P450 INDUCTION IN CRYOPRESERVED HEPATOCYTES FROM PXR AND CAR NUCLEAR RECEPTOR KNOCK-OUT MICE

Kevin P. Forbes1, Kirsten Amara2 and Albert P. Li3
1Horizon Discovery Group Company, Saint Louis, MO 63146 | k.forbes@horizondiscovery.com
2In Vitro ADMET Laboratories, Inc. (IVAL), Columbia, MD 21045 and Malden, MA 02148

Abstract
The nuclear receptor programs X receptor (PXr) and constitutive androstane receptor (Car) are closely related transcription factors that regulate the expression of phase I (cytochromes P450) and phase II (carboxylate transferases) enzymes in response to xenobiotics, including prescription drugs. Here, we report the isolation and preliminary characterization of cryopreserved hepatocytes from male Paxr and Car knockout (KO) rats and Paxr/Car double knockout (DKO) rats (Horizon Discovery: 5442 Labs). We were successful in the isolation and cryopreservation of the hepatocytes from three knockout models, yielding hepatocytes with high (97±3%) viability and plating efficiency. The cryopreserved hepatocytes from aldehyde and biotinylated rats were cultured for the evaluation of gene expression in the presence and absence of PXr and Car ligands. The cryopreserved hepatocytes were recovered using UCM (NIH, USA) and plated in 24-well collagen-coated plates. The hepatocyte monolayer contained confluent monolayer cultures with epithelial morphology typical of primary cultured rat hepatocytes. After culturing overnight, medium was changed to protein free induction medium for rat hepatocytes (RHM; Vitalab Inc.). The hepatocytes were treated with Paxr antagonist pregnonolone-3α-carboxylate (PCN) and the Car ligand, 3β,5β-tetralone-14α-biotinylglandular (TCPOBO). Our results show that PXr was required for PCP activation of Cyp2a, Cyp2b10, Cyp2b11, and Slco1a2 gene expression, and that Car was required for TCPOBO activation of Cyp2b11 and Cyp3a11 gene expression, in good agreement with data obtained from in vivo rat knockout models. As primary hepatocytes, nuclear receptor knockout cell lines and mouse knockout models, hepatocytes from the PXR and CAR knockout rats therefore represent a useful in vitro complement to the in vivo models for drug development, especially for functional studies on metabolic pathways involving nuclear receptors.

Materials and Methods
Hepatocyte Reagents and Materials:
- Vitalab Cryopreserved Rat Hepatocytes
- Male SD, Cat#: 80220, Lith.: R5012 (age, 7-10 weeks)
- Male Paxr KO, Cat#: 82125, Lith.: R5512 (age, 7-10 weeks)
- Male Car KO, Cat#: 82125, Lith.: R5512 (age, 7-10 weeks)
- UCMTM: Universal Cryopreservation Recovery Medium, 50 mL, UCM, Cat#: CK003
- RHMTM: Renal Hepatocyte Induction Medium, 50 mL (with supplement)
- UCM: CellAffix Collagen I Coated Plate, 24-well, 5/ pack, APS: Cat#: 21006

Results

Conclusions and Future Directions
Successful isolation and cryopreservation of rat hepatocytes from three knockout models, Paxr, Car, Paxr/Car, was achieved. Loss of Paxr and Car specific induction of P450 and transporter genes under compound treatment, analogous to in vivo observations, was observed. These models, both in vivo and the isolated hepatocytes, should be useful for studying metabolism of xenobiotic compounds and hepatotoxicity. These models are also critical components for the humanization of the cytochrome P450 pathways in the SD rat and subsequent isolated hepatocytes.

Acknowledgements
We would like to thank the R&D teams from Horizon Discovery and In Vitro ADMET Laboratories for their help and assistance. In addition, the business development and commercial teams of Steve Reed, Eric Rhodes, Lara Reid and Kevin Gamber.

References

Drug Metabolizing Enzyme | Substrate [μM] | Induction Time (minutes) | Metabolite Quantified | Activity (pmol/min/mg/100k cells)
--- | --- | --- | --- | ---
CYP2B | 0.1 | 0.0 | 0.1 | 0.0
CYP3A | 0.1 | 0.0 | 0.1 | 0.0

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CYP2B Activity Assay: The hepatocytes were incubated at a cell density of 0.5 million hepatocytes/mL in a 1.2-week plate (50,000 Hepatocytes/well) for the designated time durations with isoforow-hexane substrates. The metabolites were identified and analyzed using LC-MS/MS.

Gene Expression Analysis (Select genes)

Table 1. Gene List Tagged Assays

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<th>Gene Symbol</th>
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</table>

PXR/KO vs WT

* Loss of PXR mediated TCPOBO induction of Cyp2b11 and Cyp3a11. Values are fold change PCN to vehicle treated, +/- standard deviation * significantly different from vehicle treated, same genotype (@p<0.05, t-test).

PXR/CAR/KO vs WT

* Loss of PXR mediated TCPOBO induction of Cyp2b11 and Cyp3a11. Values are fold change PCN to vehicle treated, +/- standard deviation * significantly different from vehicle treated, same genotype (@p<0.05, t-test).

CONFIRMATION OF FUNCTIONAL PXR AND CAR KNOCK-OUT

Cryopreserved hepatocytes were cultured on collagen-coated plates for 4 days. On day 2, culture medium was replaced with medium supplemented with PCN 10 μM or TCPOBO 2500 nM and changed again on day 3 (48-hr treatment). Hepatocytes post-treatment RNA isolated and purified. Each compound was dissolved in 20% DMSO/RHM before adding to the RHM for culturing. The rough concentration of DMSO in the medium is 0.08%, and that concentration of RHM/DMSO is used as our vehicle/control treatment.

QVETIVE RT-PCR: Hepatocytes homogenized in Tissue Reagent (Life Technologies, USA) with ceramic beads using a PowerHybe homogenizer. Total RNA was purified from homogenate and 300μL of total RNA was DNaseI-treated and purified RNA Clean-up using an RNase Minikit (Qiagen, USA). First strand cDNA was synthesized from 500ng to 1μg of purified RNA using the RT1 First Strand Kit from Qiagen RT1 Profile PCR Array System. First strand cDNA was used in Taqman assay (Table 1). Thermalcycler, USA) for Phase II enzymes and transporters in single-pipe reactions following the manufacturer’s recommended conditions on Bio-Rad’s CFX96 Real-Time PCR Detection System. Average fold-change was calculated normalizing to three reference genes (Abll, Hprt1, GAPDH).

CONCLUSIONS AND FUTURE DIRECTIONS

Successful isolation and cryopreservation of rat hepatocytes from three knockout models, Paxr, Car, Paxr/Car, was achieved. Loss of Paxr and Car specific induction of P450 and transporter genes under compound treatment, analogous to in vivo observations, was observed. These models, both in vivo and the isolated hepatocytes, should be useful for studying metabolism of xenobiotic compounds and hepatotoxicity. These models are also critical components for the humanization of the cytochrome P450 pathways in the SD rat and subsequent isolated hepatocytes.

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