In Vitro-In Vivo Extrapolation of 7-Ethoxycoumarin Metabolism Using 3D-Organotypic Liver Bioreactor

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Introduction

- Metabolism is one of the key processes affecting overall toxicity of environmental/industrial chemicals and the therapeutic profile of drugs.
- Metabolism may lead to detoxification, excretion and also bioactivation resulting in toxicity.
- Liver is the major organ for metabolism and clearance of xenobiotics and maintenance of physiological homeostasis.
- Thus, it is essential to develop an *in vitro* liver model that mimics physiologically relevant liver function *in vivo, e.g.* metabolism, detoxification and biosynthesis for more predictive risk assessment of environmental/industrial chemicals.
- The main objectives of this study are 1) to develop a more physiologically relevant liver model under dynamic flow that maintains the viability and functionality of hepatocytes for prolonged periods, and 2) to recapitulate the biotransformation and clearance of compounds similar to the liver *in vivo.*

Methods

Chemicals:

7-Ethoxycoumarin/7-OH Coumarin & glucuronide/sulfate of 7-OH Coumarin were purchased from Sigma Aldrich (St. Louis, MO) & BD Biosciences (Woburn, MA), respectively.

Human and rat hepatocytes:

Primary rat hepatocytes were prepared at the Hamner Institutes & primary human hepatocytes were purchased from Triangle Research Labs (RTP, NC).

3D-organotypic culture system

The D^4™ Culture System was provided by RealBio Technology Inc. (Kalamazoo, MI). This system was primarily designed to mimic microenvironments found *in vivo* including the formation of nutrient and gas gradients. Together, these features were applied to provide a reproducible and controllable liver bioreactor with the ability to modulate culture conditions, e.g. ECM composition, protein binding, flow rate, etc. (Fig.1.)

Culture conditions

- Seeding density: 12, 15 & 20x10^6 per scaffold
- Extracellular matrix (ECM) protein coating: gelatin
- Flow rates: 1.0, 0.5, 0.25 mL/min
- Species specific medium for hepatocytes: for human HMM & for rat DMEM-HG/ DMEM-LG.

Functional & cellular integrity of hepatocytes

- General metabolic activity: glucose consumption, lactate production using portable glucose and lactate meters and pH change in the medium using litmus paper.
- Hepatocyte-specific functions: albumin & total protein production using clinical chemistry analyzer.
- Cellular damage: ALT & AST using clinical chemistry analyzer.

Morphological integrity of hepatocytes: H&E staining

In vitro to in vivo extrapolation for intrinsic clearance of 7-EC

- Primary rat hepatocytes inside liver bioreactor were incubated with 10 µM 7-EC.
- 7-EC, 7-OH Coumarin, glucuronide of 7-OH Coumarin were analyzed using TSQ Quantum Ultra AM QQQ MS with ESI (+) performed by OpAns (RTP, NC).
- Sulfate of 7-OH Coumarin was analyzed on an Agilent 6430 QQQ MS with ESI (-).
- *In vivo* intrinsic clearance of 7-EC was estimated using a mono-exponential decay method.

Results

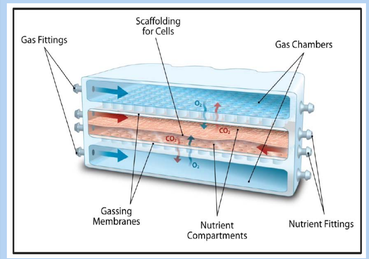

Fig. 1.The RealBio D^4 ™ Culture Chamber. *In vivo*-like conditions are promoted through the use of an open 3D cell scaffold (~10 cm^2 surface x 0.1 mm depth) and decoupled nutrient and metabolic gas supplies. Cell suspensions are injected uniformly into the upper compartment and settle onto the woven fabric of the scaffold which promotes attachment and formation of a 3D tissue-like structure across the length of the chamber.

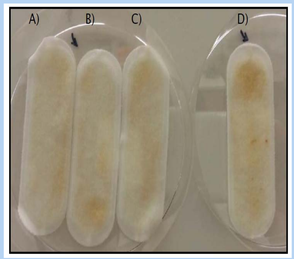

Fig.2. Effect of gelatin coating on the attachment and distribution of primary human hepatocytes after 14 days in culture with the flow rate of 0.5 mL/min. (A)12×10^6, (B) 15×10^6 & (D) 20×10^6 of primary human hepatocytes on uncoated scaffold & (C) 15×10^6 of primary human hepatocytes on 0.1% gelatin-coated scaffold. ↓ represents the inlet direction.

Fig.3. Morphological integrity of 37 day-cultured human hepatocytes inside the liver bioreactor with 4% paraformaldehyde fixation, paraffin embedding & H & E staining. An arrow represents a fiber from the scaffold.

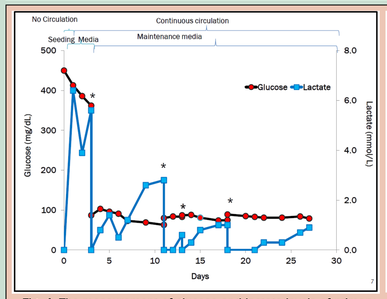

Fig. 4. The measurement of glucose and lactate levels of primary rat hepatocytes inside the liver bioreactor for 27 days. Incubation conditions are i) 12×10^6 hepatocytes on uncoated scaffold , ii) 24hr seeding stage under static conditions, iii) total 3 days in DMEM-HG seeding medium with continuous flow initiated, iv) switch to DMEM-LG maintenance medium under continuous flow, and v) a continuous flow state at 1 mL/min. *medium replacement.

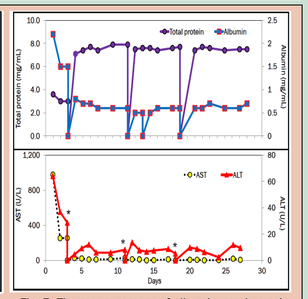

Fig. 5. The measurement of albumin, total protein, ALT and AST levels of primary rat hepatocytes (12×10^6) inside the liver bioreactor for 27 days. *medium replacement.

- The pH of the medium ranged from 7.4 to 8.0 over the 27 day culture period.

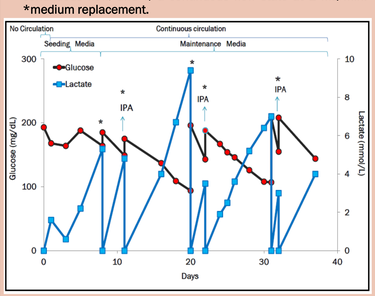

Fig. 6. The measurement of glucose and lactate levels of primary human hepatocytes inside the liver bioreactor for 37 days. Incubation conditions are i) 15×10^6 hepatocytes on a gelatin-coated scaffold, ii) 24hr seeding stage under static conditions, iii) total 8 days in HMM seeding medium with continuous flow initiated, iv) switch to HMM maintenance medium under continuous flow, and v) a continuous flow rate at 0.25 mL/min. *medium replacement. IPA:luciferin-IPA incubation for the measurement of CYP3A4 activity.

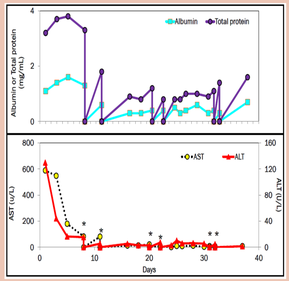

Fig. 7. The measurement of albumin, total protein, ALT and AST levels of primary human hepatocytes (15×10^6) inside the liver bioreactor for 37 days. *medium replacement.

- The pH of the medium ranged from 7.2 to 7.5 over the 37 day culture period.

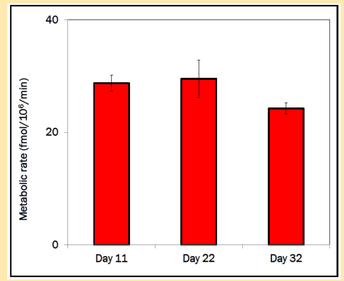

Fig.8. The measurement of CYP3A activity in primary human hepatocytes inside the liver bioreactor during a 32-day culture period using Luciferin-IPA luminescence assay (Promega).

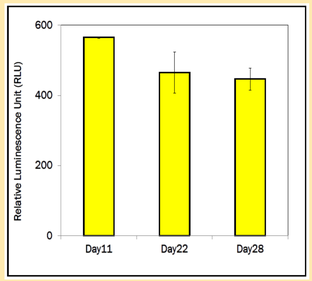

Fig.9. The measurement of CYP3A activity in primary rat hepatocytes inside the liver bioreactor during a 28-day culture period using Luciferin-IPA luminescence assay (Promega).

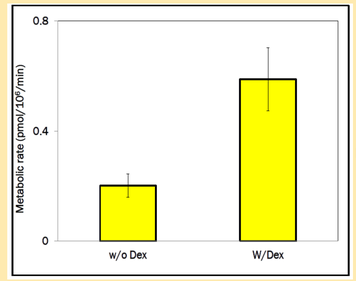

Fig. 10. The measurement of rat CYP3A activity using luciferin-IPA assay. At 28 days primary rat hepatocytes inside the liver bioreactor were treated with 5 µM dexamethasone (Dex) for 24hr followed by measuring CYP3A activity using luciferin-IPA.

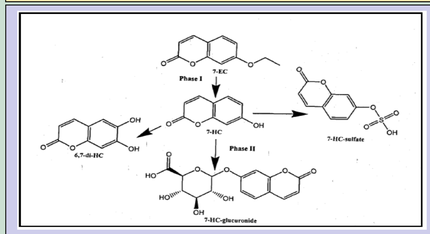

Fig.11. Proposed Phase I and Phase II biotransformation of 7-EC (ref. 1).
7-EC;7-Ethoxycoumarin, 7-HC;7-OH Coumarin, 7-HC-sulfate;7-OH Coumarin Sulfate, 7-HC-glucuronide; 7-OH Coumarin glucuronide and 6,7,-di-HC; 6,7-dihydroxy Coumarin.

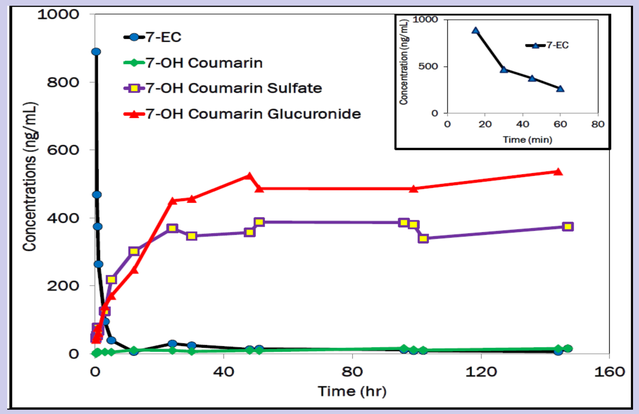

Fig.12. The measurement of 7-EC & its metabolites produced by primary rat hepatocytes inside the liver bioreactor (20-day old culture) after incubation with 10 µM 7-EC for 6 days at 37°C. Inset: Early time points for the depletion of 7-EC in the liver bioreactor .

- Overall 7-EC derived metabolite production:
  7-OH Coumarin glucuronide > 7-OH Coumarin sulfate >> 7-OH Coumarin.
- Estimated *in vitro* intrinsic clearance (CL_{int}) of 7-EC using its mono-exponential decay method: 113.3 µL/min/10^6 cells with a half life of 26 min.
- Scaling *in vitro* CL_{int} to *in vivo* CL_{int}: 135.9 mL/min/RBW*
  *RBW: rat standard body weight (250g)

Summary/Conclusion

- ECM protein coating and adjustment of flow rates improved the attachment and distribution of hepatocytes inside the liver bioreactor.
- Primary human hepatocytes inside the liver bioreactor formed 3D tissue-like structure throughout the scaffold.
- Primary hepatocytes of rat and human inside the liver bioreactor were stably maintained for over 4 weeks.
- After a 7-10 day period rat and human hepatocytes inside the liver bioreactor showed stable levels of biomarkers and CYP3A enzyme activity.
  - Albumin/protein production: for human 0.3-0.7/0.8-1.8 and for rat 0.5-0.7/7.4-7.9 (mg/mL / mg/mL, respectively).
  - ALT/AST levels: for human 0.3-10.3/1.5-81.6 and for rat 2.4-11.8/0.4-28.4 (U/L / U/L, respectively).
- Rat hepatocytes at 28 days inside the liver bioreactor treated with 5 µM Dex showed approximately 3-fold increase in CYP3A enzyme activity.
- Rat liver bioreactor successfully produced phase I and phase II metabolites of 7-EC over an extended period.
  - 7-EC was primarily metabolized to 7-OH Coumarin and further metabolized to the glucuronide and sulfate conjugation.
- Predicted CL_{int} (136.0 mL/min/RBW) was comparable with observed *in vivo* CL_{int} (137 mL/min/RBW, respectively) (ref. 2).

Future Directions

- The current liver bioreactor represents an advanced *in vitro* metabolizing system to monitor phase I and phase II biotransformation of xenobiotics similar to liver *in vivo*.
- The current liver bioreactor will be used to determine the impact of serum/tissue protein binding and altered blood flow (flow rate) on the clearance, metabolite profiles and toxicity of xenobiotics.
- The current liver bioreactor will be used to estimate transporter-mediated hepatic clearance of xenobiotics in humans from *in vitro* metabolism data.

References

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2. Carlile D., Stevens A., Ashforth E., Waghela D. and Houston J.B. 1998. Drug Metab. Dispos. 26 (3) 216-221.

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