Abstract: 360

Generation of Historical Control Database, Acceptance and Interpretation Criteria in a Comparative T4-Glucuronidation Assay Using Primary Human and Rat Hepatocyte (TruVivo®) for Regulatory Acceptance

Ahtasham Raza[1], Stephanie Kellum[1], Justin Brown[1], Suhair Al Janabi[1], Lauren Kent[2], Marco Corvaro[3], Jinlan Dong[1], Jessica LaRocca[4], Edward LeCluyse[5], Raja Settivari[1] and Shadia M. I. Catalano[1].

[1]Corteva Agriscience (DE, USA), [2]Corteva Agriscience (Oxford, U.K), [3]Corteva Agriscience (Cremona, Italy), [4]Corteva Agriscience (IN, USA), [5]LifeNet Health LifeSciences (NC, USA).

INTRODUCTION

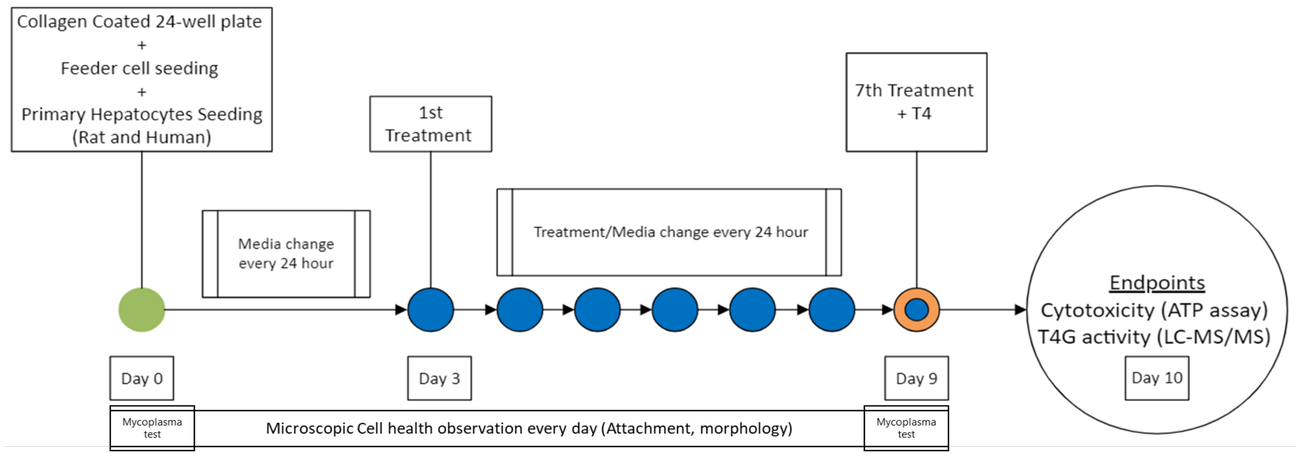
Evaluating the human relevance of liver-mediated thyroid hormone disruption remains a significant challenge within the European Union (EU) regulatory framework. Currently, no *in vitro* methodologies exist with established guidelines, acceptance criteria, or interpretation frameworks that can quantitatively assess such disruption across species. This gap hinders both applicants and regulators in determining the human relevance of chemically induced thyroid toxicity.

New Approach Methodologies (NAMs) offer a promising solution by enabling direct comparisons between human and rat hepatic responses. Among these, the comparative *in vitro* hepatic enzyme assay is specifically highlighted in the ECHA-EFSA guidance on endocrine disruptors. However, its development and implementation have proven technically complex and difficult to standardize.

This study contributes to the advancement of NAMs by utilizing TruVivo®, a tri-culture hepatic model composed of hepatocytes and feeder cells (growth-inactivated fibroblast and epithelial cells), to evaluate thyroxine glucuronide (T4G) formation, a key metabolite in thyroid hormone metabolism and a critical endpoint for assessing liver-mediated thyroid disruption. The work focuses on quantifying interspecies differences in T4G formation following exposure to nuclear receptor reference agonists (NR-RFs), while also establishing historical control data (HCD), assay acceptability criteria, a suitable positive control, and interpretation criteria.

METHODOLOGY

Test System:	TruVivo® 2D⁺ Hepatic test system
Test Species:	Human (individual donor 3 Male/3 Female), SD Rat (pooled lot 2 Male/1 Female)
Vehicle Control (VC):	Dimethyl Sulfoxide (DMSO)
Nuclear Receptor (NR) Activating Reference Compounds:	Polychlorinated biphenyl 153 (PCB153, 30µM) Pregnenolone 16α-carbonitrile (PCN, 10µM) Phenobarbital (PB, 500µM) Rifampicin (RIF, 10µM)
Duration of exposure:	7 days
Number of Independent runs:	3
Model Health check:	Imaging (microscopic), Mycoplasma (colorimetric), Cytotoxicity (ATP)
Endpoints:	T4G (LC-MS/MS), Cytotoxicity (ATP-Promega)
Statistical analysis:	Outlier detection, Variance component analysis (VCA) for source of variation, Bootstrap sampling for control limit (CL) identification (software R)

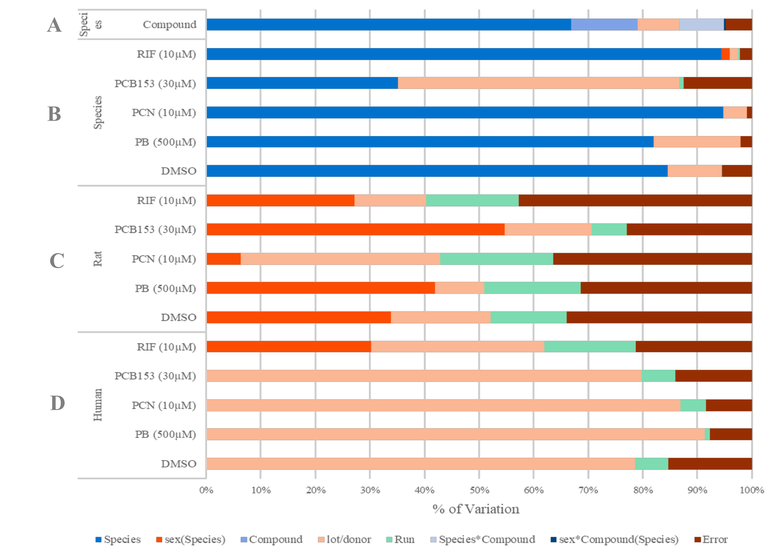


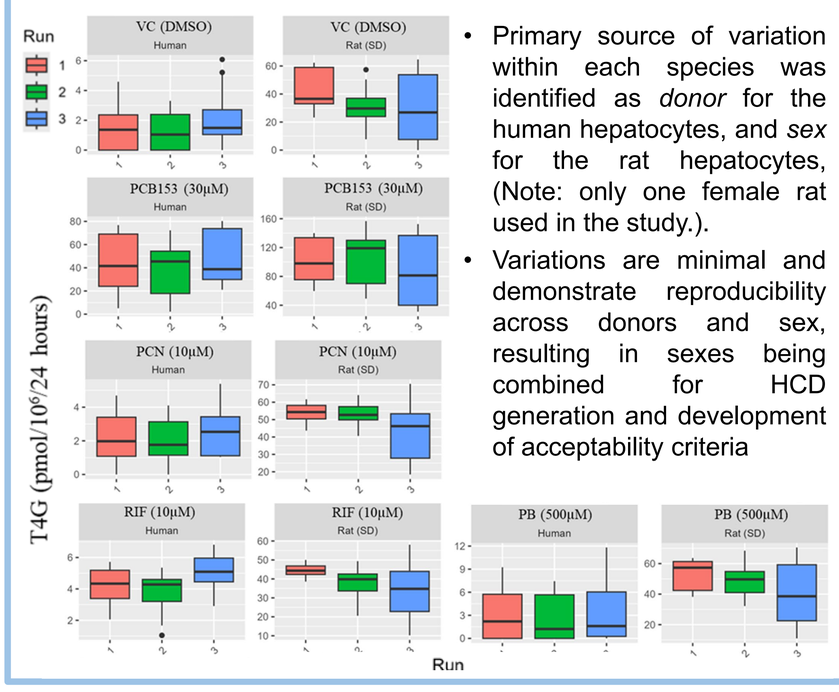
Overview of experimental design for both rat and human cultures using TruVivo® tri-culture hepatic system.

RESULTS

Variability Sources & Assay Reproducibility

- Variance component analysis (VCA) was performed to estimate the percentage of variation caused by each source.
- VCA was conducted on all combined data (A), followed by all species combined but separated by vehicle control (DMSO) and reference controls (B), then each species and separated for DMSO and the individual reference controls (C and D).
- VCA identified the major source of variation as species differences (>65%).

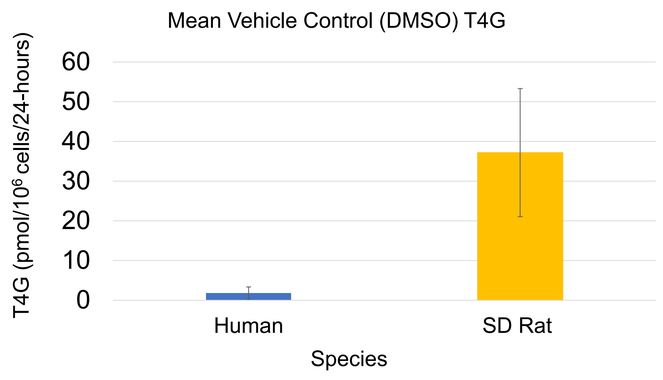




- Primary source of variation within each species was identified as *donor* for the human hepatocytes, and *sex* for the rat hepatocytes, (Note: only one female rat used in the study.).
- Variations are minimal and demonstrate reproducibility across donors and sex, resulting in sexes being combined for HCD generation and development of acceptability criteria

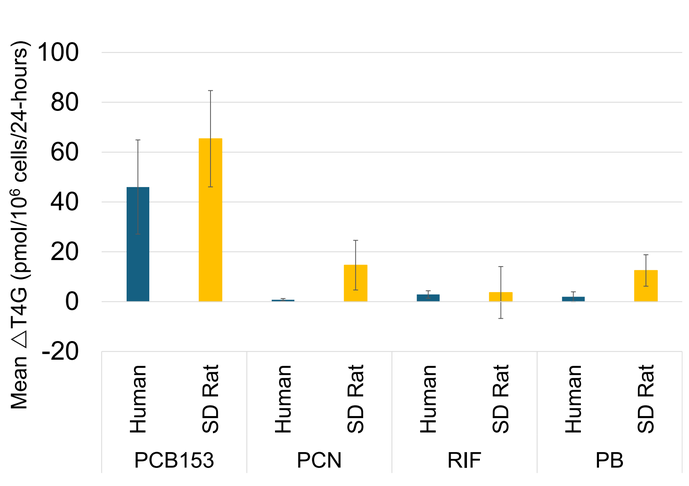
Quantitative Species Differences

- T4G induction observed in the VC indicates that basal level of T4G induction is greater in rat hepatocytes than in human hepatocytes.



Identification of Positive Control

- ΔT4G induction observed after treatment with PCB153 indicates PCB153 induces robust T4 metabolism in both rat and human hepatocytes. Unlike the induction from the other NR activating reference compounds (below), the PCB153 induction is seen across donors and lots (not shown).
- PCB153 would be an appropriate positive control for the assay.
- Use of PB, PCN, and RIF require additional testing to understand applicability in this assay.



Historical Control Database

- An HCD was generated to include species mean, species standard deviation, and species mean range, combined sexes.
- VC HCD will be based on T4G values. All NR activating reference compound HCDs will be based on ΔT4G values.

A summary of the HCD is as follows:

Vehicle Control - Human and Rat Hepatocytes (Combined sexes)

Species	Compound	Concentration (µM)	Mean T4G pmol/10⁶ cells/24h	StdDev	Min	Max	N samples	N Runs	N donors
Human	DMSO	0	1.76	1.59	0	5.4	72	3	6
Rat	DMSO	0	37.18	16.14	20.17	60.75	36	3	3

Reference Controls - Human Hepatocytes (Combined Sexes)

Species	Compound	Concentration (µM)	Mean ΔT4G pmol/10⁶ cells/24h	StdDev	Min	Max	N samples	N Runs	N donors
Human	PCB153	30	46.01	18.9	16.91	73.02	90	3	6
	PCN	10	0.77	0.48	-0.25	1.37	90	3	6
	RIF	10	2.88	1.5	0.07	5.35	90	3	6
	PB	500	1.95	2	-0.63	5.81	90	3	6

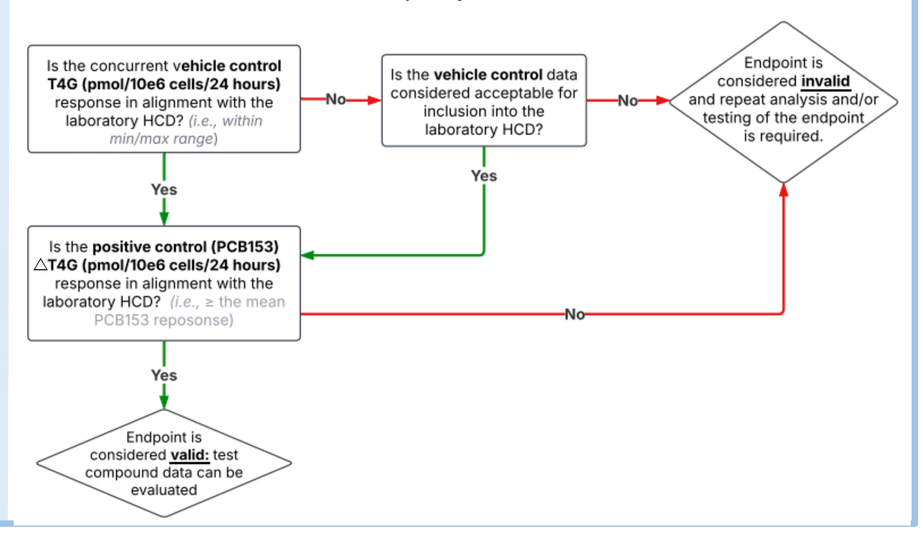
Reference Controls - Rat Hepatocytes (Combined sexes)

Species	Compound	Concentration (µM)	Mean ΔT4G pmol/10⁶ cells/24h	StdDev	Min	Max	N samples	N Runs	N donors
Rat	PCB153	30	65.39	19.33	36.88	94.59	45	3	3
	PCN	10	14.64	9.96	-0.88	25.46	45	3	3
	RIF	10	3.66	10.42	-13.15	14.94	45	3	3
	PB	500	12.51	6.32	1.38	20.07	45	3	3

Definition of Acceptance Criteria

= values used to set acceptability criteria

- Control limit testing was conducted and concluded that additional data is required prior to using control limits as a measure of study acceptability.
- The statistical recommendation is to use the means and ranges established in the HCD to define assay acceptance criteria for the comparative T4-glucuronidation in vitro assay (TruVivo®).
- Assay acceptability criteria will be set based on the T4G values for the VC and ΔT4G values for PCB153 for each species.



CONCLUSIONS

The data generated in this testing resulted in:

- Identification of sources of assay variability
- Demonstration of assay reproducibility
- Demonstration of quantitative species differences with illustration of clear differences in basal T4G levels (i.e., human demonstrate quantitatively lower basal T4G than SD rat)
- Results from treatment with PCB153 have identified PCB153 as a positive control for both human and rat hepatocytes
- Generation of an HCD
- Defined acceptability criteria and accompanying flow chart for application

It is recognized that the HCD and acceptability criteria outlined in this study are a direct reflection of the data generated from this study alone and will be updated as additional data is generated.

The T4G TruVivo® assay has demonstrated the potential to be a robust and reliable tool for evaluating human relevance in liver-mediated thyroid hormone disruption, supporting its potential regulatory acceptance within the EU endocrine disruptor assessment framework.

ONGOING WORK

Evaluation of additional compounds in the T4G TruVivo® assay to define interpretation criteria. This evaluation involves two key steps:

(1) Identifying candidate molecules through literature review focused on liver-mediated thyroid effects in humans and/or rats.

(2) Screening these candidates in the T4G TruVivo® assay using a standardized SOP, HCD, and predefined acceptance criteria.

CONTACT INFORMATION

Ahtasham Raza
Senior Molecular Toxicologist
ahtasham.raza@corteva.com

Shadia Catalano
Genetics and Molecular Toxicology Leader
shadia.catalano@corteva.com