High-Throughput Assessment of Increased Chemical Toxicity Due to Hepatic Steatosis

Nyssa N. Tucker[1,2 †], Gail M. Nelson[2], Joshua A. Harrill[2], Brian N. Chorley[2*]

[1]Oak Ridge Institute for Science and Education, Oak Ridge, TN 37831

[2]Center for Computational Toxicology & Exposure, US Environmental Protection Agency, RTP, NC 27709

† Biological and Biomedical Sciences Program, University of North Carolina at Chapel Hill, CH, NC 27707 (current address)

*Brian N. Chorley I Chorley.Brian@epa.gov I 919-541-2329

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Abstract

Hepatic steatosis alters native liver xenobiotic metabolism, impacting the bioactivation or detoxification of chemicals and sensitivity to chemical toxicity. Here, we assess the impact of steatosis on chemical toxicity in a quantitative and high-throughput manner. We induced steatosis in HepaRGs by dosing maintenance media with 1 mM of 1:2 oleic:palmitic free fatty acid for 1 week. Cytochrome P450 (CYP) gene expression and metabolic activity (CYPs 1A1, 1A2, 2B6, 2C9, 2E1, 3A4) were significantly altered in the steatotic culture condition. Relative culture viability was determined by CTG, LDH, and multiplexed fluorometric measurements of nuclear morphology using the Opera Phenix high-content screening (HCS) system. Naive and steatotic HepaRG cells were exposed to known hepatotoxicant (rotenone) over a 5-point dose range for 24 hrs. Rotenone toxicity (IC50) shifted from a baseline 0.64 µM to 0.48 µM in steatotic cells as measured by CTG, from 0.83 µM to 0.57 µM measuring LDH, and 0.80 µM to 0.62 µM using cell counts derived from HCS (data significantly different between treatments at each dose level [p <= 0.05]). Additional high-throughput toxicity measures – including morphology, ROS generation, and mitochondrial membrane potential – as well as additional and chemicals known CYP-mediated chemicals known to be impacted by CYP-mediated metabolism are currently being assessed. The results of this study will help outline methods to quantitate the impact of pre-existing conditions on environmental chemical toxicity in a high-throughput manner. These efforts will contribute to ongoing Agency efforts to assess environmental exposure risks in susceptible subpopulations.

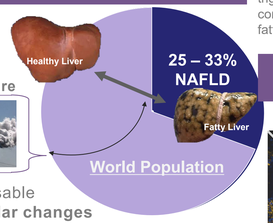
This poster does not necessarily reflect EPA policy.

Introduction

Hepatic Steatosis = Fatty liver

- **Prevalent** ~1/3rd of the world
- Multiple causes →

A) Genetic **B) Behavioral C) Exposure**



- Often **asymptomatic** & reversable
- Causes **morphological cellular changes**
- Disease state (altered lipid metabolism + ↑ triglyceride retention) **may increase susceptibility** to environmental chemicals.

In Vitro Model

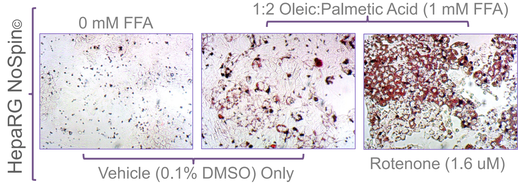


Figure 1. Establishing significant fat build-up in HepaRG cells. Oil red O staining indicated intracellular accumulation of lipid droplets after free-fatty acid [**FFA**] exposure in the media for 1 week. The addition of 1.6 uM rotenone, a known hepatotoxicant and mitochondrial respiratory chain complex I inhibitor, significantly increased fat retention due to decreased fatty acid metabolism.

U.S. Environmental Protection Agency
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Center for Computational Toxicology & Exposure
Biomolecular & Computational Toxicology Division
Alternative Experimental Toxicology Models Branch

R1: Model Characterization

Steatotic Cells – Fluorescence

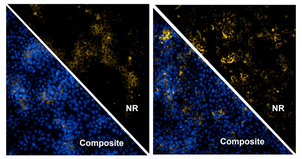

Figure 2. Fluorometric indication of hepatic steatosis in HepaRG cells. Hoechst [HO] nuclear stain and Nile Red [NR] triglyceride stain following 48h exposure to media containing 1 mM of a 1:2 oleate:palmitate free-fatty acid [FFA].

Lipid Accumulation – Spot Count / Cell

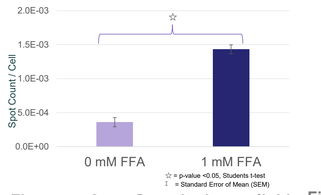

Figure 3. Quantitative lipid accumulation with NR staining. Lipid accumulation due to 1 week of FFA exposure in media was assessed by measuring fluorescence of lipid-bound Nile Red dye normalized to cell count determined by Hoechst staining.

CYP Gene Expression 24h

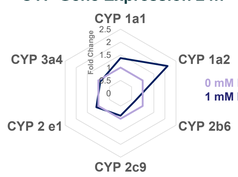

Figure 4. CYP gene expression changes due to hepatic steatosis in HepaRG. Real-time qPCR measured 6 common CYP enzymes active in human liver. Metabolic enzyme expression shown as fold-change following 7-day incubation of 1 mM 1:2 oleate:palmitate FFA.

Metabolic Activity 24 & 48h

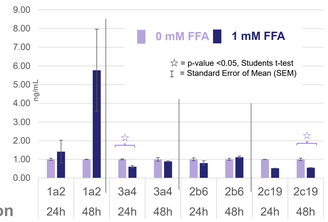

Figure 5. CYP activity perturbation due to hepatic steatosis in HepaRG. HPLC measurement of 3 CYP activity levels using targeted substrates. Metabolic enzyme activity measured following a 1h substrate incubation, shown in ng/mL and normalized to 0mM FFA control.

R2: Altered Susceptibility + Toxic Fingerprint

High-throughput Profiling of Cellular Toxicity Indicators

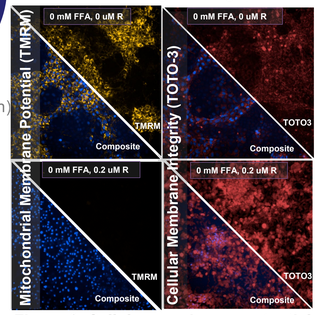

Figure 6. Cellular membrane potential change with low-concentration test chemical exposure. HepaRG with no FFA exposure, 48h 0.2 uM rotenone with a HO nuclear stain and TMRM (mitochondrial membrane potential) / TOTO-3 (cellular membrane integrity).

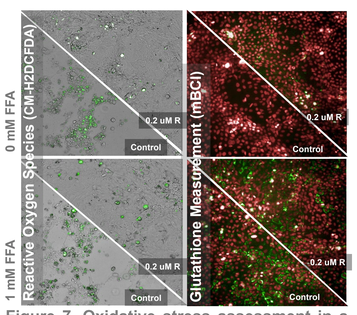

Figure 7. Oxidative stress assessment in a low-concentration test chemical exposure in steatotic HepaRG. HepaRG incubated with FFA & 48h 0.2 uM rotenone, visualized with brightfield and CM-H2DCFDA (ROS); or FFA, 24h 0.2 uM rotenone using DRAQ5 nuclear stain, and mBCI (GSH).

Mechanistic Indicators – Comparative Quantification

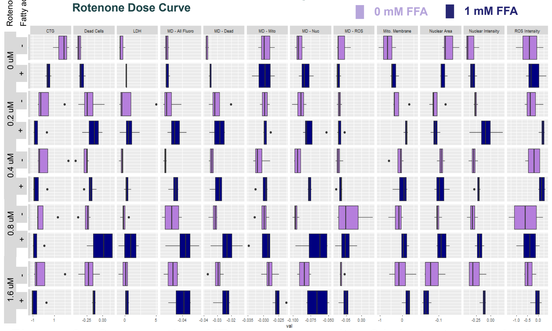

Figure 8. Comparative HT measures with 24 h test chemical exposure over dose range. Using the Opera Phenix imager and Harmony software, fluorescent stains are reduced to values representing single cell data resolution. Further analysis using R, R studio, and the TidyVerse suite result in quantitative outputs confirming visual and complementary assay outcomes.

R3: Correlation of Low- and High- Throughput Measures

Rotenone Exposure - ATP IC50

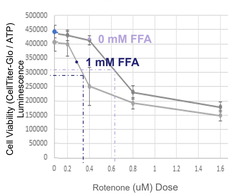

Figure 9. Rotenone-mediated toxicity increases when cells are steatotic. Data points derived from an ATP-proxy viability assay were fit to a non-linear least-squares model and IC50 values were determined (Figure 10).

Comparative IC50s

	0 FFA	1 FFA
CTG	0.62	0.35
LDH	0.83	0.57
MD	0.19	0.10

Figure 10. IC50 Calculations from Different Toxicity Measurements. IC50 results from 4 assay types examining rotenone toxicity. Mahalanobis distance (MD) derived from measured nuclear features (see Figure 11). CellTiter-Glo (CTG) and Lactate Dehydrogenase (LDH) release assays from same plate, MD from independent experiment.

Correlation of Features with CTG/LDH

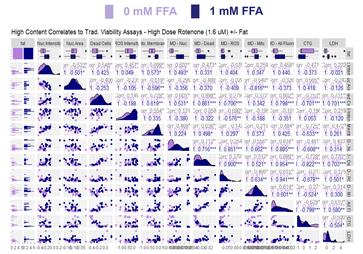

Figure 11. Feature information correlated with nuclear features, latent variables, and low-throughput methods. Imaging output quantified using Harmony software and analyzed using R(ggpairs).

WIP: Interrogating CYPs with Targeted Chemicals

Fluorescence HO / NR – Acetaminophen Exposure

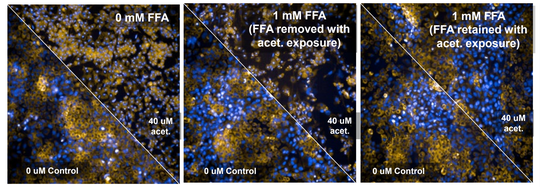

Figure 12. Nuclear morphological assessments with 24 h high concentration test chemical exposure. Two methods of FFA exposure with test chemical produce visually distinct outcomes, potentially due to differences in chemical uptake and processing. Examining nuclear morphology indicates potential use of fluorescent intensity as proxy measures of cell viability. Further analysis of textural features are potentially useful indicators of sub-toxic responses.

Test Chemical Set

CYP	Toxic Parent	Toxic Metabolite	Non Toxic
1a2	Clozapine	B[a]P	Melatonin
2a6	Coumarin Nifedipine	Metronidazole Phenacetin	Cisapride Montelukast
2b6	Efavirenz	Ciclopidine Cyclophosphamide	Bupropion
2c8	Paclitaxel Troglitazone	Isotretinoin Amiodarone	Loperamide Rosiglitazone
2c9	Fluvastatin	Ibuprofen Phenytoin	Glimepiride Warfarin
2c19	Chloramphenicol Clomipramine	Nelfinavir	Primidone Warfarin
2d6	Fluvoxamine Metoclopramide	Mexiletine Phenacetin	Diltiazem Propranolol
2e1	Cisplatin	Acetaminophen Benzene	Chlorzoxazone Sevoflurane
3a4/5	Docetaxel Rotenone	Dapsone Flutamide	Midazolam Testosterone

Figure 13. Panel of evaluated CYP-targeting chemicals. A selection of 50+ chemicals via literature review and will be assessed for mechanistic profile.

Conclusion and Future Directions

- Steatotic HepaRG are viable but have altered CYP metabolism.
- This state **alters the hepatoxicity** of some chemical exposures, underscoring the importance of assessing hepatic steatosis as a **common susceptibility factor** for chemical toxicity.
- We can assess the impact of steatosis on **CYP enzyme activity** by examining reference toxicants targeted by specific CYP metabolism:
  - High-content imaging/analysis, especially **topographical measures,** will help us assess the impact of hepatic steatosis on chemical-mediated toxicity**.**
- Mitochondrial or cellular membrane potentials coupled with GSH/ROS measures may indicate **additional parameters** of **mechanistic toxicity** using similar workflow processes.

Fatty liver changes liver metabolism.
The resultant impact on chemical toxicity can be quantified in a high-throughput, hepatic cell culture.

Greatest thanks to Denise MacMillan and the chemistry core at the EPA for HPLC ID of CYP activity! Same to my collaborators / mentor! Literally couldn't do it alone.