AN IN VITRO TRI-CULTURE SYSTEM TO ASSESS COMPOUND-INDUCED HEPATIC CLEARANCE OF THYROXINE IN HUMANS

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LifeNet Health® LifeSciences

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ABSTRACT

Currently, there is a need for a reliable human cell-based *in vitro* assay that recapitulates alterations in thyroxine clearance by thyroid-disrupting chemicals (TDCs). The purpose of this study was to develop and validate an *in vitro* strategy to assess compound-induced alterations in thyroxine metabolism and clearance using a novel human hepatocyte tri-culture system. Pre-validation studies were designed to determine minimum lot qualification, stability of T4 glucuronidation rates over time and optimal exposure period to nuclear receptor agonists in tri-cultures of human primary hepatocytes and feeder cells of stromal and endothelial origin over a 2-week culture period. Using this framework, additional studies were performed in tri-cultures from pre-qualified hepatocyte lots (n=3) to validate a working protocol to assess the impact of known CAR and PXR agonists on phase 1 and 2 enzyme gene expression by qPCR and glucuronidation of 7-ethoxycoumarin (7EC, 100 µM) and T4 (0.1 µM) by LC-MS/MS. Tri-cultures from 3 separate qualified hepatocyte lots were treated daily for 7 days (days 3-10) with PB, CITCO, RIF, PCB153 or PCN. The results of the pre-validation study showed T4 glucuronidation rates were consistent and stable between hepatocyte lots from 7 to 14 days in culture when normalized to cell number (1.5-2.0 pmol/h/10^6 cells). Pre-qualified hepatocyte lots that showed >3-fold increases in T4 glucuronidation rates after exposure to PCB153 exhibited optimal responses to CAR and PXR agonists (e.g., phenobarbital (PB), CITCO and rifampicin (RIF)) after 7 days of exposure (ending on day 10). T4 glucuronidation exhibited linear kinetics at physiologic levels (0.1 µM) between 4 and 24 hours of incubation. *CYP2B6* and *UGT1A1* were induced >5- and >2-fold respectively, by PB, CITCO, RIF and PCB, and *CYP3A4* and *UGT2B4* were induced >5- and >2-fold, respectively, by PB and RIF. Glucuronidation of 7-HC from 7-EC was increased by PB (6-fold), CITCO (2.7-fold), RIF (4-fold), and PCB (1.9-fold), while glucuronidation of T4 was increased by PB (2.2-fold), RIF (2-fold) and PCB (3.2-fold). The results showed that a robust and consistent induction of CYP and UGT gene expression was observed across donor lots for PXR and CAR agonists. Notably, PCB caused the greatest increases in T4 clearance mechanisms compared to RIF, CITCO and PB, which was distinct from the fold changes in CYP and UGT gene expression or 7-EC metabolism. In conclusion, the human hepatocyte tri-culture model represents a reproducible and reliable *in vitro* platform for assessing compound effects on T4 metabolism and clearance.

INTRODUCTION

- Research on endocrine-disrupting chemicals (EDCs), including their targets and effects, has been ongoing for decades. EDCs were mainly thought to exert effects on their target organs, the reproductive system and additional endocrine glands, such as the thyroid, through nuclear hormone receptors. However, it has become widely accepted that EDCs also can affect enzymatic pathways and additional receptor types.[1,2]
- Thyroid hormones are critical in neurodevelopment, both cognitive and neuronal functions, and physical development and health. However, they can also have negative effects, such as tumor formation. Production and release of these hormones is regulated by the hypothalamic-pituitary-thyroid (HPT) axis.[3,4]
- Thyroid-disrupting chemicals, a subgroup of EDCs, can disrupt HPT axis regulation at all levels. Chemicals that have been implicated include phenobarbital, plasticizers, pesticides, and flame retardants.[2,3,5,6]
- Alterations in the homeostasis of the HPT axis is typically characterized by six key events.

Induction of Hepatic Enzymes (UGTs) → Increased Hepatic T4/T3 Metabolism, Biliary Excretion → Decreased Serum T4/T3 Half-life, Concentration → HPT Axis Activation → Increased Serum TSH Concentration → Thyroid Pathology

- Increased hepatic thyroxine (T4) metabolism typically involves glucuronidation via UGTs, which are regulated by the nuclear receptors AhR, CAR, and PXR. Some thyroid-disrupting chemicals target these nuclear receptors, resulting in altered thyroid hormone homeostasis.[7,8,9,10]
- Traditionally, thyroid-based research has been completed in *in vivo* rodent models. However, these models do not directly translate to human outcomes and are resource intensive.
- Current *in vitro* models have proven unstable over time and unreliable as robust *in vitro* assays to screen potential compounds for effects on hepatic T4 clearance via different modes of action.
- LifeNet Health's all-human tri-culture system extends the longevity of hepatocytes in culture, while maintaining key morphological and functional properties critical to T4 clearance mechanisms.

The objective of this study was to develop and validate an *in vitro* strategy to assess compound-induced alterations in T4 metabolism and clearance using a novel human hepatocyte tri-culture system.

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MATERIALS & METHODS

Culture Model

- Tri-cultures were created by first seeding feeder cells of stromal and endothelial origin in serum-containing Tri-Culture Plating Medium (TCPM) in 24-well collagen I-coated BioCoat® plates (Corning, Corning, NY); human hepatocytes (0.6 x 10^6 cells/mL) were added 1-2 hrs later. Medium was replaced daily, starting at 4 hrs post-hepatocyte plating, with fresh Tri-Culture Culture Medium (TCCM). [For additional details about the design of the human tri-culture model, please visit Poster #4439 (P439).]
- Cryopreserved primary human hepatocytes (**Table 1**) were pre-qualified using T4 glucuronidation rates following PCB153 exposure (>3-fold increase).

Table 1. *Characteristics of hepatocyte donors.*

Donor	Characteristics								
	Gender	Race	Age	BMI	Tobacco History	Alcohol History	Drug History	Medication	Cause of Death
1916422	Female	Caucasian	48	24	No	No	No	Unknown	Respiratory Arrest
1921756	Male	Caucasian	56	29	1 ppd	6/day	No	None	CVA/Stroke
1922211	Male	Caucasian	40	28	½ ppd	12/week	Yes	Yes	Anoxia

Treatments and Assays

- *Induction*: Tri-cultures were exposed to reference compounds [Phenobarbital (PB; 100-1000 µM), CITCO (0.25 µM), Dexamethasone (DEX; 10 µM), Rifampicin (RIF; 10 µM), PCN (10-20 µM), PCB153 (30 µM)] or vehicle [DMSO; 0.1% (v/v)] for 3, 5, or 7 days, with the start time adjusted to ensure exposure ended on Day 10 of culture. Media was replaced daily with fresh compound-containing media.
- *T4 metabolism and clearance*: T4 was either added at saturating (1 µM) or physiologically relevant (0.1 µM) conditions, dependent upon the endpoint, to tri-cultures and samples collected for up to 24 hours. LC-MS/MS analysis was used to determine T4, T4-glucuronide (T4-Gluc), T4-sulfate (T4-SO_4), and T3 levels.
- *Phase I and II metabolism:* 7-Ethoxycoumarin (7-EC; 100 µM) was added to tri-cultures and samples collected for up to 60 minutes. LC-MS/MS analysis was used to determine 7-EC, 7-hydroxycoumarin (7-HC), 7-HC glucuronide (7-HCG), and 7-HC sulfate (7-HCS) levels.
- *Urea synthesis*: Urea levels were measured in media samples collected over time in culture (Stanbio Laboratory, Boerne, TX).
- *Gene expression analysis*: RNA was isolated from samples using Qiagen RNeasy mini kits with DNase treatment (Qiagen, Germantown, MD). Reverse transcription was carried out using High-Capacity cDNA Reverse Transcription kits (Applied Biosystems/Life Technologies, Grand Island, NY). qPCR was performed using TaqMan™ Universal PCR Master Mix and TaqMan™ Gene Expression Assays for the genes of interest (Applied Biosystems/Life Technologies).
- *Hepatocyte attachment determination*: Cell counts were performed on media samples collected at each media change through Day 2 of culture. Average % attachment for each hepatocyte lot was determined after accounting for cells lost due to non-attachment and/or death.

RESULTS

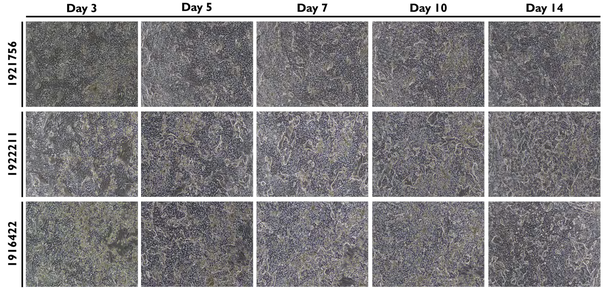

Figure 1. Representative phase contrast images of tri-cultures from 3 individual human hepatocyte donors over 14 days in culture. All images shown are at 100x magnification.

ACKNOWLEDGEMENTS

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RESULTS

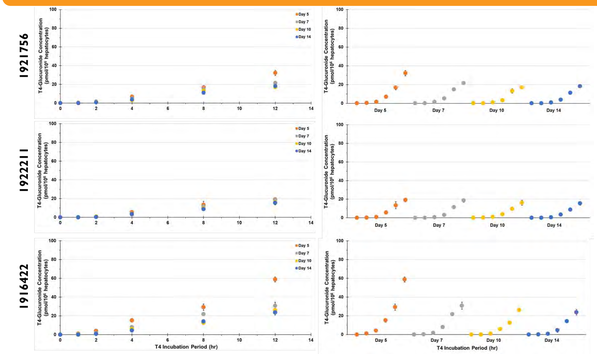

Figure 2. T4 metabolism over time in culture. Tri-cultures were incubated with T4 (1 µM) on Days 3, 5, 7, 10, and 14 of culture. Samples were collected up to 12 hours post-T4 addition for LC-MS/MS analysis. Results were normalized to the number of hepatocytes added for plating. (n = 3 wells)

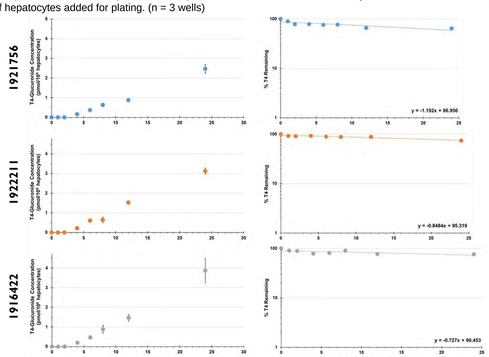

Figure 3. T4 clearance. Tri-cultures were incubated with T4 (0.1 µM) on Day 10 of culture. Samples were collected up to 24 hours post-T4 addition for LC-MS/MS analysis. Results were normalized to the number of attached hepatocytes. (n = 3 wells)

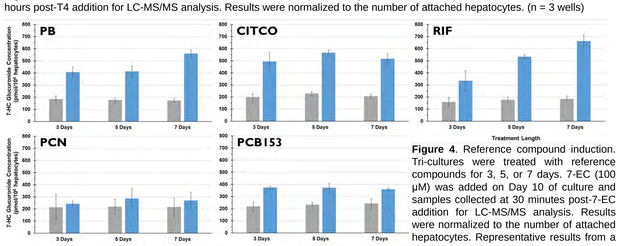

Figure 4. Reference compound induction. Tri-cultures were treated with reference compounds for 3, 5, or 7 days. 7-EC (100 µM) was added on Day 10 of culture and samples collected at 30 minutes post-7-EC addition for LC-MS/MS analysis. Results were normalized to the number of attached hepatocytes. Representative results from a single human donor are shown.

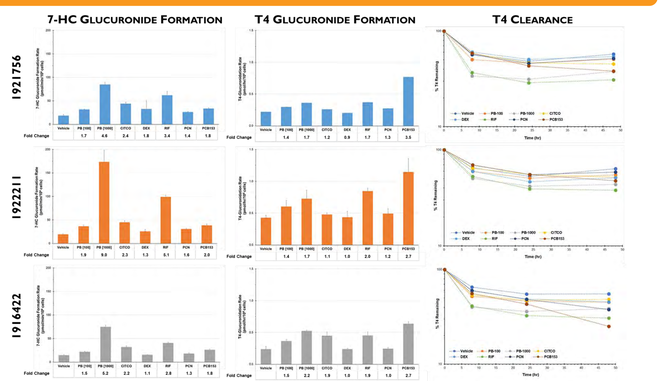

Figure 5. 7-EC metabolism and T4 metabolism and clearance following reference compound treatment. Tri-cultures were treated with reference compounds for 7 days. On Day 10 of culture, 7-EC (100 µM) was added to a set of wells and samples collected up to 60 minutes post-addition for LC-MS/MS analysis. T4 (0.1 µM) was added to second set of wells and samples collected up to 48 hours post-addition for LC-MS/MS analysis. 7-EC and T4 results from 60 minute and 48 hour samples, respectively, are shown. Results were normalized to the number of attached hepatocytes. (n = 3 wells)

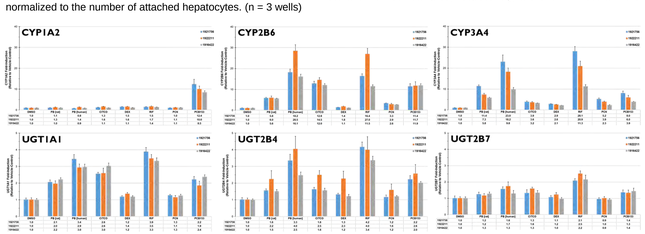

Figure 6. CYP and UGT induction following reference compound treatment. Tri-cultures were treated with reference compounds for 7 days. Samples were collected on Day 10 of culture for RT-qPCR analysis. (n = 3 wells)

CONCLUSIONS

- Stabilization of the tri-culture model occurs over the initial 3-5 days of culture.
- The analytical method is highly sensitive with limits of detection being 10 pg/mL for T4 metabolites (T4-Gluc, T4-SO_4).
- T4 metabolism (T4-Gluc, T4-SO_4) was consistent in the tri-culture model and stable between days 5 and 14 of culture.
- T4 clearance was consistent across individual human hepatocyte lots in the tri-culture model.
- Tri-culture responses to reference compounds, both gene expression and functional activity, were reflective of known *in vivo* responses.
- 7-HC glucuronidation was affected by reference compounds differently than T4 glucuronidation. PB and Rif had a greater effect on 7-HC glucuronidation, whereas PCB153 had a greater effect on T4 glucuronidation. These results suggest that different pathways are involved in 7-EC and T4 clearance.
- T4 parent compound clearance was affected in tri-cultures by reference compounds in a selective manner, according to the expected rank order (e.g., RIF≈PB>CITCO≈PCB153) at 8h. However, T4 clearance demonstrated non-linear kinetics while altering the rank order of PCB153, suggesting additional elimination mechanisms involved in T4 clearance in the presence of PCB153.
- Human tri-culture data shows good fidelity between trials with expected responses for gene expression and 7-HC Gluc rates.
- Thyroxine hormone conjugation alone may not fully reflect what is involved in T4 clearance and warrants further investigation.