

COMPARISON OF HUMAN AND CYNOMOLGUS MONKEY HEPATOCYTE CYTOCHROME P450 LEVELS IN INDUCTION STUDIES

Neil S. Jensen, Rebecca C. M^cGee, and Paul M. Silber*

In Vitro Technologies, Inc., 1450 South Rolling Road, Baltimore, MD 21227



Abstract

Purpose: To evaluate and compare the effect of inducers of cytochrome P450 enzyme activities *in vitro* using both human and cynomolgus monkey (*Macaca fascicularis*) hepatocyte monolayers. Few studies have addressed which compounds induce cytochrome P450 enzymes *in vitro* in the cynomolgus monkey hepatocyte model.

Methods: Human and cynomolgus monkey (CM) hepatocytes were isolated by a standard two-step collagenase procedure. Hepatocytes were plated on collagen-coated 48-well plates using serum-containing *In Vitro*GRO™ CP medium. Plates were incubated in a 37°C / 5% CO₂ incubator for 48 hours to establish hepatocyte monolayers. Serum-free medium containing inducers of CYP450 activities (β-naphthoflavone [33 μM], rifampin [25 μM], or phenobarbital [2 mM]) were then added and plates were incubated for an additional 48 hours. Substrates in modified KHB buffer (7-ethoxyresorufin [CYPIA], and testosterone [CYP3A]) were added to the monolayers. HPLC, mass spectrometry, and fluorescent spectrophotometry were used to measure parent and metabolite levels after incubation. In addition, MTT assays were used to assess monolayer viability.

Results: CM and human hepatocytes demonstrated similar induction profiles. Rifampin induced CM hepatocyte CYP3A activities by 2 – 4 fold, which was similar to the inductions observed with human hepatocytes. β-naphthoflavone induced 1A activity in CM hepatocytes 10 – 20 fold, and phenobarbital induced CYP3A in both CM (2 – 4 fold) and human hepatocytes. MTT values of hepatocyte monolayers indicated cells were viable and metabolically active.

Conclusion: Many of the same inducers of human CYP enzymes also result in induction in CM hepatocytes including rifampin, phenobarbital and β-naphthoflavone. However, a more thorough screening of compounds will be needed to completely understand the correlation of CYP450 induction between human and CM hepatocytes.

Introduction

The use of hepatocytes in a variety of *in vitro* studies, including drug metabolism, inhibition, induction, and cytotoxicity, has continued to increase. The results are then correlated with *in vivo* data to help predict undesirable side effects with new drugs. Hepatocytes isolated from monkeys (cynomolgus, rhesus) have been used in *in vitro* studies because of their close relationship to humans. Limited data is available on the use of cynomolgus monkey (CM) hepatocytes in drug-drug interaction studies, and on the similarity or difference of the results obtained using hepatocytes from monkeys and humans. In addition, little is known regarding what effect exposure to traditional inducers might have on the expression of cytochrome P450 enzymes in monkey hepatocytes. In this study, we examined the expression of cytochrome P450 CYP1A and CYP3A enzymes in both fresh human hepatocyte monolayers and fresh CM hepatocyte monolayers after exposure to rifampin, phenobarbital, or β-naphthoflavone.

Materials and Methods

Hepatocyte Cultures. Fresh Cynomolgus Monkey Hepatocytes (FCMH) and Fresh Human Hepatocytes (FHH) were isolated by a two-step collagenase procedure, and plated on collagen coated 48-well flat bottom plates using *In Vitro*GRO™ CP medium. Plates were incubated overnight in a 37°C, 5% CO₂, humidified incubator to allow attachment of the hepatocytes. After overnight incubation, loose cells were washed off and fresh *In Vitro*GRO™ CP medium was added to the wells.

Induction of CYP1A and CYP3A. At 48 hours, medium was removed and β-naphthoflavone (BNF, 33 μM), rifampicin (RIF, 25 μM), or phenobarbital (PB, 2 mM) in *In Vitro*GRO™ HI medium were added to the plated hepatocytes, along with vehicle control dosing solutions (0.1% DMSO or 1% ACN). At 72 hours, new medium containing inducers or vehicle controls were added to the plated hepatocytes, and cells were incubated an additional 24 hr.

MTT Assay. MTT (10X) was added to wells containing compounds, vehicle controls, or medium controls (final concentration: 0.5 mg/ml). Plates were incubated in a 37°C, 5% CO₂, humidified tissue culture incubator for three hours, then medium from all wells was removed, and 0.2 ml of acidified isopropanol was added to each well to dissolve the MTT formazan. Absorbance of MTT formazan was measured at 572 nm and 690 nm on a Wallac Victor² multilabel counter. The corrected absorbance was determined by subtracting the 690 nm value from the 572 nm value.

Metabolism of Testosterone (CYP3A). Medium was removed and testosterone (100 μM) in modified Krebs Henseleit Buffer was added to plated hepatocytes induced with the compounds above, or vehicle controls. Incubations were performed in a 37°C, 5% CO₂, humidified incubator for one hour, and stopped by addition of an equal volume of cold methanol. Metabolites were identified by HPLC.

Metabolism of Ethoxyresorufin (CYPIA). Medium was removed and ethoxyresorufin (10 μM) in modified Krebs Henseleit Buffer containing 3 mM salicylamide was added to plated hepatocytes induced with the compounds above, or vehicle controls. Incubations were performed in a 37°C, 5% CO₂, humidified incubator for one hour, and stopped by addition of an equal volume of cold methanol. The amount of metabolite was determined by fluorescence on Wallac Victor² multilabel counter at excitation 530 nm and emission 590 nm and compared to a standard curve of resorufin.

Percent Induction. The concentration of metabolites from the wells containing induced plated hepatocytes were divided by the concentration of metabolites from the vehicle control wells and multiplied by 100 to determine the percent of induction. If the vehicle control was below the lower limit of detection, the lowest point of the standard curve was used for calculations instead of the vehicle control value.

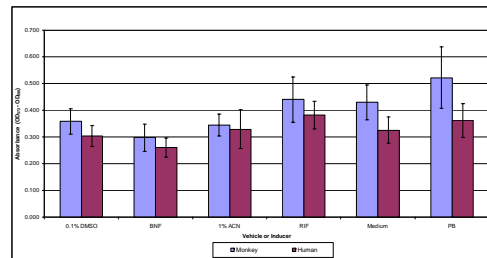


Figure 1. Viability of hepatocyte monolayers as measured by MTT reduction.

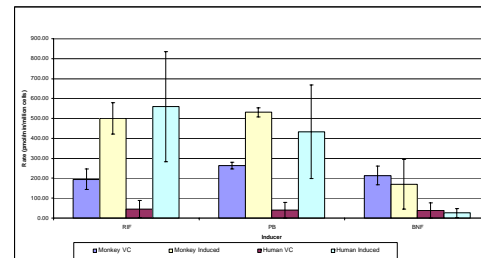


Figure 2. Induction of CYP3A activity as measured by testosterone 6β-hydroxylation reaction rates.

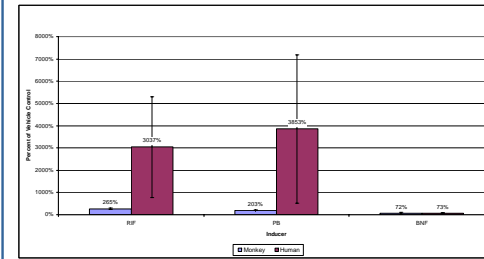


Figure 3. Induction of CYP3A activity as measured by testosterone 6β-hydroxylation and expressed as a percent of vehicle control.

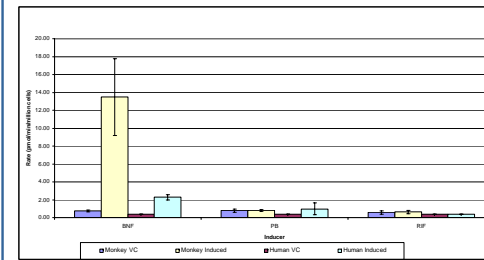


Figure 4. Induction of CYPIA activity as measured by ethoxyresorufin O-deethylation reaction rates.

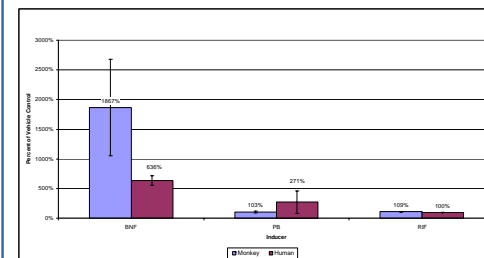


Figure 5. Induction of CYPIA activity as measured by ethoxyresorufin O-deethylation and expressed as a percent of vehicle control.

Results

Viability of Hepatocyte Monolayers. All hepatocyte monolayers maintained viability after 5 days of incubation as measured by MTT assay (Fig. 1). In addition, all hepatocyte monolayers were > 70% confluent by microscopic inspection after the 5 day incubation period. No significant differences were observed in treated vs. control samples, or between FCMH and FHH monolayers.

CYP3A Induction and Metabolism. CYP3A activity was determined by incubating hepatocyte monolayers with testosterone and measuring the resulting levels of 6β-OH testosterone. Incubation of hepatocytes with RIF and PB resulted in increases in CYP3A activity (Fig. 2), and induction of CYP3A activity in both FCMH and FHH as measured by % of vehicle control (Fig. 3). FHH had significantly higher levels of % increase over vehicle control. However, this was due to low levels of average CYP3A activities in vehicle controls in FHH samples.

No induction of CYP3A activity was demonstrated in hepatocytes incubated with BNF (Fig. 2 and 3).

CYP1A Induction and Metabolism. CYP1A activity was determined by incubating hepatocyte monolayers with ethoxyresorufin and measuring the resulting levels of resorufin. Incubation of hepatocytes with BNF resulted in an increase in CYP1A activity (Fig. 4), and significant induction of CYP1A activity as measured by % of vehicle control (Fig. 5). FCMH incubated with PB demonstrated moderate CYP1A activity (Fig. 4), with no enzyme induction present (Fig. 5). FHH incubated with PB resulted in a small increase in CYP1A activity, and a 271% increase over vehicle control enzyme levels, indicating induction had occurred. No difference was observed between RIF incubated cells and vehicle controls (Fig. 4 and 5) indicating no induction of CYP1A activity occurred under those incubation conditions.

Conclusion

- Fresh cynomolgus monkey hepatocytes can be cultured in monolayer, and maintain viability for at least 5 days *in vitro*.
- Induction of CYP1A and CYP3A can be demonstrated with fresh cynomolgus monkey hepatocytes using standard compounds for induction.
- CYP1A and CYP3A induction profiles are similar for various compounds when using fresh cynomolgus monkey or fresh human hepatocytes, with the exception of cells incubated with phenobarbital.
- Screening with a larger number of potential inducers would be necessary to better evaluate the induction profiles of fresh human hepatocytes compared with fresh cynomolgus monkey hepatocyte.