2003), is contrasted by relatively low variability observed in immortalized hepatocytes. On average, the rates of phenacetin O-dealkylation and midazolam 1´-hydroxylation in Fa2N-4 cells were 25% and 5% of the average rates in primary cultures of human hepatocytes. In contrast, the activity of HepG2 cells was only 5% and 1% of the CYP1A2 and CYP3A4 activity seen in primary cultures of human hepatocytes, respectively (Fig. 3).
3. The concentration-response curve for CYP3A4 induction by rifampin in Fa2N-4 cells is comparable to that in primary cultures of human hepatocytes, as shown in Fig. 4. Similar results were seen with the prototypical CYP3A4 inducer dexamethasone. No CYP3A4 induction was seen in with the negative control probenecid in either primary or immortalized hepatocytes (Fig. 5).
Similarly, Fa2N-4 cells were able to differentiate CYP1A2 inducers (*e.g.* β-naphthoflavone, 3-methylcholanthrene) from non-inducers (*e.g.* probenecid) (Fig. 6).
4. Cells grown at 37°C for 72 hours without feeding prior to treatment with prototypical inducers (*i.e.*, a weekend feeding) responded to inducers to the same extent as did cells that were fed every 24 hours (*i.e.*, by daily changes in the medium) (Fig. 7).

Discussion

1. Fa2N-4 is a stably transformed cell line that responds like primary cultures of human hepatocytes even after 10 months in culture (representing more than 100 post-crisis population doublings). These cells have been frozen and thawed multiple times. This stability over time allows for higher throughput screening of NMEs for their enzyme-inducing potential.
2. The activity of CYP1A2 and CYP3A4 in control (DMSO treated) Fa2N-4 cells are substantially higher than those in HepG2 cells, and is approximately 4-10% of the activity seen in primary cultures of human hepatocytes. Because of the broad range of CYP activity in primary cells, some cultures of human hepatocytes had enzyme rates equal to or even lower than those seen in Fa2N-4 cells. These comparisons favor the Fa2N-4 cells as an alternative system to primary hepatocytes to screen NMEs as inducers of multiple CYP enzymes.
3. We have demonstrated that Fa2N-4 cells can go 72 hours (over the weekend) without maintenance (*i.e.*, without daily medium changes) prior to dosing without the loss of CYP enzyme activity or inducibility. This allows for maintenance-free weekends when conducting enzyme induction in Fa2N-4 cells.

REFERENCES

LeCluyse E, Madan A, Hamilton G, Carroll K, DeHaan R, and Parkinson A (2000). Expression and regulation of cytochrome P450 enzymes in primary cultures of human hepatocytes. *J. Biochem Molecular Toxicology* **14 (4)** 177-188.

Luo G, Cunningham M, Kim S, Burn T, Lin J, Sinz M, Hamilton G, Rizzo C, Jolley S, Gilbert D, Downey A, Mudra D, Graham R, Carroll K, Xie J, Madan A, Parkinson A, Christ D, Selling B, LeCluyse E, and Gan LS (2002) CYP3A4 induction by drugs: Correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug Metab Dispos* **30:** 795-804.

Lyon K, Czerwinski M, Perry M, Toren P, and Parkinson A (2004) The use of immortalized hepatocytes in induction studies. Presented at Society of Toxicology Annual Meeting in Baltimore, MD, March 21-25, abstract 1685.

Madan A, Graham RA, Carroll KM, Mudra DR, Burton LA, Krueger LA, Downey AD, Czerwinski M, Forster J, Ribadeneira MD, Gan LS, LeCluyse EL, Zech K, Robertson P, Jr., Koch P, Antonian L, Wagner G, Yu L, and Parkinson A (2003). Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultural human hepatocytes. *Drug Metab Dispos* **31:** 421-431.

Mills J, Rose K, Sadagopan N, Sahi J and de Morais S (2004). Induction of drug metabolizing enzymes and MDR1 using a novel human hepatocyte cell line. *J Pharm Exp Ther* **309:** 303-309.

Morris A, Awwal E and Frank K (2003) In vitro induction of cytochrome P450s and drug transporters using the Fa2N-4 immortalized hepatocyte line. *Drug Metabolism Reviews* **35:** suppl. 1 #249.

Silva JM and Nicoll-Griffith DA (2002) In vitro models for studying induction of cytochrome P450 enzyme, in *Drug-Drug Interaction* (Rodriguez AD ed) pp 189-216, Marcel Dekker, New York, NY.

2004