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

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Abstract

Limited information is available on the induction of cytochrome P450 enzyme activities *in vitro* in fresh and cryopreserved cynomolgus monkey (*Macaca fascicularis*) hepatocytes. In this study, fresh cynomolgus monkey hepatocytes (FCMH), cryopreserved cynomolgus monkey hepatocytes (CCMH) and fresh human hepatocytes (FHH) were plated on collagen-coated 48-well plates. After a 48-hour preincubation period in medium, hepatocyte monolayers were incubated for 48 hours with vehicle control, 8 µM 3-methylcholanthrene (3-MC), 33 µM β-naphthoflavone (BNF), 2 mM phenobarbital (PB), 25 µM rifampicin (RIF), or 50 µM omeprazole (OMEP). At the end of the 5-day incubation period, all plates had hepatocyte monolayers of 70% or greater confluence. After the induction period, hepatocytes were evaluated for 7-ethoxyresorufin O-deethylase (CYP1A) and testosterone 6β-hydroxylase (CYP3A) enzyme activities. CYP1A was induced greater than 2-fold in FCMH, CCMH, and FHH by BNF, 3-MC, and OMEP. RIF did not induce CYP1A activity in any of the hepatocyte monolayers. CYP3A was induced greater than 2-fold by RIF and PB in all hepatocyte monolayers, while CYP3A activity was not induced by 3-MC or BNF. OMEP significantly induced CYP3A in FHH but did not induce in CCMH or FCMH. Of the five compounds in this study, all except OMEP demonstrated a similar induction profile for both CYP1A and CYP3A in human and cynomolgus monkey hepatocytes. In addition, CCMH responded in the same manner as FCMH, demonstrating the utility of this cryopreserved non-human primate hepatocyte model.

Introduction

Human hepatocytes have been increasingly utilized in a broad range of *in vitro* studies including drug metabolism, drug-drug interactions, and cytotoxicity. These results are then correlated with *in vivo* data to help predict undesirable side effects with new drugs. Hepatocytes isolated from monkeys (cynomolgus, rhesus) have also been used in these *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, most data from *in vitro* studies with CM hepatocytes have used freshly isolated hepatocytes. Traditionally, cryopreserved cynomolgus monkey hepatocytes (CCMH) have been used in suspension studies. However, induction studies require the use of plated hepatocytes. CCMH have not been shown to reliably attach to collagen-coated plates in numbers sufficient to be used in 5-day cytochrome P450 induction studies. Also, the availability of fresh cynomolgus monkey hepatocytes (FCMH) is limited due to challenges in acquiring the monkey livers. In this study, we demonstrate the use of plateable CCMH. These hepatocytes form a monolayer (> 70% confluence) when plated on collagen-coated plates. Induction studies were carried out using these plateable CCMH, compared with studies using FCMH and fresh human hepatocytes (FHH). The availability of plateable CCMH provides a valuable *in vitro* model that avoids the burdensome requirements of obtaining fresh monkey tissue, and preparing fresh hepatocytes.

Materials and Methods

Hepatocyte Cultures. **Fresh Cynomolgus Monkey Hepatocytes (FCMH), Cryopreserved Cynomolgus Monkey Hepatocytes (CCMH), and Fresh Human Hepatocytes (FHH) were obtained from BioreclamationIVT.**

Plating of Cryopreserved Cynomolgus Monkey Hepatocytes. **Vials were thawed in a 37°C water bath, and cells were diluted in *InVitro*GRO™ CP medium. Cell counts and viability were determined by Trypan blue exclusion. The cell suspension was then diluted to 700,000 viable cells per ml with *InVitro*GRO™ CP medium, and transferred to a collagen coated 48-well plate (140,000 cells per well). Plates were incubated overnight in a 37°C, 5% CO_2, humidified incubator to allow attachment of the hepatocytes.**

Induction of CYP1A and CYP3A. **At 48 hours, medium was removed and ß-naphthoflavone (BNF, 33 µM), 3-methylcholanthrene (3-MC, 8 µM), rifampicin (RIF, 25 µM), phenobarbital (PB, 2 mM), or omeprazole (OMEP, 50 µM) in *InVitro*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.**

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 control. Incubations were performed in a 37°C, 5% CO_2, humidified incubator for one hour, and stopped by addition of an equal volume of cold methanol. Metabolites were identified by HPLC.**

Metabolism of Ethoxyresorufin (CYP1A). **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 control. Incubations were performed in a 37°C, 5% CO_2, 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 Victor^2 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.**

MTT Assay. **MTT (10X) was added to each well containing compounds or vehicle controls (final concentration: 0.5 mg/ml). Plates were incubated in a 37°C, 5% CO_2, 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 Victor^2 multilabel counter. The corrected absorbance was determined by subtracting the 690 nm value from the 572 nm value.**

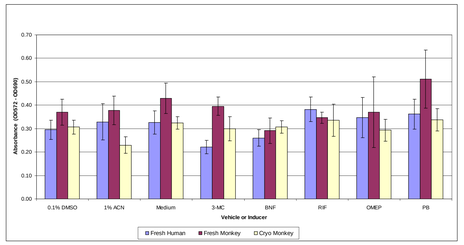

Figure 1. Viability of hepatocyte monolayers as determined by MTT reduction.

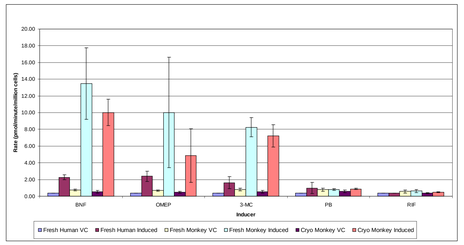

Figure 2. Induction of CYP1A activity as measured by ethoxyresorufin *O*-deethylation reaction rates.

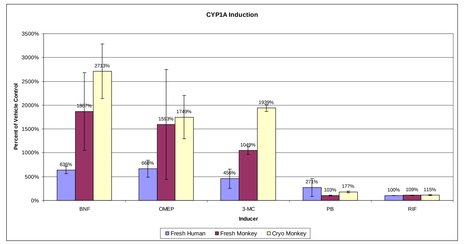

Figure 3. Induction of CYP1A activity as measured by ethoxyresorufin *O*-deethylation and expressed as percent of vehicle control.

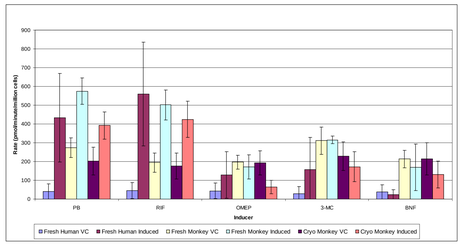

Figure 4. Induction of CYP3A activity as measured by testosterone 6β-hydroxylation reaction rates.

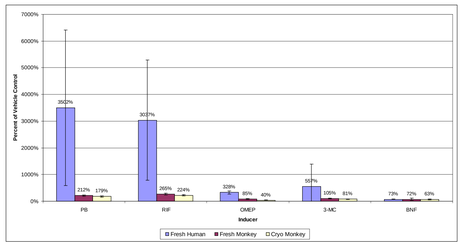

Figure 5. Induction of CYP3A activity as measured by testosterone 6β-hydroxylation and expressed as percent of vehicle control.

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 after the 5 day incubation period. No significant differences were observed in treated vs. control samples, or between FCMH, CCMH, and FHH monolayers.**

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, OMEP, and 3-MC resulted in increases in CYP1A activity (Fig. 2), and significant induction of CYP1A activity as measured by % of vehicle control (Fig. 3). PB incubation gave variable increases in CYP1A activity (Fig. 2), and no clear indication of induction (Fig. 3). No difference was observed between RIF incubated cells and vehicle controls (Fig. 2 and 3) indicating no induction of CYP1A activity occurred.**

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. 4), and induction of CYP3A activity as measured by % of vehicle control (Fig. 5). 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.**

Incubation of hepatocytes with OMEP and 3-MC resulted in low levels of CYP3A induction in FHH, but no induction in FCMH or CCMH samples (Fig 4 and 5). Again, some of this effect was due to low average vehicle control enzyme activities in the FHH samples.

No induction of CYP3A activity was demonstrated in hepatocytes incubated with BNF (Fig. 4 and 5).

Conclusions

- **Plateable cryopreserved cynomolgus monkey hepatocytes (CCMH) can be cultured in monolayer, and maintain viability for at least 5 days *in vitro*.**
- **Induction of CYP1A and CYP3A can be demonstrated using plateable CCMH.**
- **CYP1A and CYP3A induction profiles are similar for various compounds when using plateable cryopreserved cynomolgus monkey, fresh cynomolgus monkey, or fresh human hepatocytes.**
- **Plateable CCMH offer a reliable and convenient alternative to freshly plated cynomolgus monkey or human hepatocytes.**