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Immortalized Human Hepatocytes: A New Advance in Convenience and Performance

Human hepatocytes support numerous preclinical drug development disciplines, including *in vitro* assessment of the likely clinical effects of drugs and chemicals on the liver and, conversely, assessment of possible effects of the liver on candidate compounds. Cryopreserved or plated human hepatocytes offer many advantages, but their utility is hampered by unpredictable supply and significant inter-individual differences in the expression of Drug Metabolizing Enzymes (DMEs) and responses to toxicants. New, immortalized human hepatocytes with near-normal morphology and function are available and should dramatically improve efficiency, reproducibility and predictive value of human hepatocyte studies.

MultiCell Technologies of Providence, RI used their patented technique to produce a line of immortalized human hepatocytes, which was recently licensed by XenoTech, headquartered near Kansas City. Evaluations of the cells by scientists from XenoTech and two major pharmaceutical companies have demonstrated the presence and activity of numerous DMEs; responses to toxicants; production of metabolites and adult proteins; and induction of Phase I and II DMEs and of transporters. The results are reproducible between experiments and across different passages, and the magnitude and specificity of responses correspond to those seen in typical preparations of normal human hepatocytes. The morphologies of immortalized cells and of a preparation of high-quality, human hepatocytes are depicted in ***F1***. The morphology of immortalized hepatocytes resembles that of many preparations of normal hepatocytes, which is a significant finding because hepatocellular morphology is tied to hepatocellular function, reflecting expression of highly differentiated properties of hepatocytes.

Appropriate Types of Studies Using Immortalized Human Hepatocytes

Induction of Drug Metabolizing Enzymes and Transporters

Moderate-to-potent induction of cytochrome P450 (CYP) and other DMEs, or of efflux transporters, is a well-recognized cause of clinically significant drug interactions through the following mechanisms:

a. Bioavailability and efficacy of a co-administered drug is reduced by virtue of increased activity of the DMEs involved in its metabolism.
b. It can cause pharmacokinetic tolerance (or auto-induction): a compound inducing its own hepatic metabolism.
c. Recent evidence implicates enzyme induction as an important influence in drug-induced hepatotoxicity (1). The correlation between toxicity and induction may increase regulatory agencies' expectations for intensified scrutiny of compounds that demonstrate moderate to potent induction potential in order to ensure a low risk of production of reactive intermediates of the candidate or of concurrently administered drugs.
d. Increased activity of efflux transporters may be important, suggesting that a candidate compound poses risk of reducing the efficacy of other co-administered drugs.

Distinguishing the risk of clinical induction among various candidate compounds should provide for reduced expenses by elimination of potential problem compounds early in development, or by improvements in planning, and study and trial designs, as inducers progress through development.

The suitability of the immortalized hepatocytes as a substitute for primary human hepatocytes was studied by Mills *et al.* (2), using prototypical inducers of expression of the mRNA of the major inducible cytochrome P450 DMEs; the Phase II DME, UGT1A; and the efflux transporter, MDR1. They found that the Fa2N-4 cells responded "as expected" using a 24-well plate format.

Morris *et al.* (3) studied both the induction of mRNA, using a different technology from that employed by Mills *et al.*, and the metabolic activity of CYPs 1A2, 2C9, 3A4, and 3A5, as well as transporters MDR-1 and MRP2. They commented that:

"Appropriate induction was observed, i.e., Rifampin and Phenobarbital induced both CYP2C9 and CYP3A4, whereas Omeprazole induced CYP1A2. Comparable induction was obtained in the 6-well and 96-well formats. **F (line) cells performed much like fresh human hepatocytes**" (emphasis added).

They also assessed the specificity of responses, using PCR (TaqMan) Primers and Probes, and demonstrated only 3A4 positivity with 3A4 plasmid DNA, only 3A5 positivity with 3A5 plasmid DNA, and strong 3A7 positivity with a slight 3A5 response using 3A7 plasmid DNA.

XenoTech recently published a summary of the results obtained from more than 60 enzyme induction studies conducted with primary cultures of human hepatocytes (4). Using that information as a reference, we analyzed

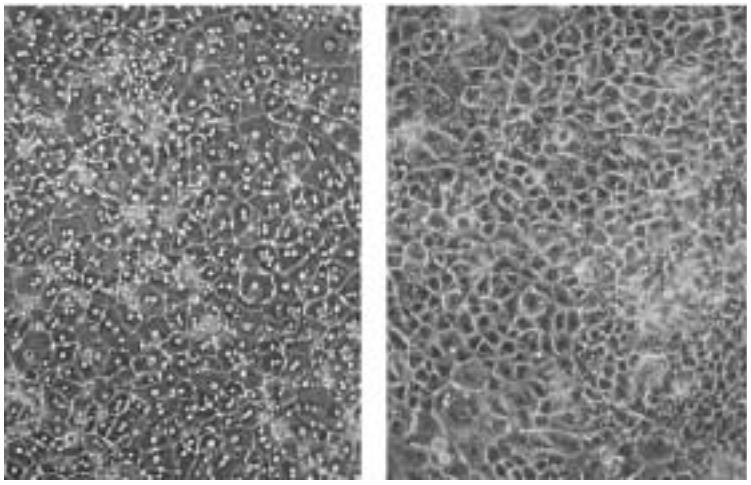
increases of enzymatic activity of the immortalized cells treated with prototypical inducers, to compare them with primary cultures. While experimental conditions were not identical (some older reference methods have been changed), they were sufficiently similar to determine if the response of the immortalized cells supports their use as a replacement for primary cultures. Results of the comparison are depicted in ***T1.*** Our conclusion is that the immortalized hepatocytes respond appropriately to enzyme inducers, with a magnitude of response equaling or exceeding that of most preparations of fresh human hepatocytes.

The results are listed in general order of importance of these CYPs. The significant difference among modal, median, and mean numbers reflects the influence of a very small number of individuals. Using fold induction of CYP3A4 as an example, 65% of all preparations of primary hepatocyte cultures will demonstrate a maximum of 5-fold induction when treated with 20µM of Rifampin for three days; a very limited number (less than 5%) will exceed 50-fold. Thus, the mean induction of 3A4 activity reflects the dramatic and unpredictable contribution of a few "outlier" individuals and is not a useful index of expected induction, the distribution of results being non-Gaussian. Inter-individual variation may also be reflected in the fold induction of 1A2 in the immortalized hepatocytes, because it is higher than most primary cultures. Our overall conclusion is that the immortalized cells perform as well as the majority of preparations of primary cultures of human hepatocytes in studying the induction of major, inducible CYPs.

Our scientists have also investigated other components of the immortalized cells' response to inducers and found:

- ***F2*** depicts the inter-experimental reproducibility achieved with immortalized hepatocytes; this is far less variance than we have observed in studies with primary cultures.
- The time-course of induction (days of treatment with inducer) is similar to that with primary cultures.
- The responses in 6-, 12-, 24- and

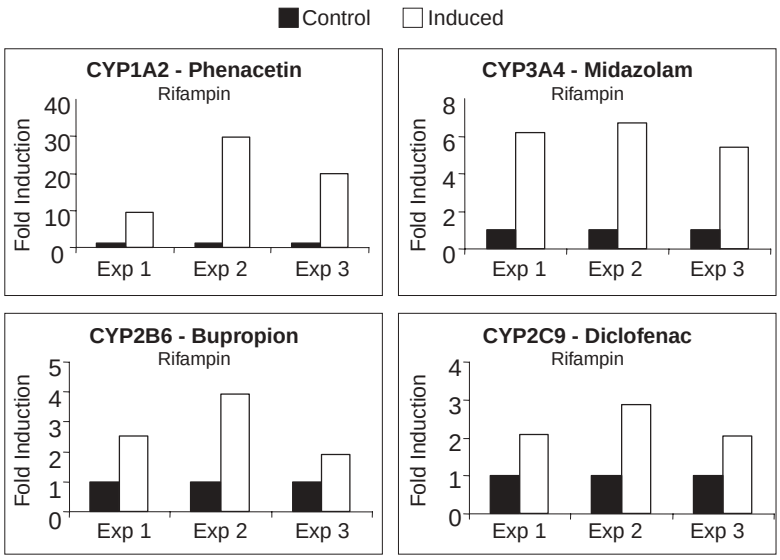
F1. Morphology of human hepatocytes (left) and Fa2N-4 cells (right) at the light microscopy level. Note that Fa2N-4 cells look remarkably similar to human hepatocytes.



T1. Comparison of CYP enzyme induction in Fa2N4 cells and in primary cultures of human hepatocytes.

Enzyme *(Inducer used)*	**Fold induction in F cells** *(Mean value, 96-well)*	**Fold induction in primary human cultures**		
		Mode	Median	Mean
CYP3A4 *(Rifampin)*	5	3	4	10
CYP1A2 *(Omeprazole in F cells, BNF in primary cells)*	20	n/a	8	13
CYP2C9 *(Rifampin)*	2	n/a	3	3.5
CYP2B6 *(Rifampin; F cell studies used different endpoint than primary)*	2.5	n/a	2.9	4.1

F2. Reproducibility of CYP enzyme induction in Fa2N-4 cells.



96-well formats are similar.
- Cryopreservation of the cells, followed by thawing and plating, has no noticeable effect on their induction responses.
- Concentration response curves are similar to those of primary cultures.
- Magnitude of induction response is essentially the same across 10 passages, as shown in ***F3***.
- The specificity of CYP3A4 induction is as expected; no induction is measured with compounds not known to induce 3A4 mRNA.

Other studies are planned or ongoing to examine induction of these cells relative to other responses. Overall, we are satisfied with their performance to the extent that we now use them in contract induction screening.

Toxicity Testing

Numerous options are routinely used for comparison of the *in vitro* toxicity of candidate compounds, such as cell lines of tumorigenic origin (HepG2 and H4IIE). Such cells are unlikely to retain many or most of the factors that predict cell-specific toxicity *in vivo*. For instance, most immortalized cells are not highly differentiated; they rapidly proliferate in culture, which requires enormous energy (ATP consumption) and which may increase their sensitivity to cellular insult compared to non-proliferative cells. The Fa2N4 immortalized hepatocytes also divide (which yields an essentially unlimited supply of them), but at a slower rate than tumor cells, and they exhibit numerous properties of differentiated hepatocytes. They produce metabolites, which is required for conversion of active drugs to reactive metabolites and is the mechanism implicated in the toxicity of the recently-recalled drug, troglitazone, as well as numerous other adverse reactions (1).

Any cell line can offer some utility in analysis of candidate compounds' potential for cytotoxicity, one being availability of cells. The immortalized hepatocytes may provide "the best of both worlds"—retention of some metabolic and transporter function, to allow for potentially superior predictive value in toxicity screening, as well as unlimited availability. XenoTech scientists measured loss of the intracellular protein *a*GST (kit from Biotrin, Inc., Dublin, Ireland) by the immortalized hepatocytes following exposure to compounds whose *in vivo* toxicity risks are well established (***F4***).

The results compared favorably with expectations. Compounds known to have relatively low risk of *in vivo* hepatotoxicity (rifampin, omeprazole, felbamate, probenecid, and acetaminophen) all caused release of aGST from the Fa2N4 cells approximating that of vehicle control (DMSO, 0.1%v/v). It bears mention that, while felbamate and acetaminophen are associated with reports of hepatotoxicity, felbamate toxicity is idiosyncratic in nature, and toxicity in both appears to require doses much higher than the "screening" ones used here. For instance, acetaminophen toxicity is associated with doses exceeding 4 g/day as well as other concurrent environmental conditions. Exposure of the immortalized hepatocytes to the known toxicants 3-methylcholranthene, methotrexate, menadione, rotenone, and troglitazone produced significantly greater release of *a*GST from the cells.

In conclusion, these studies lead us to expect that these immortalized hepatocytes will be suitable for specific *in vitro* toxicity screens. We also expect that some characteristics of the immortalized hepatocytes' response to toxicants may differ from some primary human hepatocytes, but the immortalized hepatocytes offer the distinct advantages of reproducibility and access. Their basal metabolism is lower than the average in normal hepatocytes, so experiments would need to be optimized for detection of metabolism-dependent toxicity.

Metabolic Stability

Guidelines for assessing metabolic stability *in vitro* have been outlined by Tucker *et al.* (5), including measuring parent compound disappearance. The fact that CYP mRNA and activities, and UGT and transporter mRNA, are detectable and inducible suggests that the hepatocytes can be used to measure loss of parent compound. Our related experiments at this point have included measuring the amount of metabolite produced by the Fa2N4 cells, and post-thaw viability in suspension. Production of metabolites of CYPs 1A2, 2C9, 2C19, and 3A4, measured over six hours by LC/MS, was relatively linear, although lower than the average metabolite production in typical human hepatocytes. The Fa2N4 cells did demonstrate an advantage in that their viability in suspension appears to exceed that of almost all preparations of cryopreserved human hepatocytes. This suggests that it may be possible to optimize study conditions to enable the cells to support reproducible, efficient studies.

Conclusion

Our induction, toxicity, and metabolism studies with these immortalized human hepatocytes have yielded very promising results as described above, and we anticipate completion of all remaining work in early 2004. Cells (cryopreserved, plated, or both) can be obtained for evaluation under a limited use license, along with MFE® medium, which we recommend for their support, and you can email the author at: *dsteen@xenotechllc.com* for additional information. We should mention that we do not currently recommend the use of these immortalized hepatocytes in studies submitted to regulatory agencies, particularly in absence of any supporting data from normal human preparations. The immortalized hepatocytes promise to contribute dramatically to improvements in candidate qualification and preclinical development. Drug companies may soon benefit from longitudinal comparison data across many candidates and years, all using the same near-normal, predictable, immortalized human hepatocytes.

® *MultiCell Technologies, Inc.*

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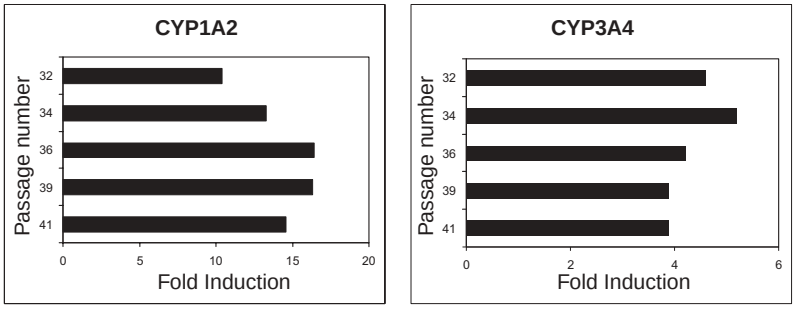
F3. Induction response across passages.



F4. *a*GST release in Fa2N-4 Immortalized Hepatocytes.