NTP
National Toxicology Program

# The Evolution of Tox21: Enhancing Physiological Relevance & Interpretability with Emerging Toxicological Approach Methods (TAMs)

Stephen S. Ferguson, Ph.D.


- I have no financial relationships to disclose.
- The statements, opinions or conclusions contained therein do not necessarily represent the statements, opinions or conclusions of NIEHS, NIH or the US government.

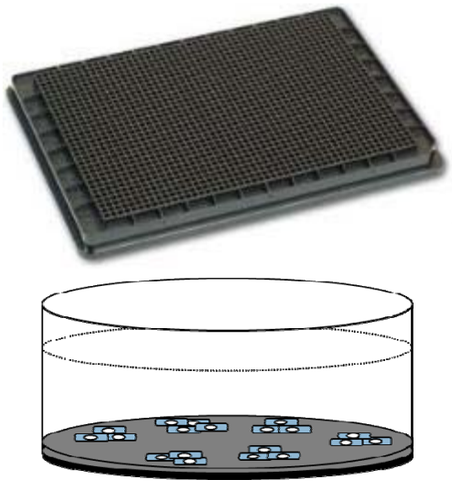
# NIEHS Focus on Environmental Exposures & Disease Risk

- Numerous research programs addressing public health challenges
- EPA has >85,000 chemicals listed in Toxic Substances Control Act (TSCA)
- Tox21 Program (NIEHS, US EPA, NCATS, & FDA)
  - Address data-poor chemicals in context with pharmaceuticals and well-studied environmental chemicals
  - Prioritize chemicals for further study
  - ~9,000 chemicals evaluated
  - ~70 high-throughput assays
  - >125 biological targets/processes
  - >120 million data points
  - Publicly Available Resources:
    - PubChem
    - Tox21 Data Browser
    - EPA CompTox Chemicals Dashboard
  - Quantitative potency estimation through in vitro to in vivo extrapolation (IVIVE)

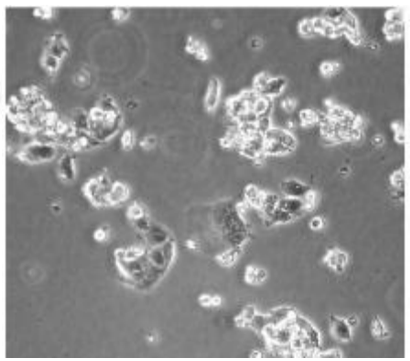
https://tox21.gov/overview/about-tox21/

- Emerging technologies are providing tools estimate human bioactivity & toxicity potential using human cells and mechanistic signaling pathway interactions

# Tox21 (NCATS, EPA, FDA & NTP)



In the beginning, there were HepG2.



ATCC Number: **HB-8065**
Designation: **Hep G2**

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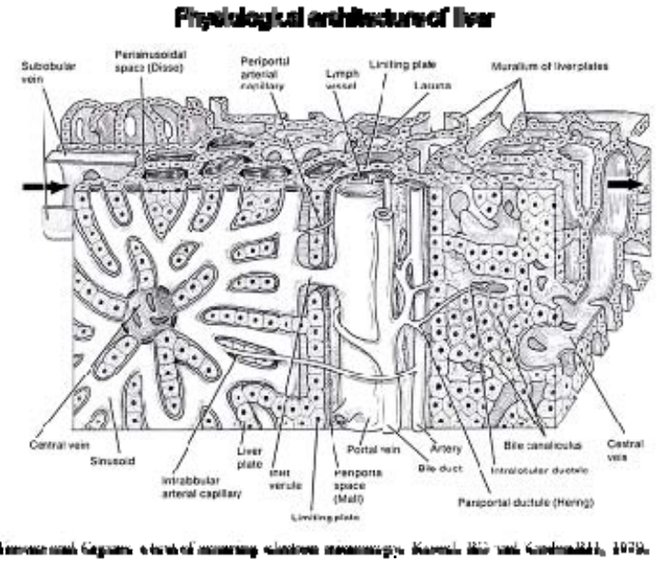
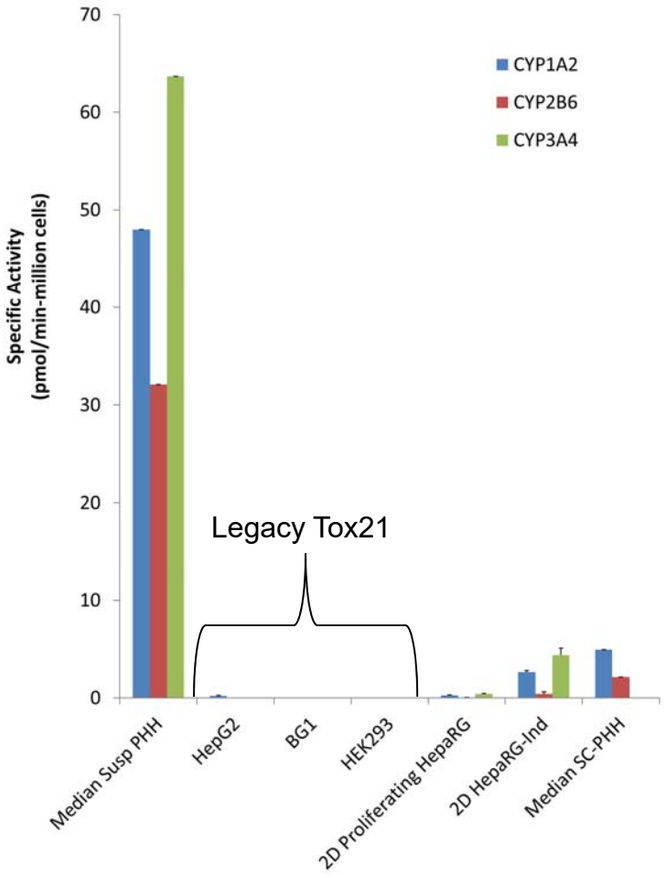


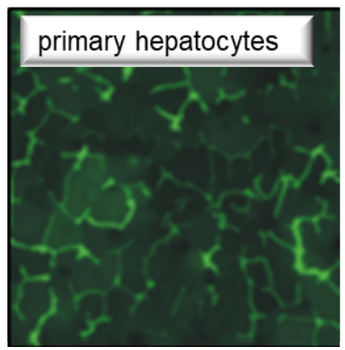

Limitations of Tox21 qHTS:

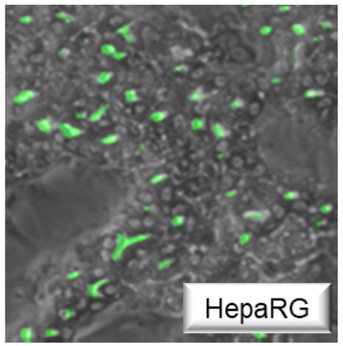
- Limited capability for xenobiotic metabolism
- Limited pathway coverage
- Focus on 'individual' cellular pathways lacking integrated biological/tissue-like functionality
- Use of immortalized and transformed cell lines
- Addition-only assays with ≤40h exposure
- linking chemicals to AOPs, pathologies, and disease



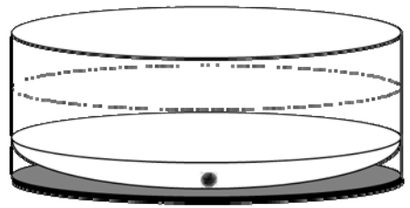
# Evolution of Tox21: Predictive Toxicology Screening

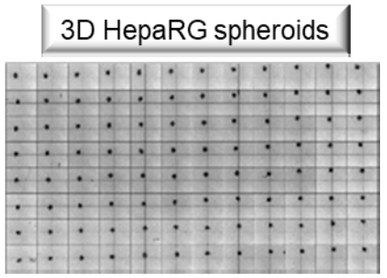
- Physiologically-relevant in vitro screening models
  - improved cellular differentiation/functionality
  - xenobiotic metabolism & bioactivation/detoxification
  - longevity to model progressions towards apical outcomes
    - pathological outcomes
    - xenobiotic clearance and drug interactions
    - pharmacology analogue case comparisons
  - species-specific response comparisons
- Multi-dimensional assay platforms (time/concentration)
  - cellular imaging
  - high throughput transcriptomics
  - metabolomics
- Quantitative translation to humans
  - IVIVE (e.g., BMC, $AC_{50}$, $CL_{int}$, $f_{ub}$)
- Extend approach:
  - Extrahepatic tissues: kidney, lung, cardiovascular, intestine
  - Susceptibility models: developmental, disease, population

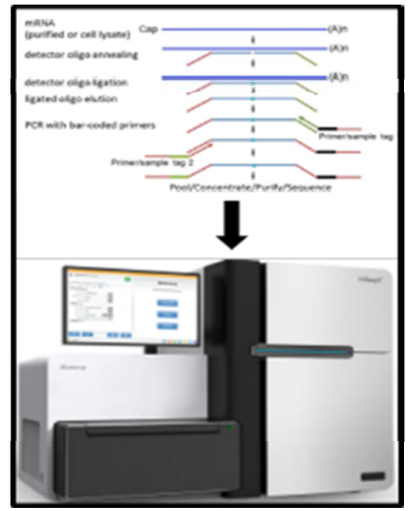


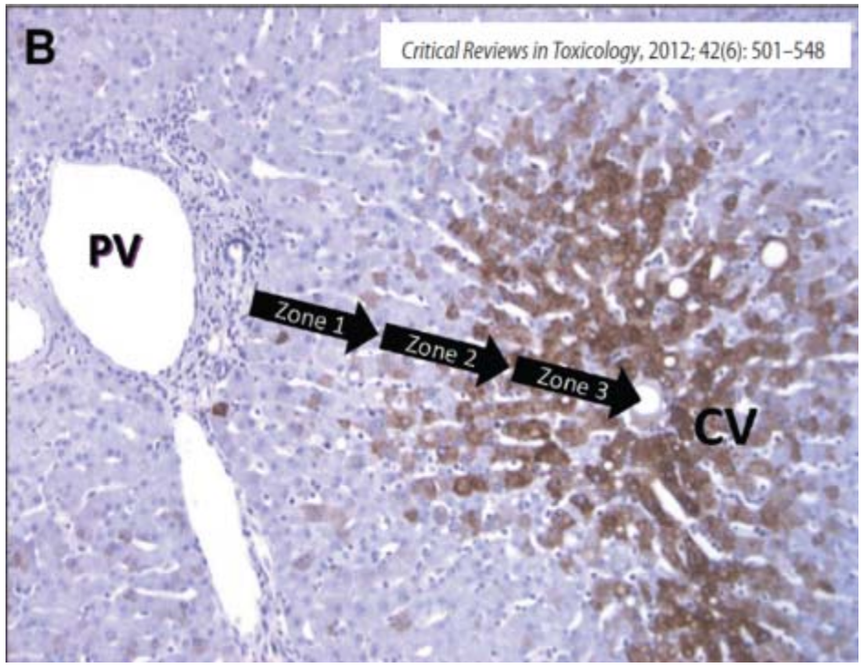







# Isolated Primary Liver Cells Rapidly De-differentiate Once Removed from Liver Tissue



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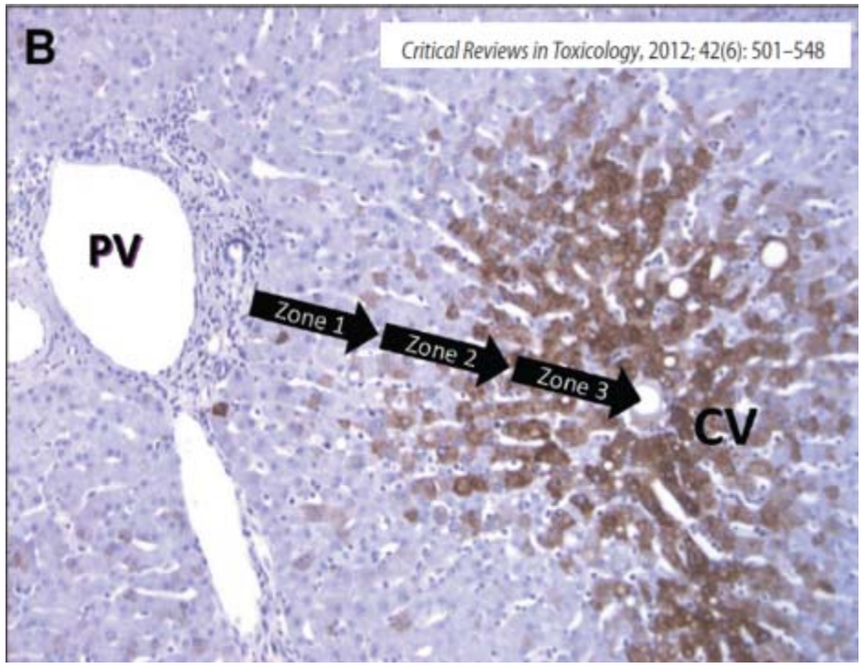


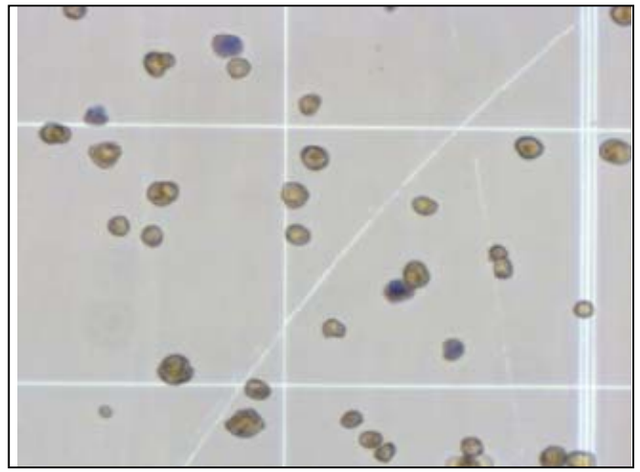

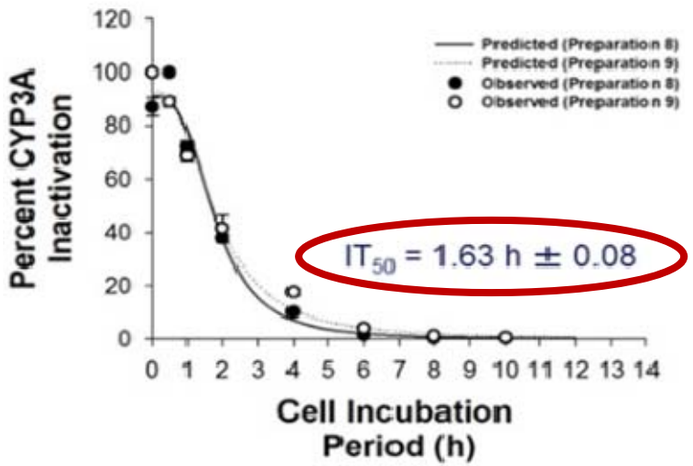


Smith et al. J. Pharm. Sci. 2012. v.101(10):3898.

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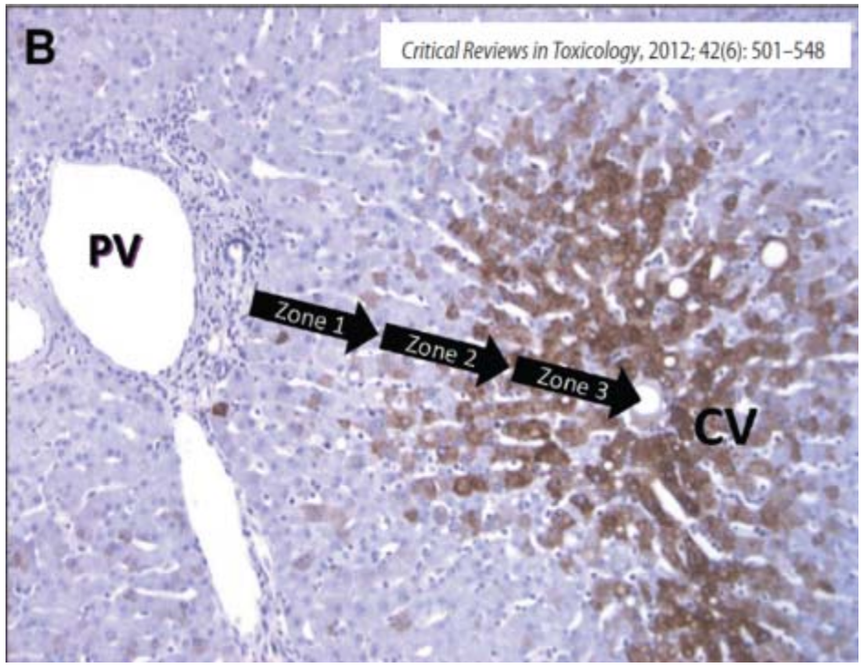


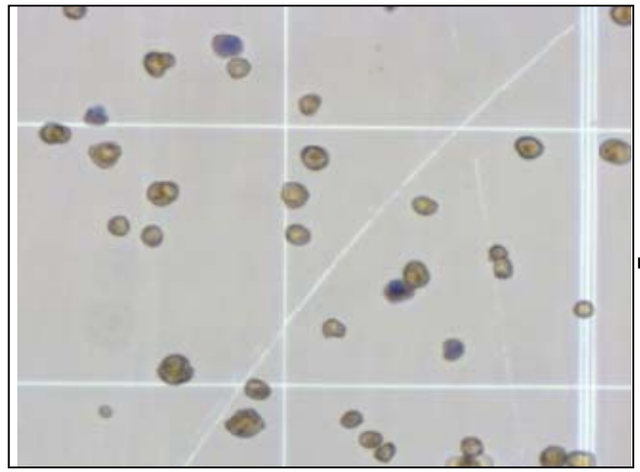

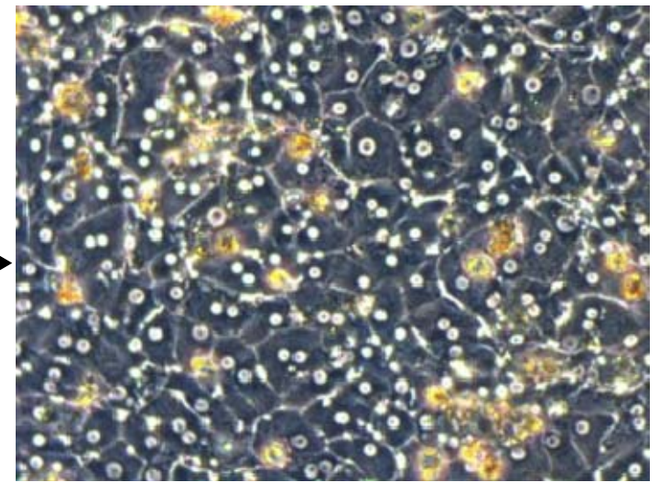

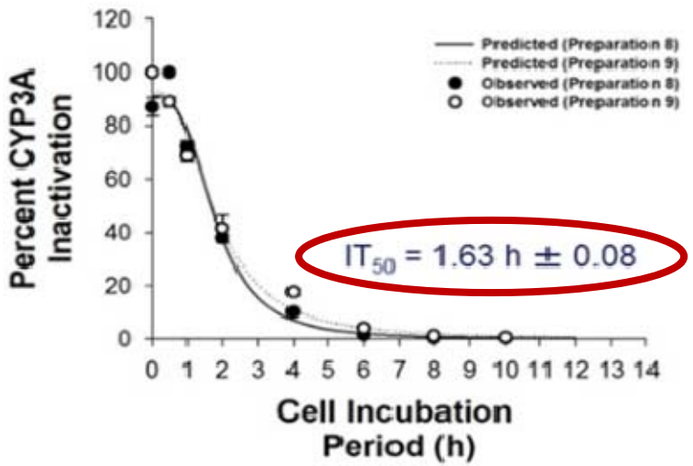


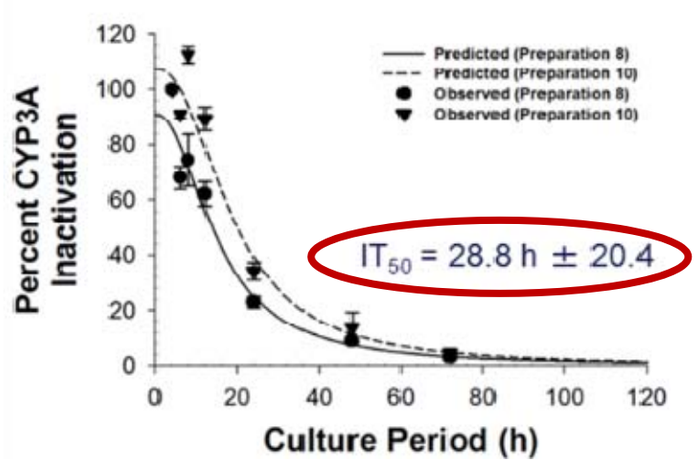


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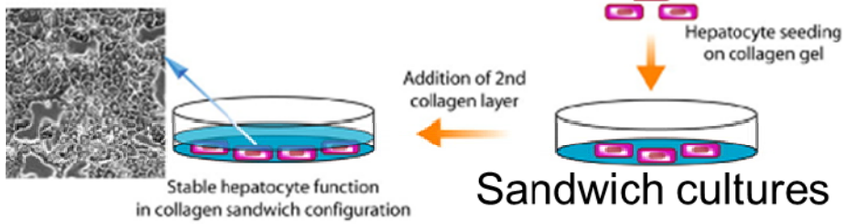
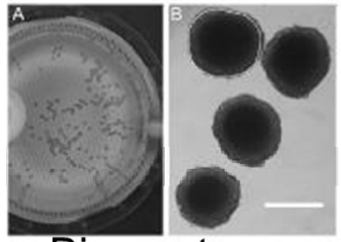
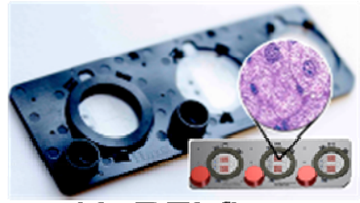


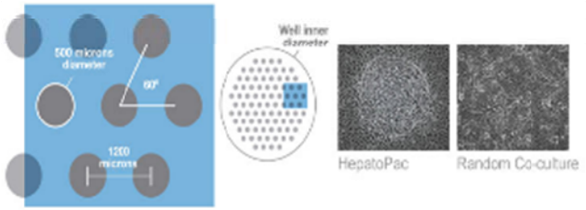

Sandwich cultures



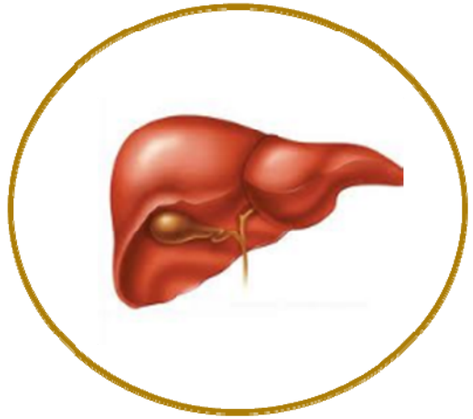
Bioreactors



HμRELflow

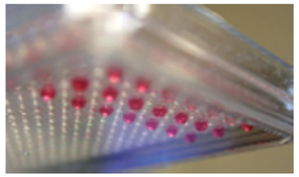



Hepatopac



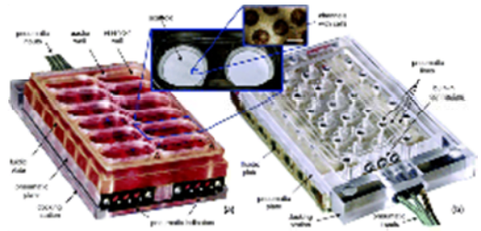
Emulate/
Wyss

3D Bioprinting
(e.g., Organovo)

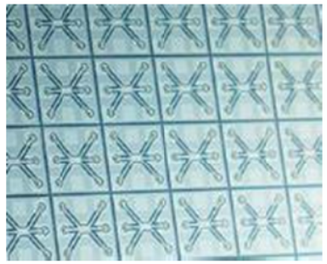


Hanging drop
(Insphero)

ULA microplate
(Corning)

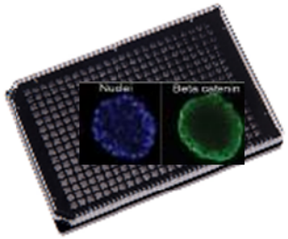


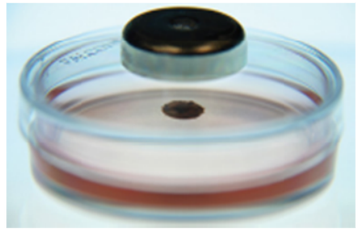
Perfused bioreactors



Mimetas

Hydrogel-based
3D models



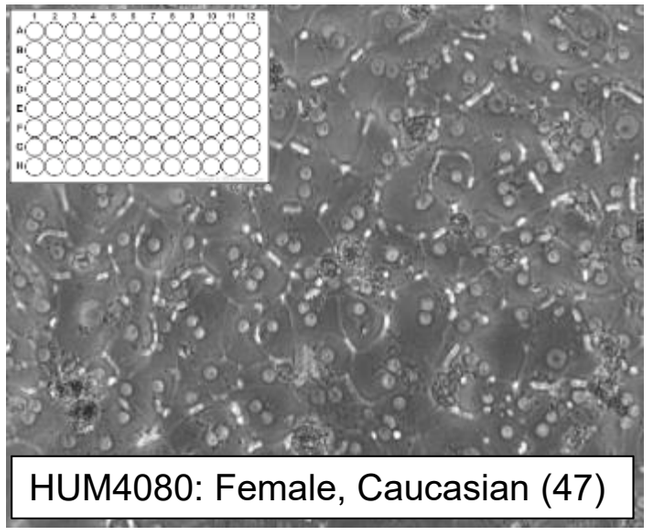



Magnetic levitation

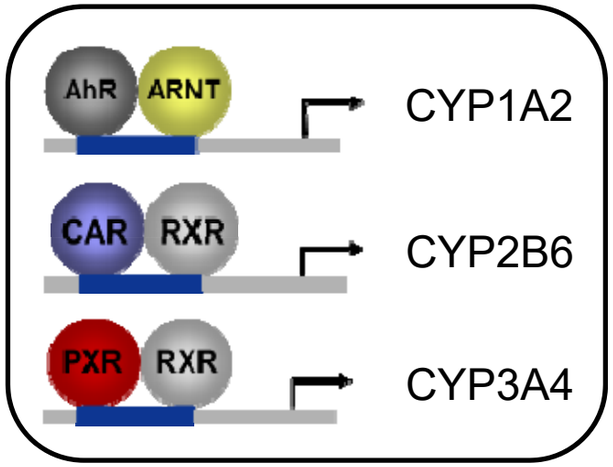
Throughput

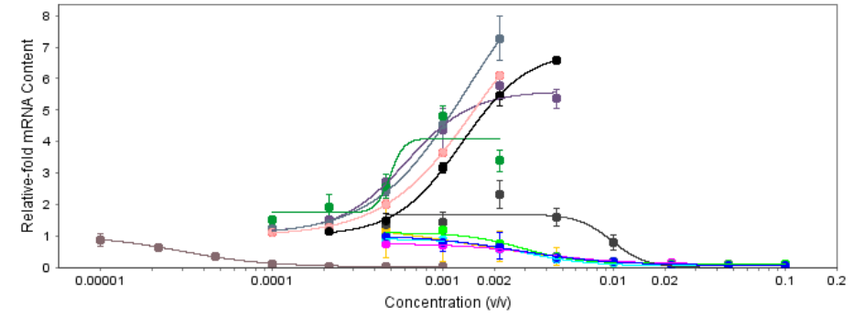
# Ginkgo Biloba Extract Liver Effects Modeling

- Sandwich cultures of PHHs (SC-PHHs)

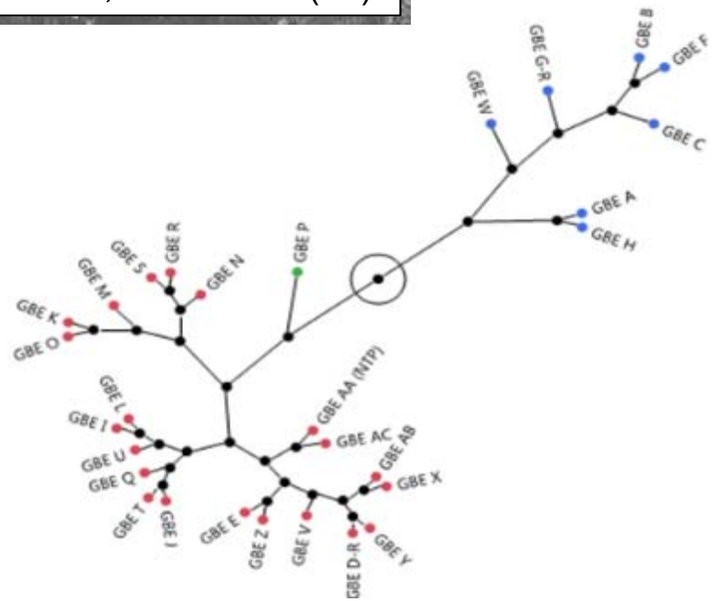


HUM4080: Female, Caucasian (47)

- Modeling liver weight increases, enzyme Induction
  - Cell Health
  - CYP450 induction/receptor activation
    - mRNA (TaqMan)
    - Liver enzymatic activity
  - Linking constituents to biological responses



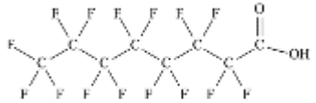

GBE-AA | CYP3A4 | EMAX: 6.90 | EMIN: 1.01 | EC50: 0.00129
GBE A | CYP3A4 | EMAX: 1.03 | EMIN: 0.0715 | EC50: 0.00266
GBE B | CYP3A4 | EMAX: 1.11 | EMIN: 0.120 | EC50: 0.00291
GBE C | CYP3A4 | EMAX: 1.69 | EMIN: 0.00106 | EC50: 0.00972
GBE F | CYP3A4 | EMAX: 0.765 | EMIN: 0.115 | EC50: 0.00365
GBE G | CYP3A4 | EMAX: 0.914 | EMIN: 0.0506 | EC50: 0.00294
GBE H | CYP3A4 | EMAX: 1.47 | EMIN: 0.0228 | EC50: 0.00142
GBE I | CYP3A4 | EMAX: 4.10 | EMIN: 1.74 | EC50: 4.91E-4
GBE J | CYP3A4 | EMAX: 9.12 | EMIN: 1.00 | EC50: 0.00156
GBE J | CYP3A4 | EMAX: 9.12 | EMIN: 1.00 | EC50: 0.00156
GBE K | CYP3A4 | EMAX: 10.2 | EMIN: 1.03 | EC50: 0.00136
GBE L | CYP3A4 | EMAX: 5.60 | EMIN: 1.11 | EC50: 6.11E-4
GBE W | CYP3A4 | EMAX: 1.02 | EMIN: 0.0248 | EC50: 2.94E-5



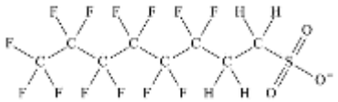
# Environmental Exposures with Differentiated HepaRG Cultures

- PFAS & AFFFs

PFOS

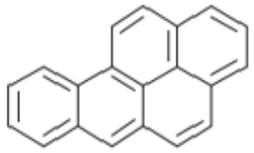


PFOA

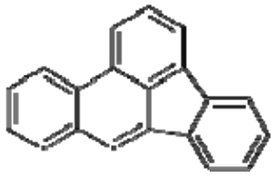


6,2-FTS

- PACs

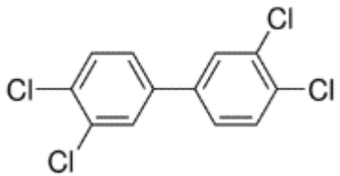


benzo(a)pyrene

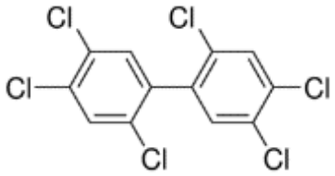


benzo(b)fluoranthene

- PCBs

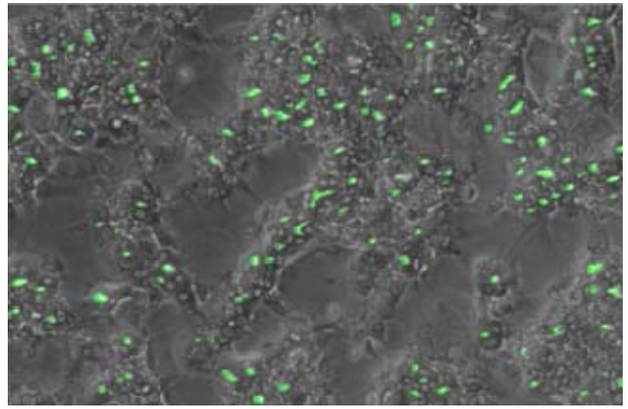


PCB126

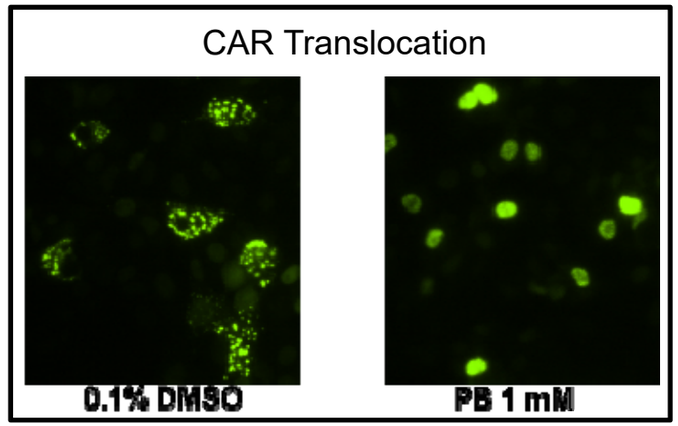


PCB153

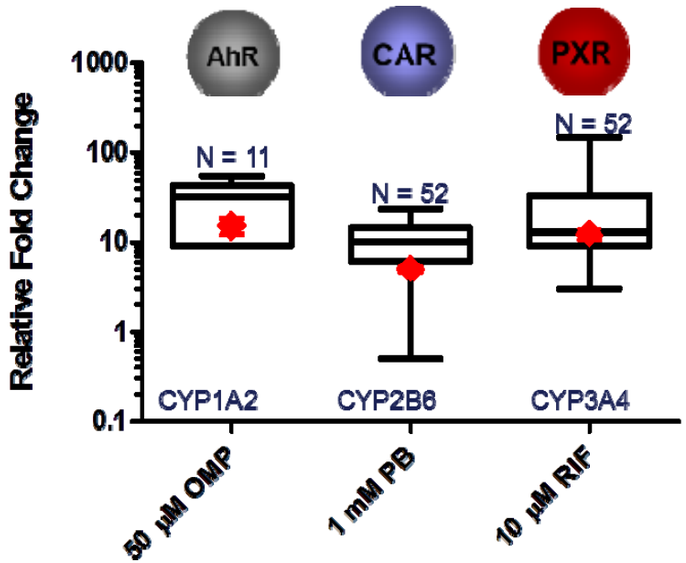
HepaRG: Hepatic Progenitor Cells



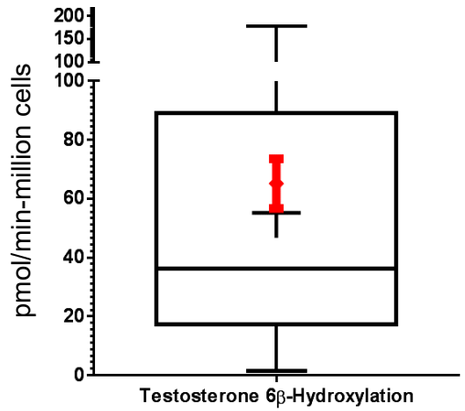
Hallmarks of Hepatocyte Functionality




Jackson et. al, Drug Metab Disp, (2016) v.44(9): 1463-79.

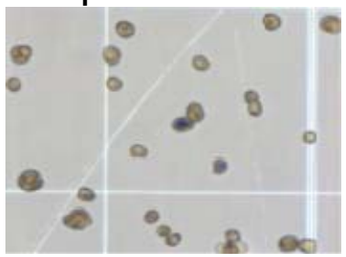



CYP3A4/5 Metabolism



Metabolic Competence

Suspension PHHs



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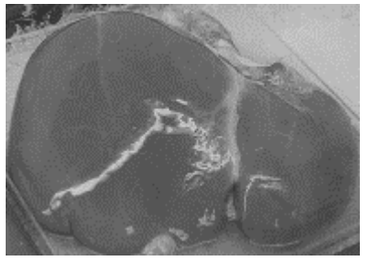
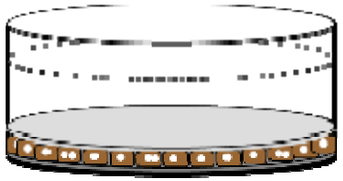
Human Liver



SC-PHHs



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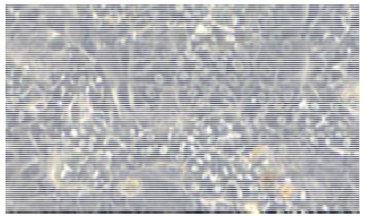
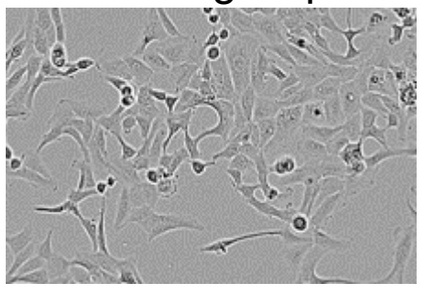
2D HepaRG

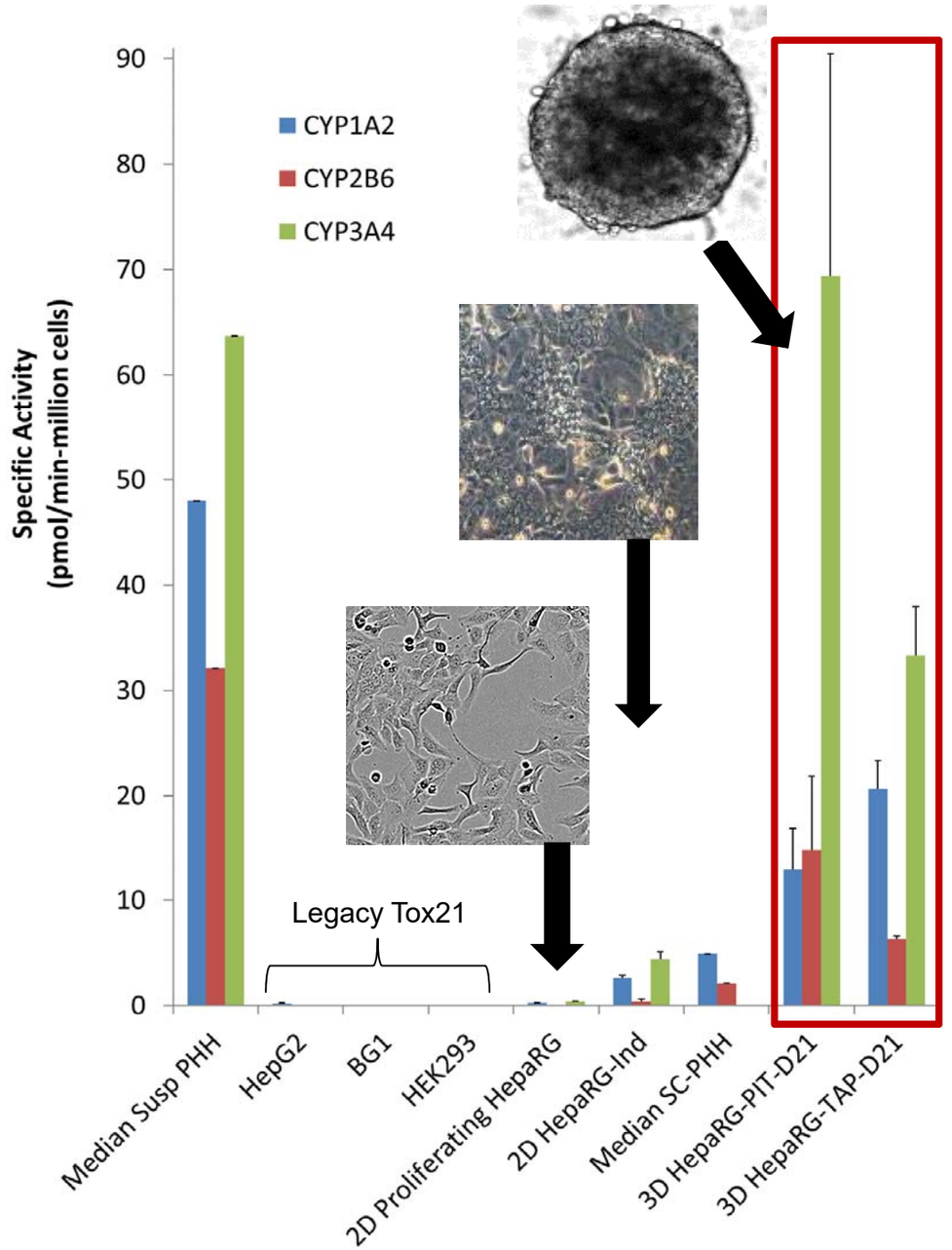


Proliferating HepaRG

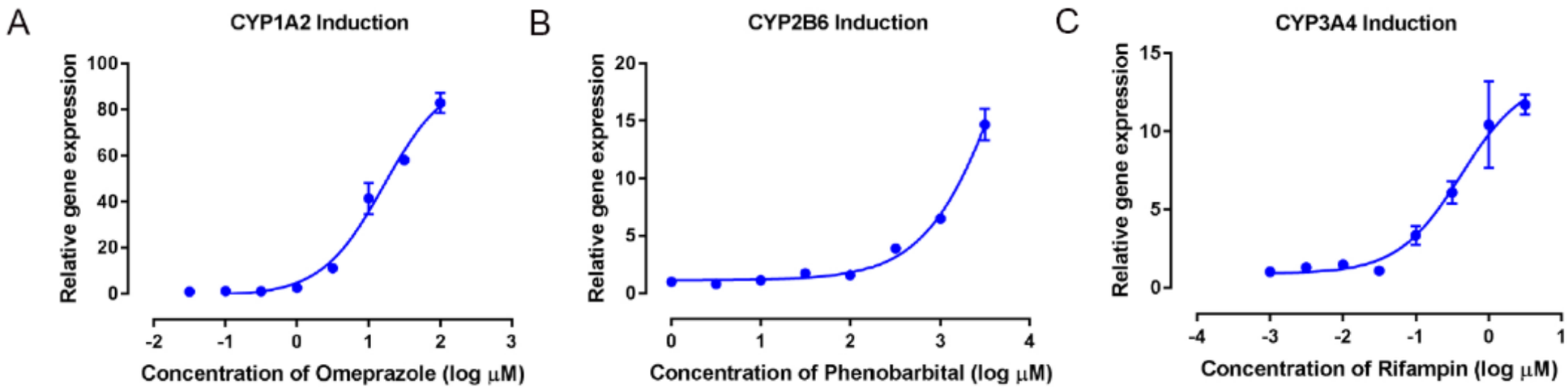


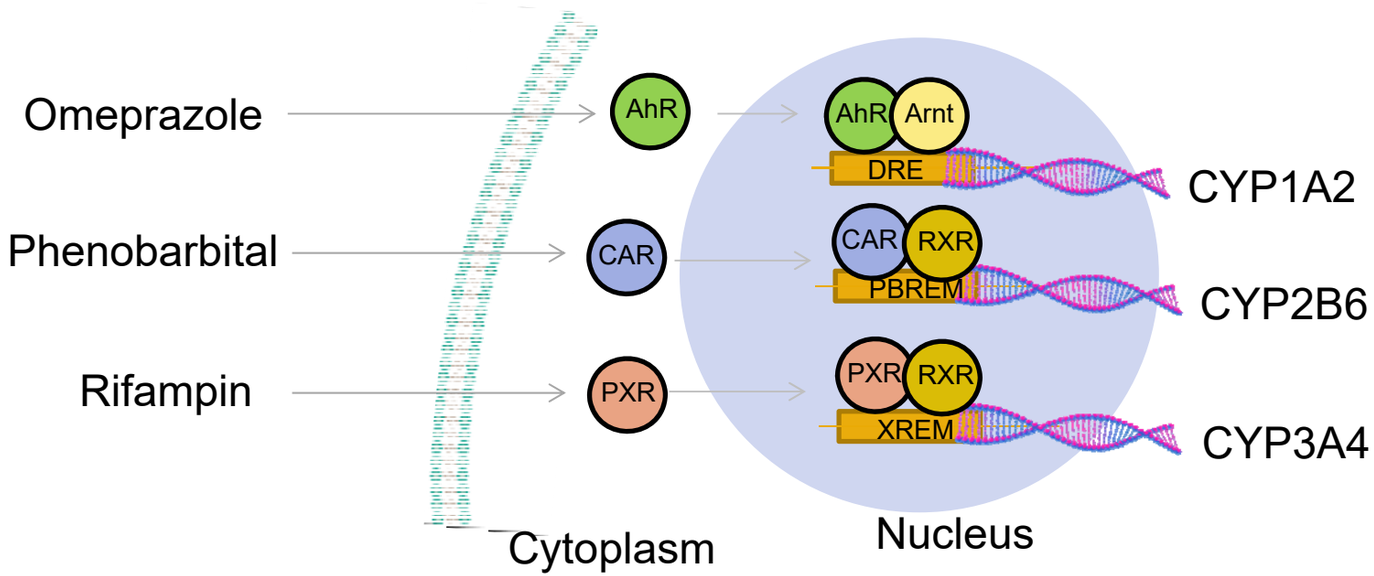
iPSC-derived hepatocytes

Transformed cell lines
(e.g., HepG2)



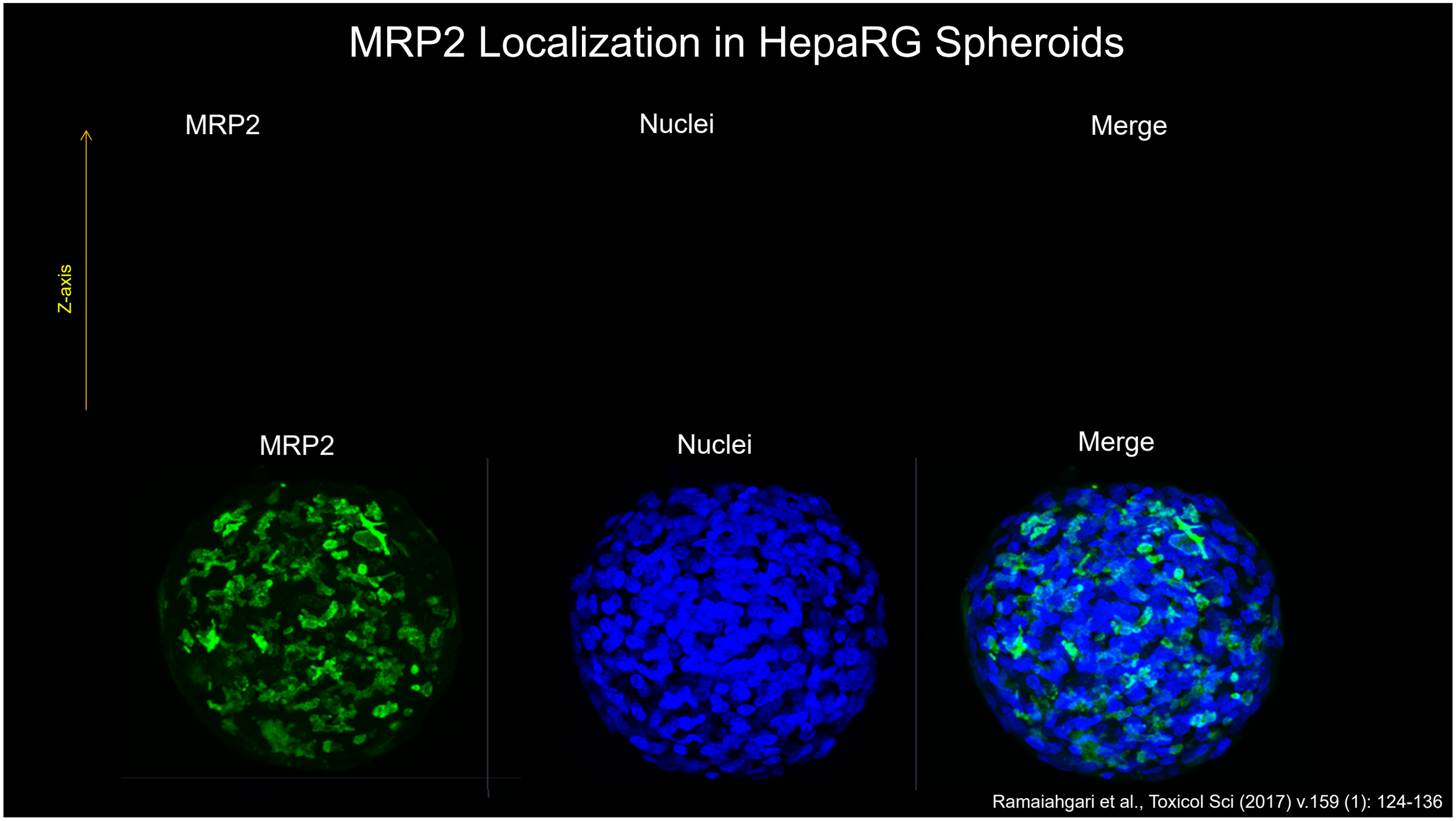
# AhR-, CAR-, & PXR-Mediated Liver Enzyme Induction



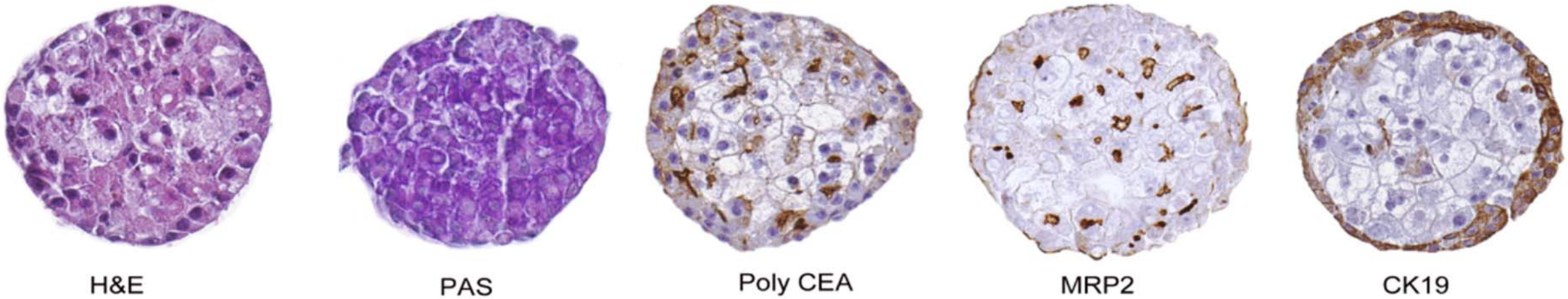

Ramaiahgari et al., Toxicol Sci (2017) v.159 (1): 124-136

# MRP2 Localization in HepaRG Spheroids




Ramaiahgari et al., Toxicol Sci (2017) v.159 (1): 124-136

# HepaRG cells form polarized spheroids




| | |
|---|---|
| PAS: | Glycogen storage |
| Poly CEA: | Glycoprotein-1 on Bile Canaliculi (BC) |
| MRP2: | Luminal transporter found at BC surfaces |
| CK19: | Marker for Cholangiocytes |

Collaboration with Darlene Dixon of NTP Labs

Ramaiahgari et al., Toxicol Sci (2017) v.159 (1): 124-136

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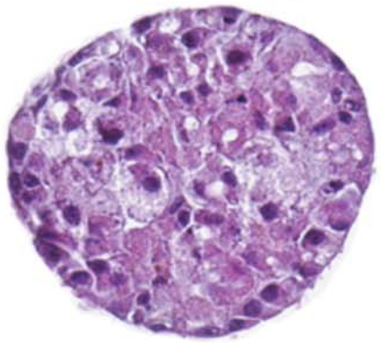
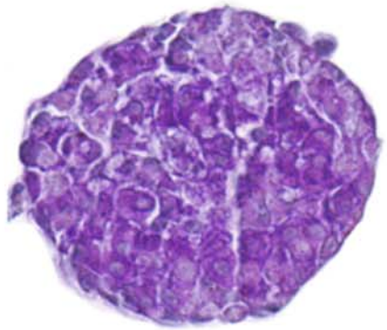
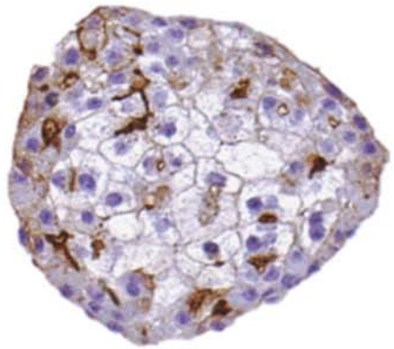


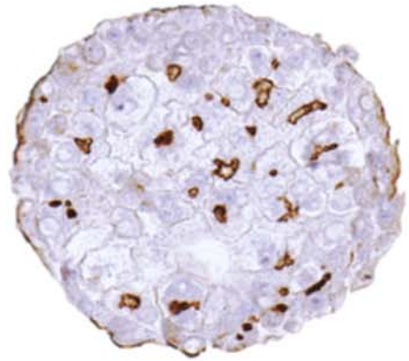
H&E



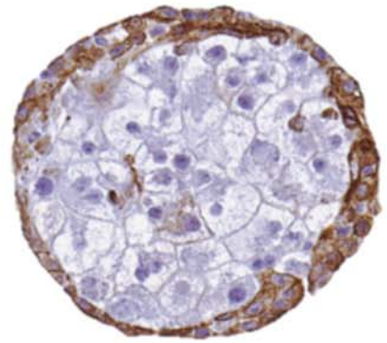
PAS



Poly CEA



MRP2



CK19

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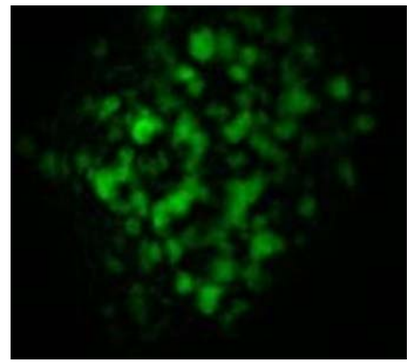

Live-cell CLF

Collaboration with Darlene Dixon of NTP Labs

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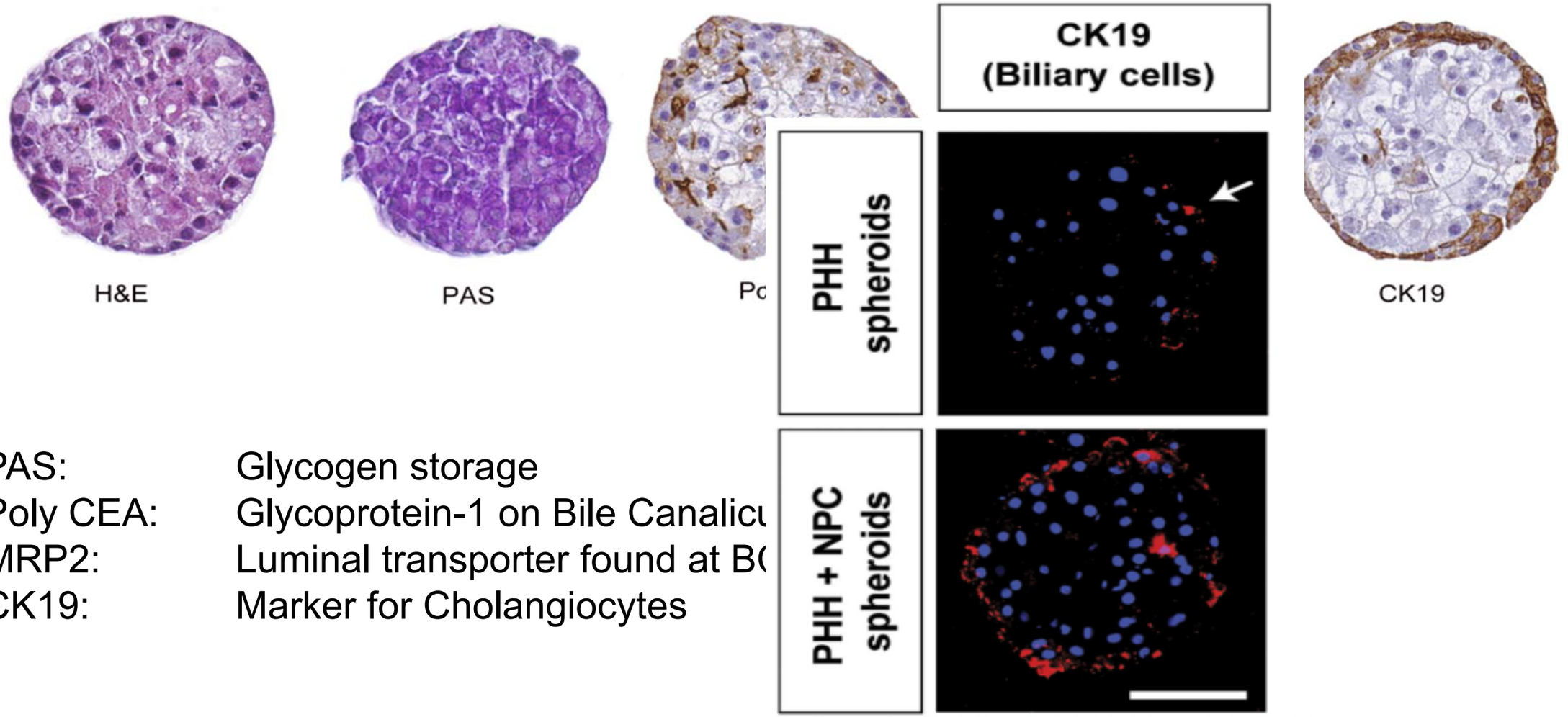
# HepaRG cells form polarized spheroids




PAS: Glycogen storage
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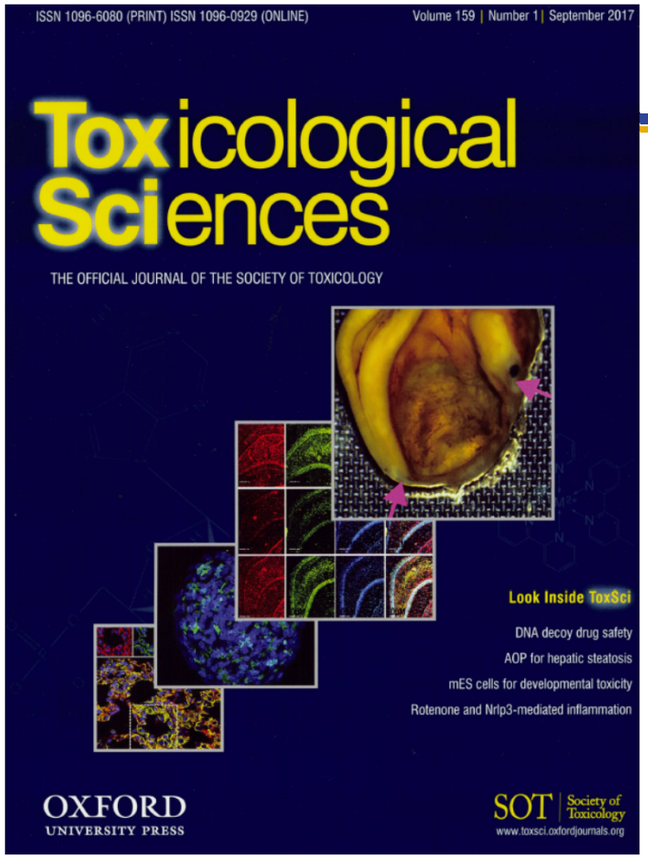
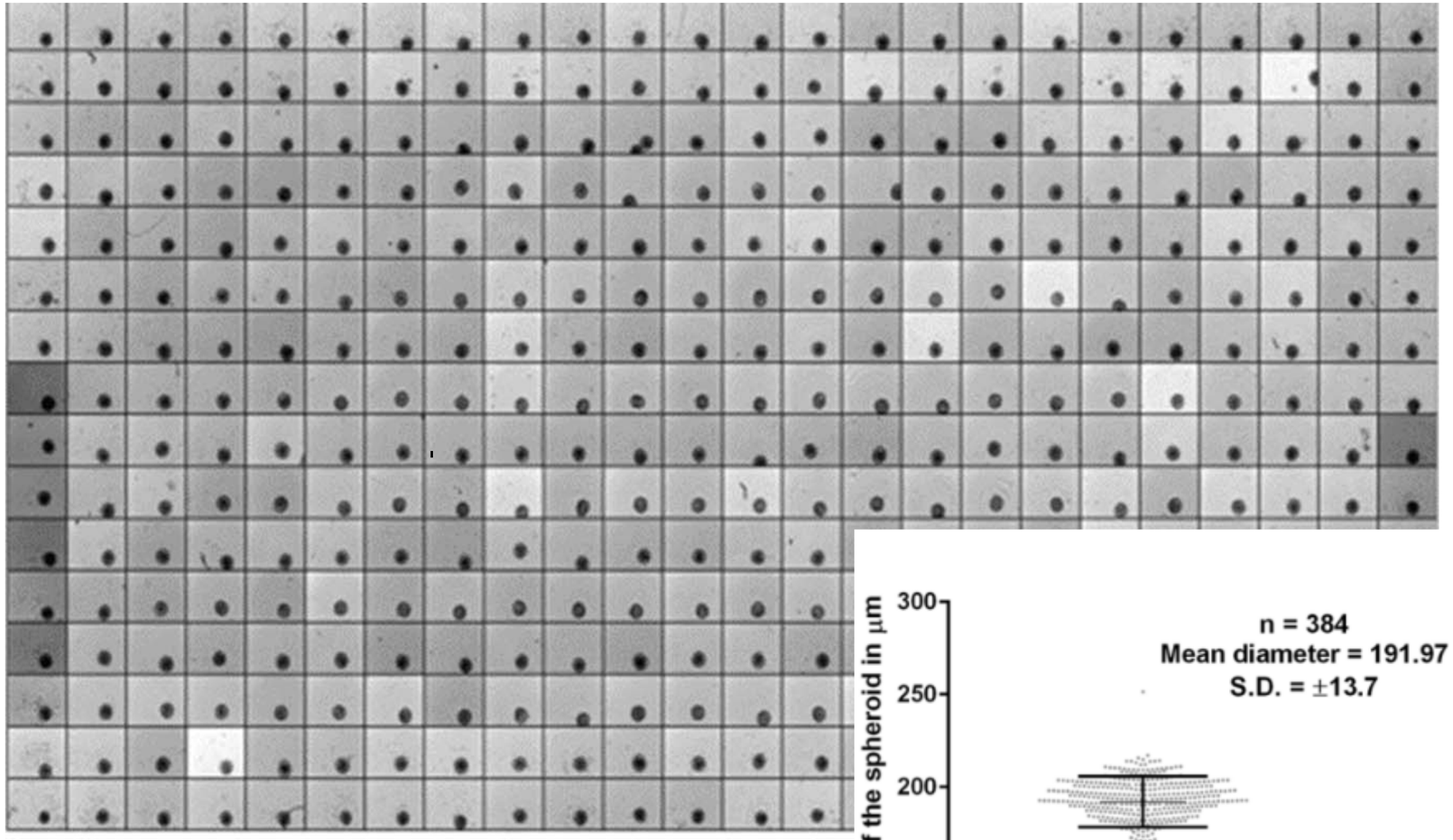
Bell et al., Sci Rep. 2016 May 4;6:25187.

Collaboration with Darlene Dixon of NTP Labs

Ramaiahgari et al., Toxicol Sci (2017) v.159 (1): 124-136

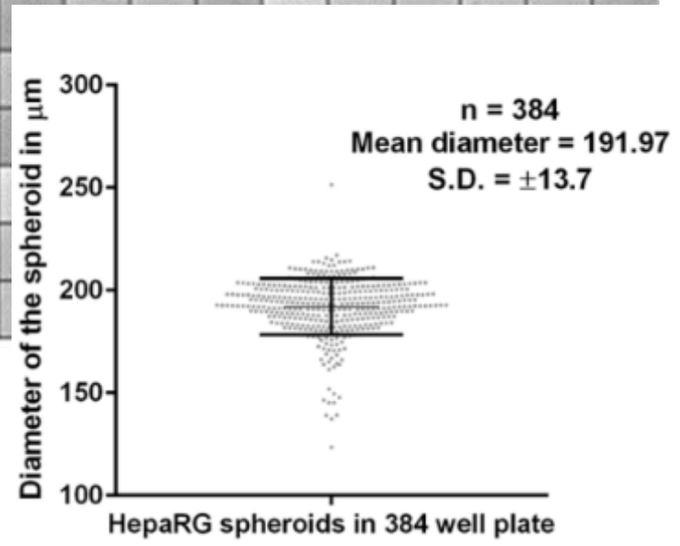



# 3D HepaRG Spheroids (384-well)



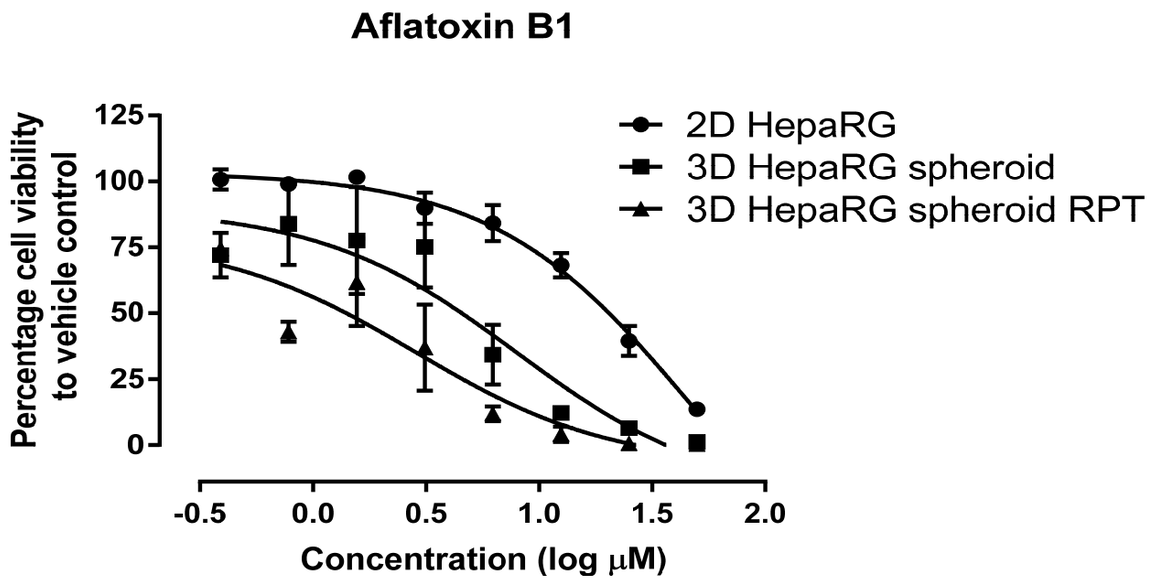
Dr. Sreenivasa Ramaiahgari

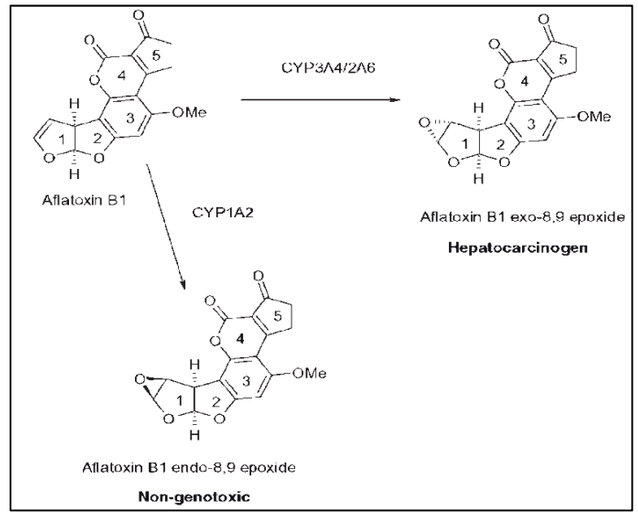
1 vial of 10 million cells
= 1 X 2D 384-well plate
= 12-25 X 384-well plates



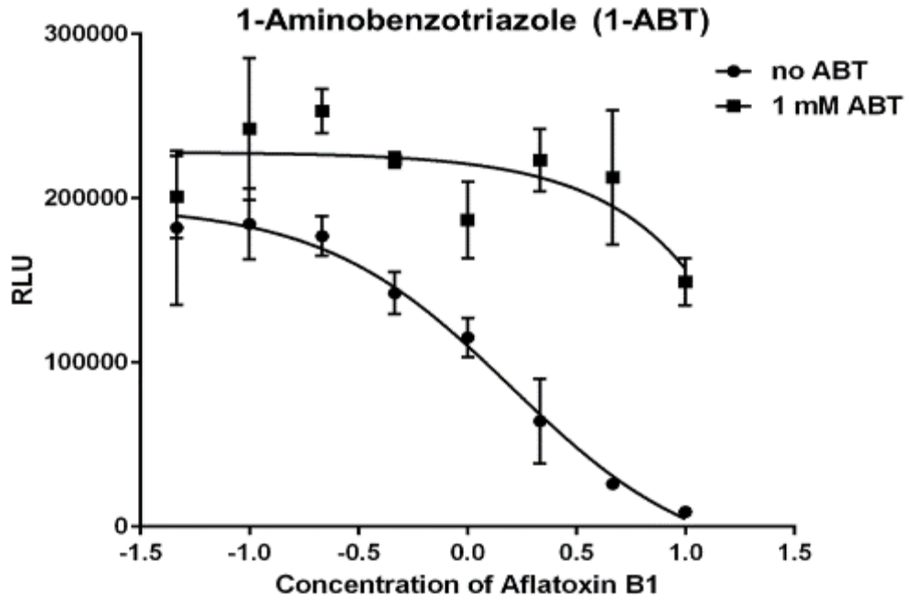

From the Cover: Ramaiahgari et al., Toxicol. Sci (2017) v.159 (1): 124-136

# HepaRG spheroids sensitive to metabolically-activated aflatoxin B1 cytotoxicity



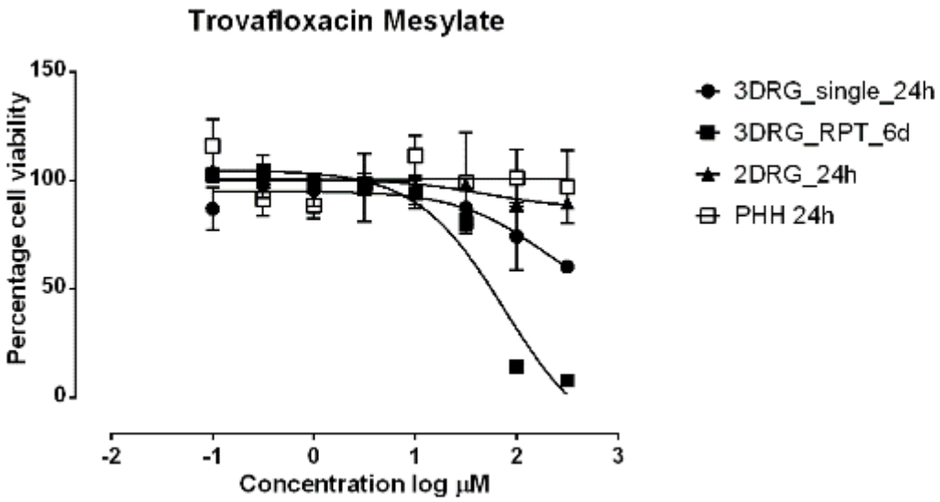



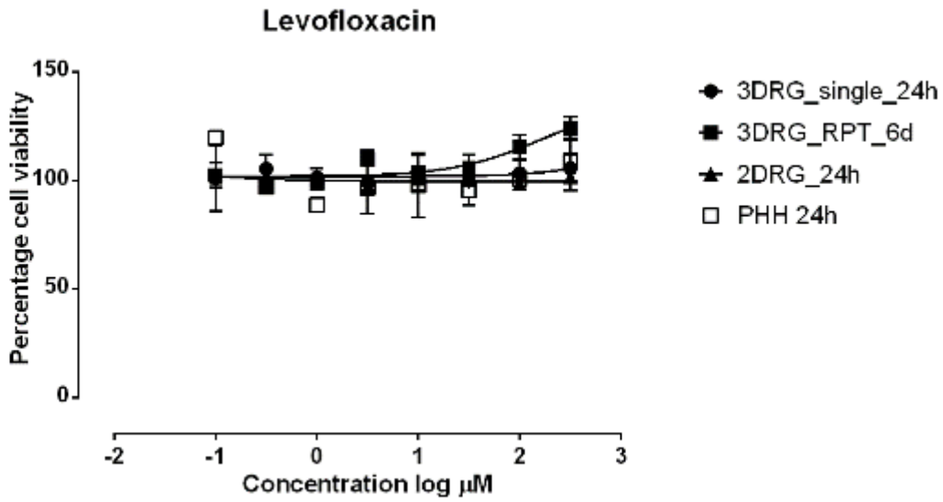

| | 2D HepaRG | 3D HepaRG | 3D HepaRG repeat exposure |
|---|---|---|---|
| $TC_{50}$ | 46.54 μM | 7.94 μM | 2.83 μM |

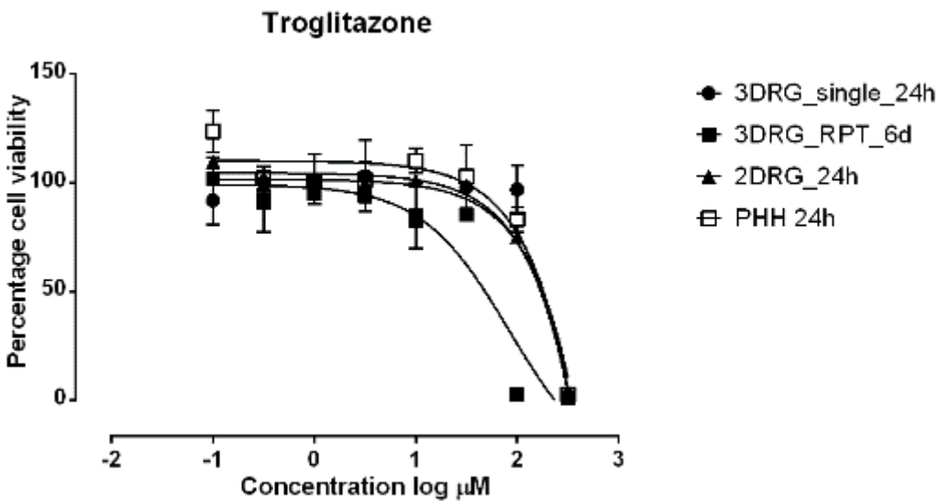


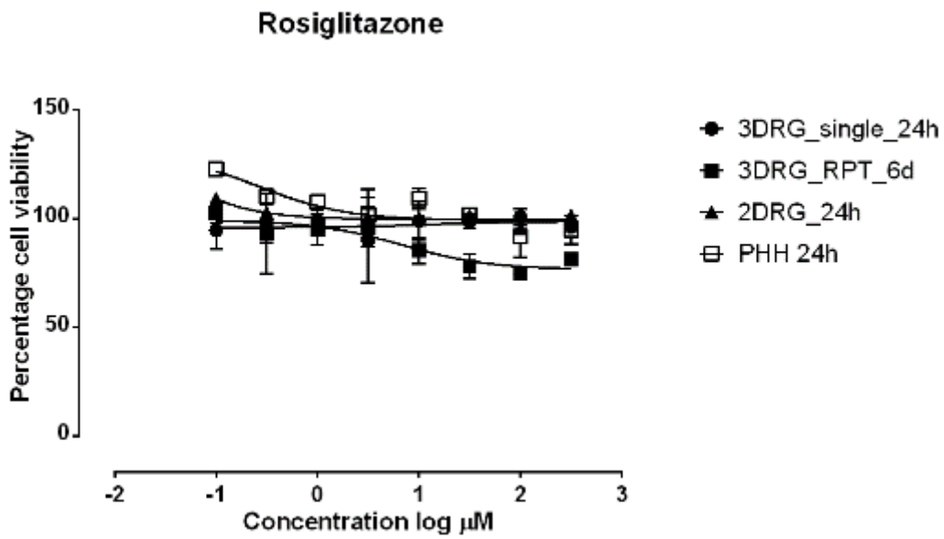

Ramaiahgari et al., Toxicol Sci (2017) v.159 (1): 124-136

# 3D HepaRG Spheroid Responses to Drug Analogues



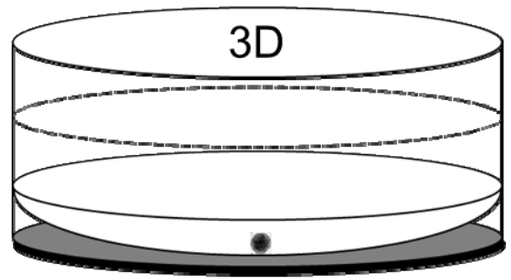

Ramaiahgari et al., Toxicol Sci (2017) v.159 (1): 124-136

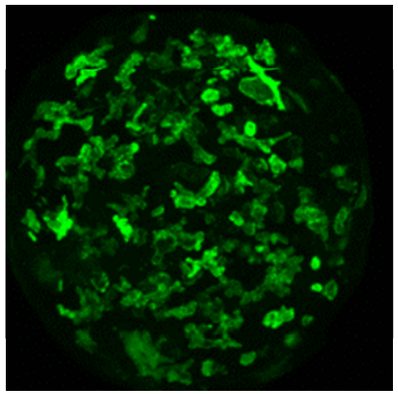
# What's So Special About 3D HepaRG Spheroids?

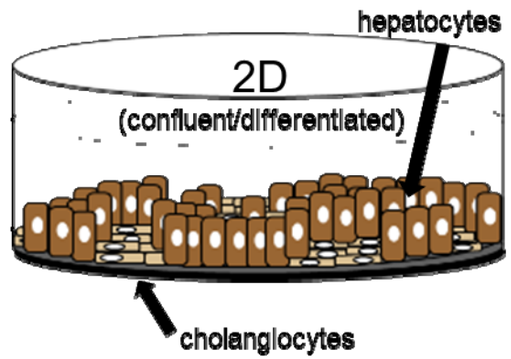
- 3 Culture Configurations of HepaRG Cells (384-well formats)
- 24 Compounds
  - Liver injury/metabolically-activated toxicity
  - Hepatic receptor activators
  - Drug analogue comparisons
  - 'Negatives' for liver injury

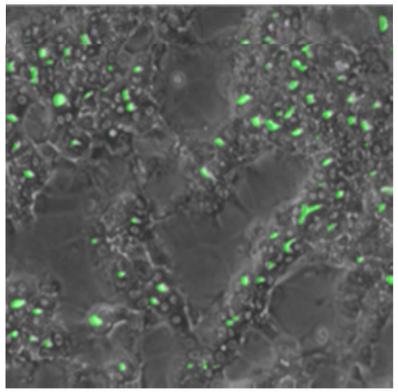
| | | | | | |
|---|---|---|---|---|---|
| acetaminophen | caffeine | diphenhydramine | DMN | rifampicin | tamoxifen |
| aflatoxin B1 | CDCA | fenofibric acid | omeprazole | ritonavir | troglitazone |
| aspirin | chlorpromazine | levofloxacin | phenobarbital | rosiglitazone | trovafloxacin |
| benzo(a)pyrene | cyclophosphamide | menadione | KCl | sucrose | valproic acid |

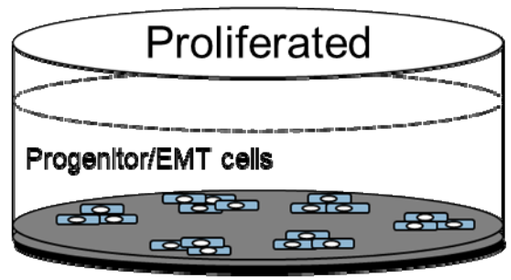
- Assays:
  - cell morphology (Incucyte, daily for each culture well)
    - Image classifications, quantitative masking of confluence
  - cytotoxicity (LDH leakage)
  - high throughput transcriptomics (HTT with S1500+, TempO-Seq)

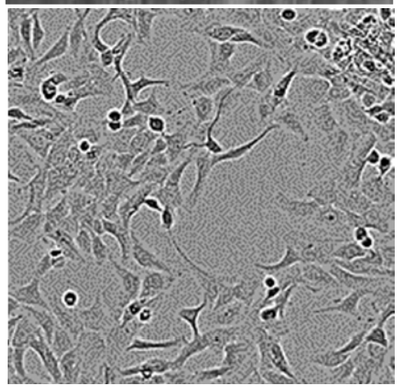


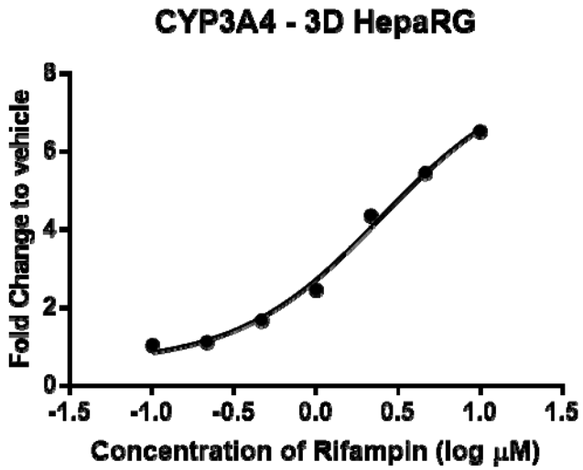


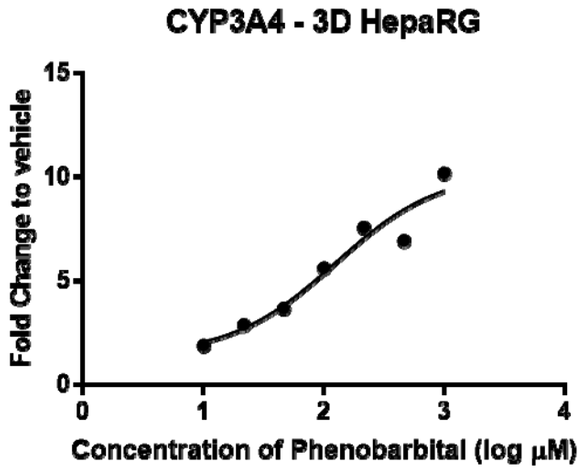



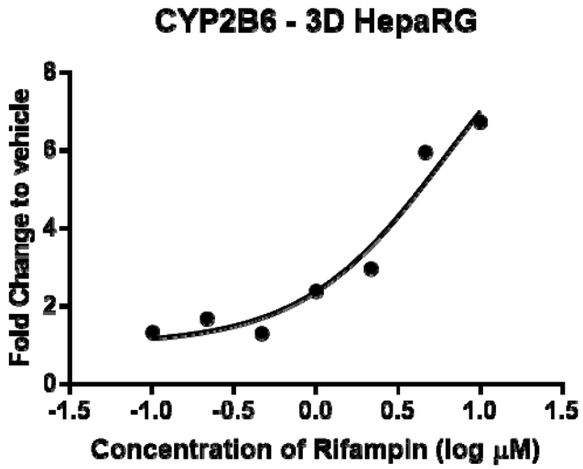


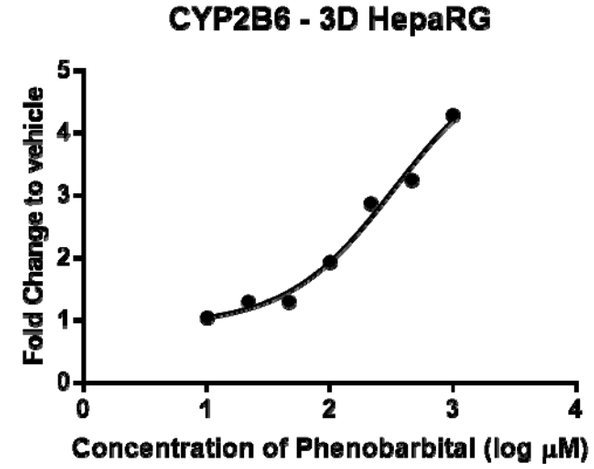



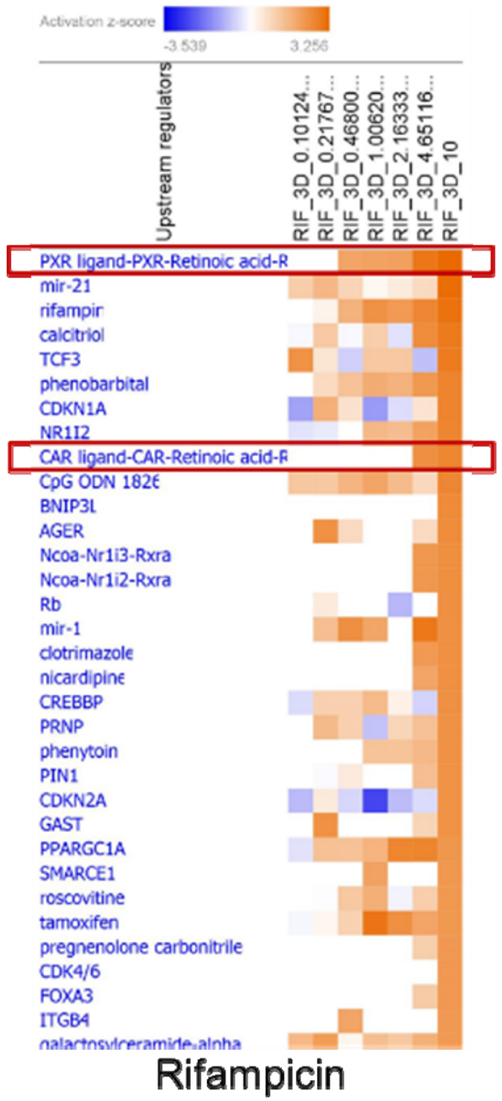
# 3D HepaRG Spheroids Model Gene- and Pathway-level Transcriptomic Functionality

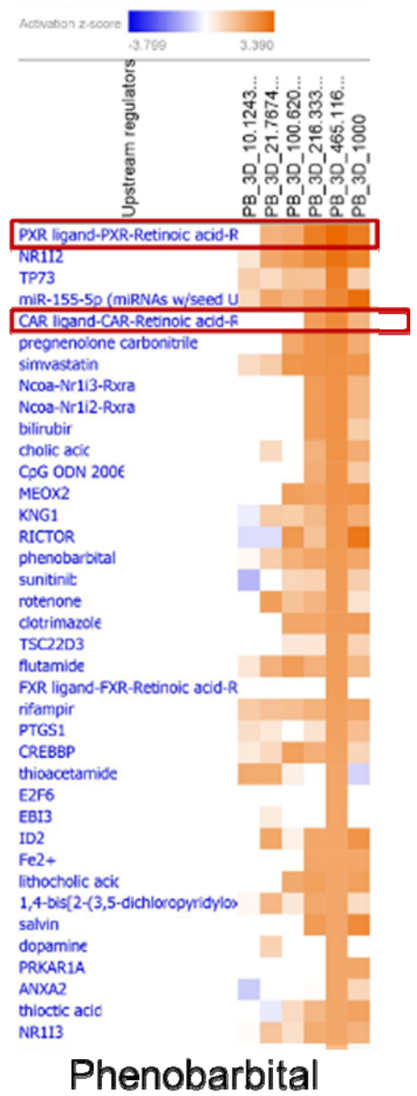


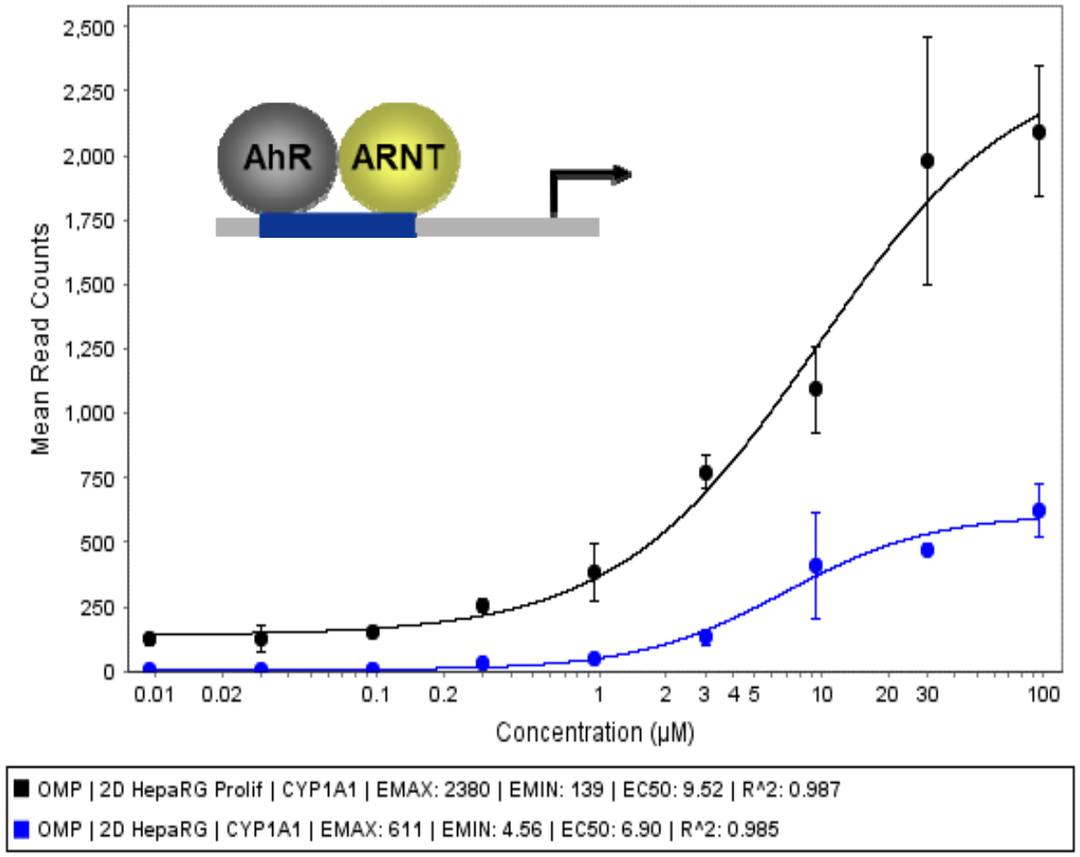



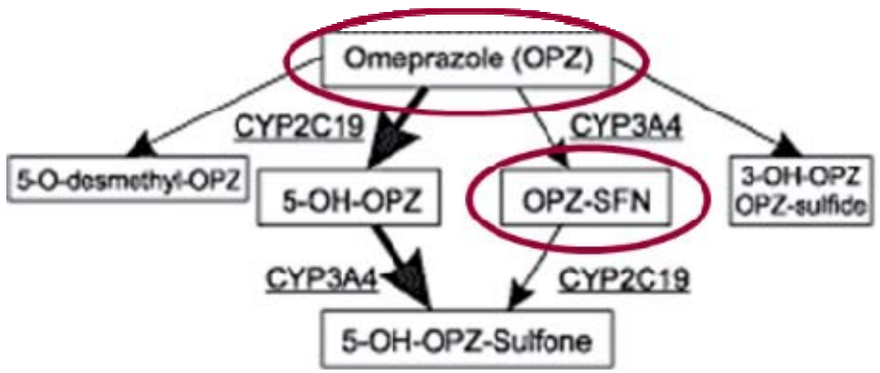



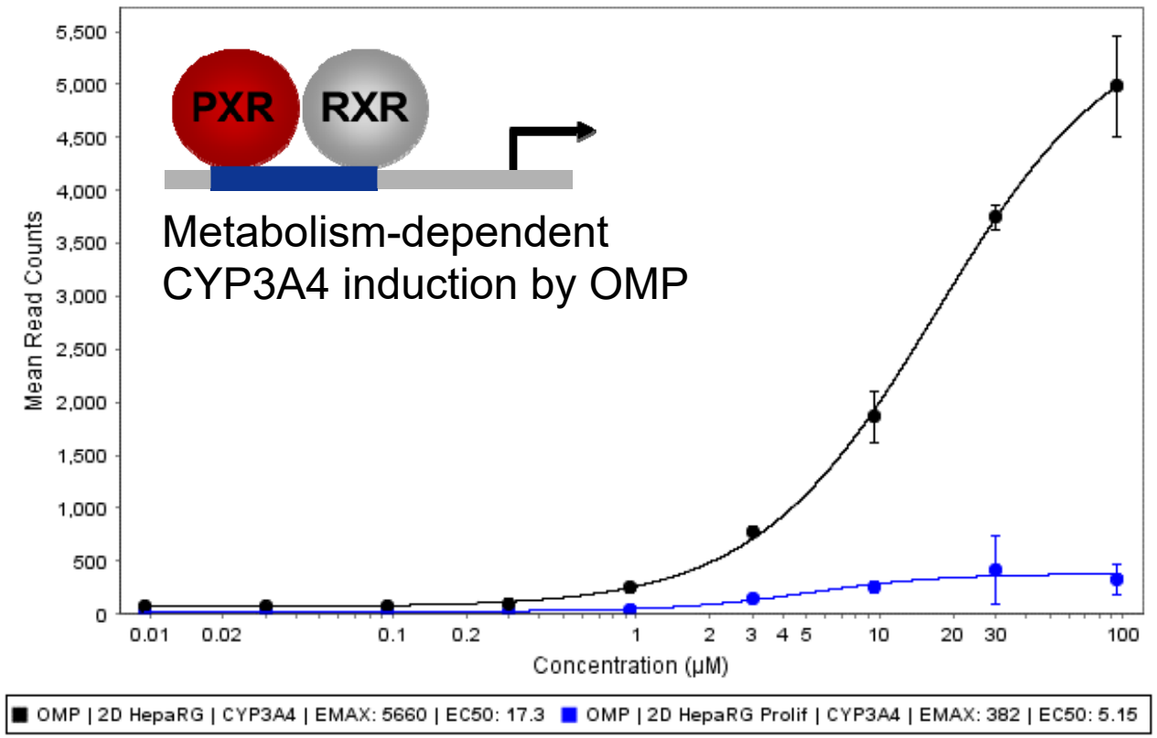



# Omeprazole

- Elevated basal CYP1A1 expression in PROLIF HepaRG; linked to liver development
- AhR functionality in 2D & PROLIF
- Reduced xenobiotic metabolism competence impacts CYP3A4 inducibility (PXR)



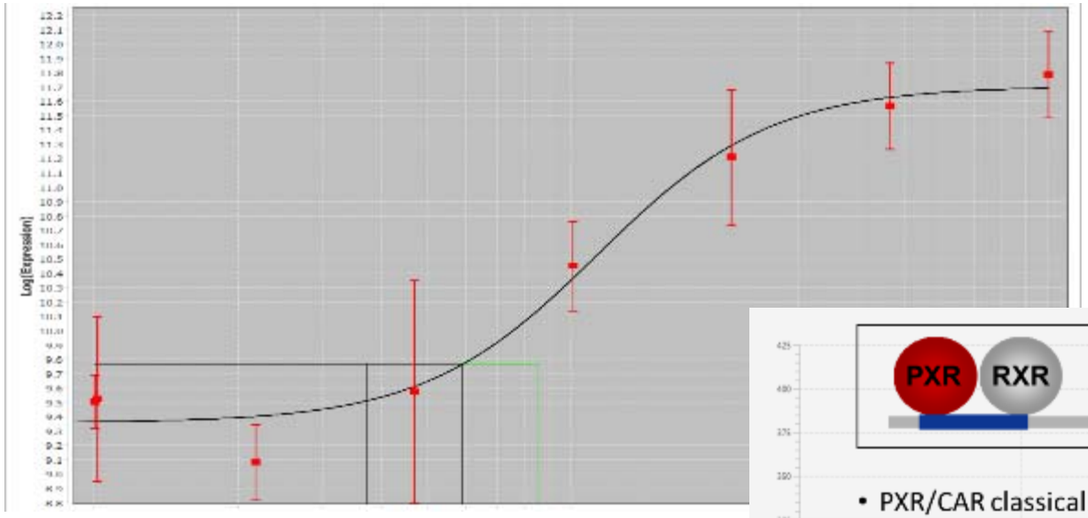



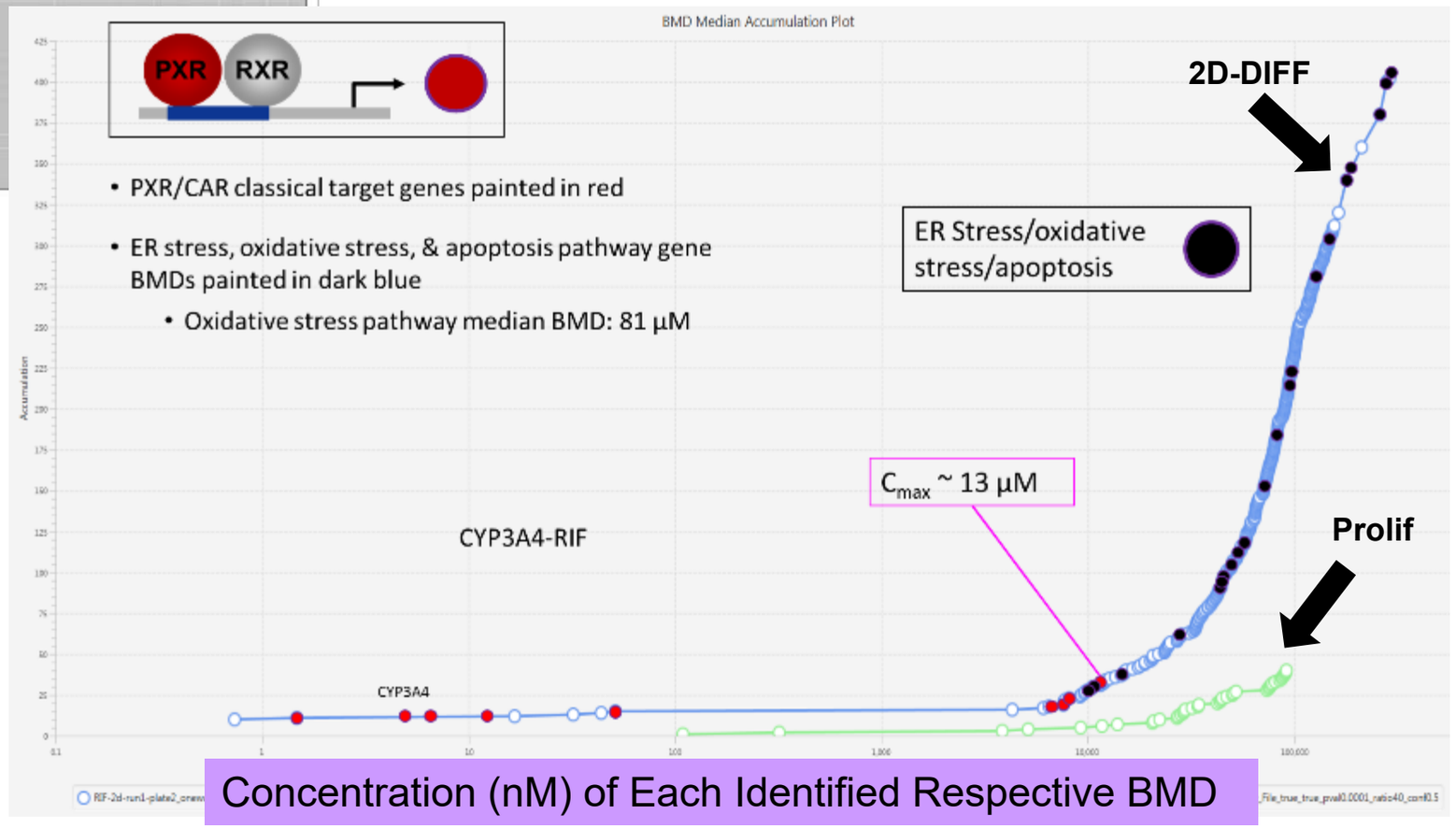

Ramaiahgari et al., 2019 (Jun) Toxicological Sciences, v.169 (2), 553-566.

# Benchmark Concentration Analysis of Transcriptomic Response

CYP3A4 BMC Curve



BMC Accumulation Plot (each circle an individual gene BMC)

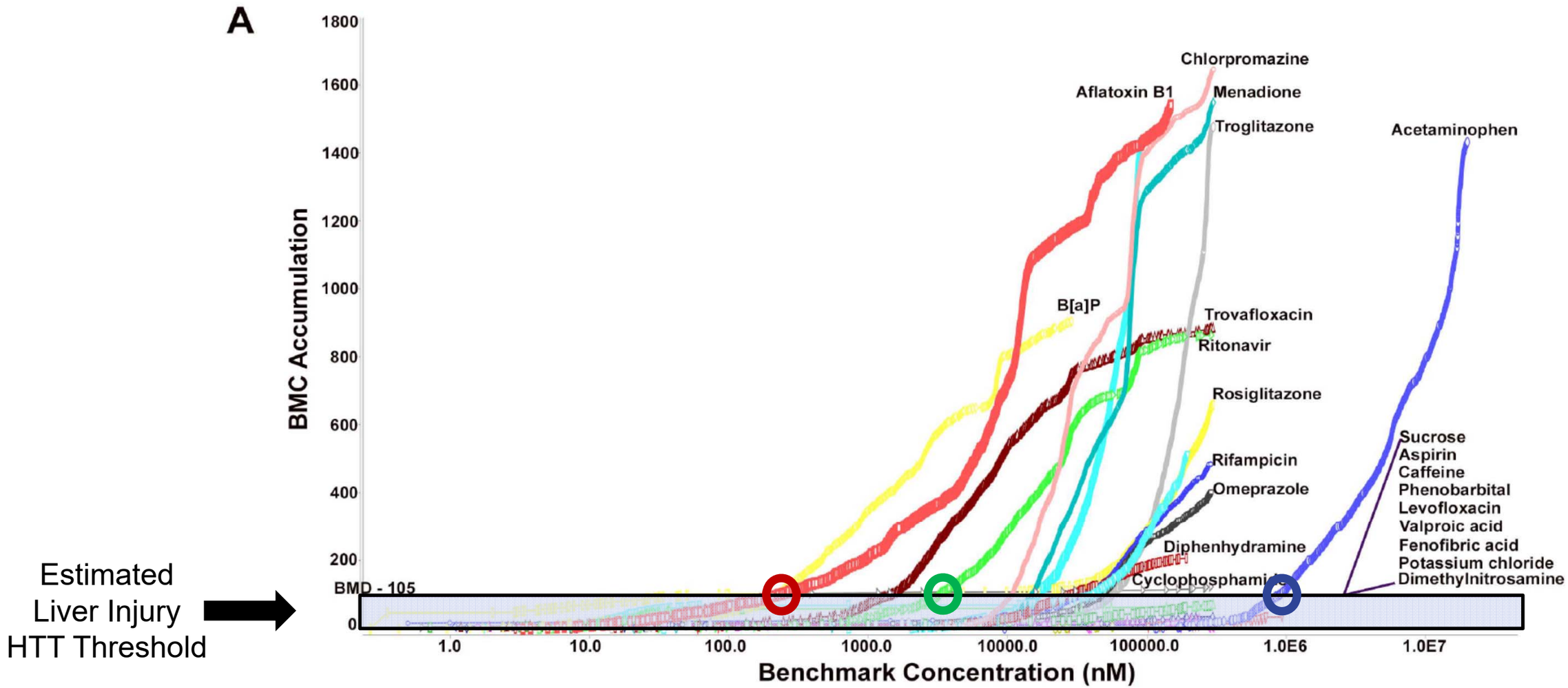




Scott Auerbach & Sciome

Concentration (nM) of Each Identified Respective BMD

# Estimation of Liver Injury Potential with Benchmark Concentration Analysis of High Throughput Transcriptomics (S1500+) with 2D Differentiated HepaRG

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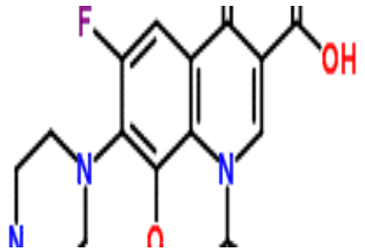
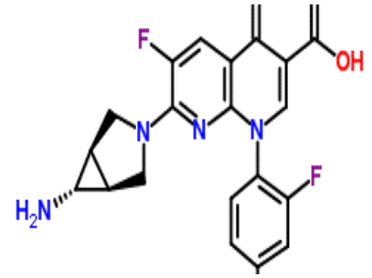
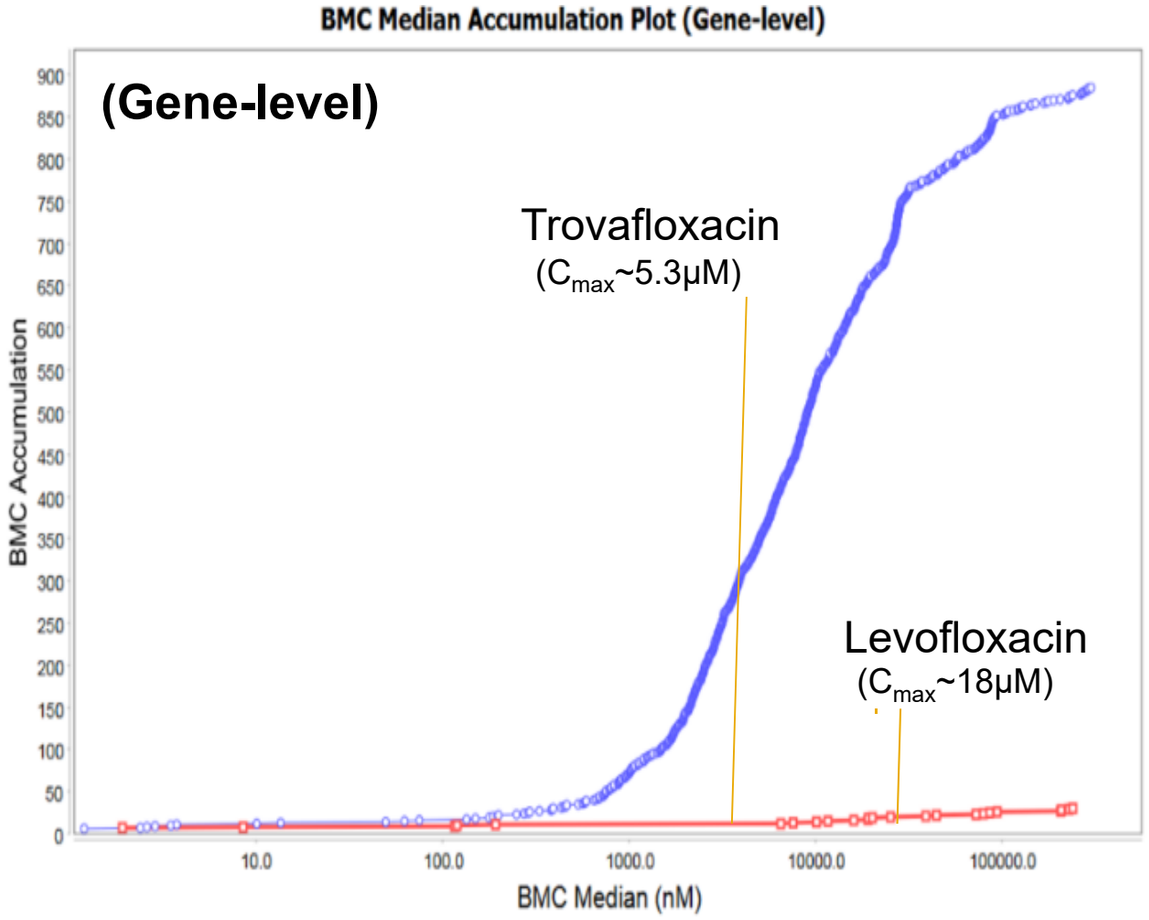
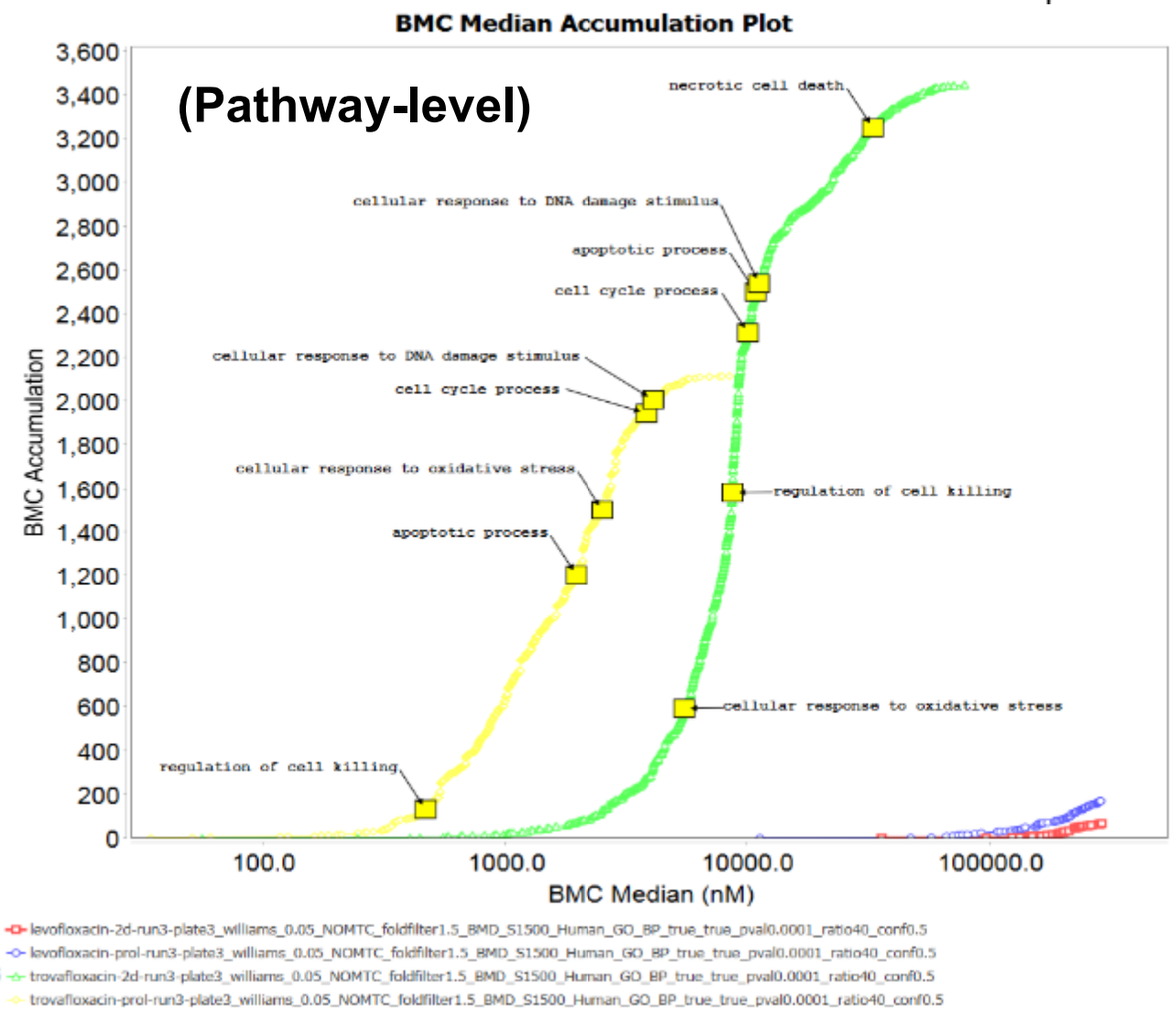


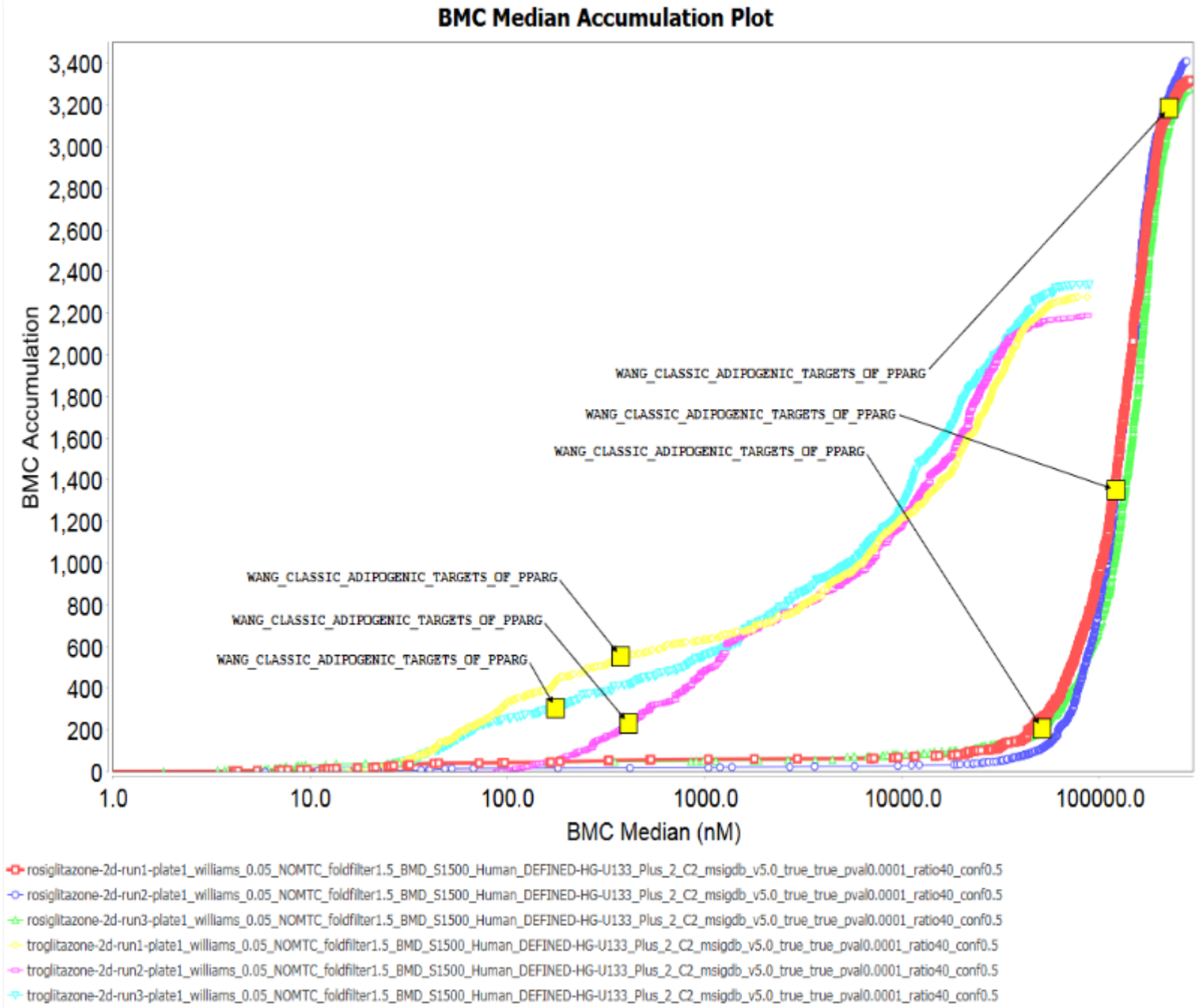

# trovafloxacin vs. levofloxacin




Ramaiahgari et al., 2019 (Jun) Toxicological Sciences, v.169 (2), 553-566.




Ramaiahgari et al., 2019 (Jun) Toxicological Sciences, v.169 (2), 553-566.

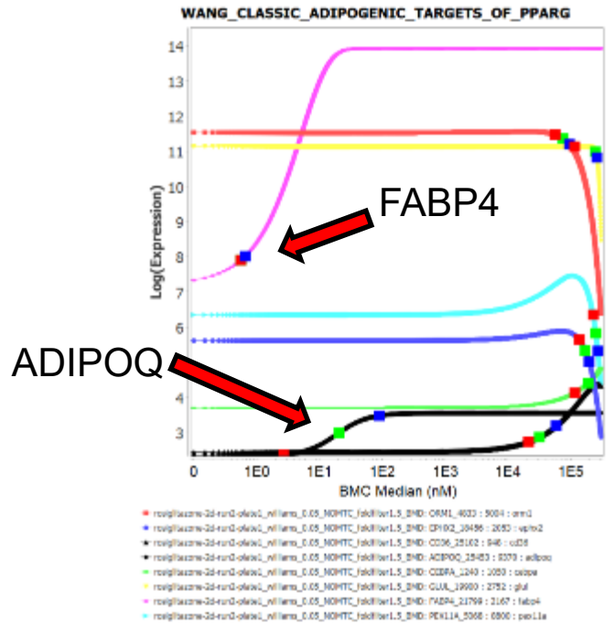
# Therapeutic Target ID via BMC Modeling

PPARγ report gene assay potencies:

Troglitazone: $EC_{50}$ = ~550 nM

Rosiglitazone: $EC_{50}$ = ~18 nM

Chen, R. et al. Pharm Biol 55, 503-509 (2017).




NOT all genes within a historical 'pathway' (i.e., gene set) are diagnostic, correct relative potency for FABP4 and ADIPOQ

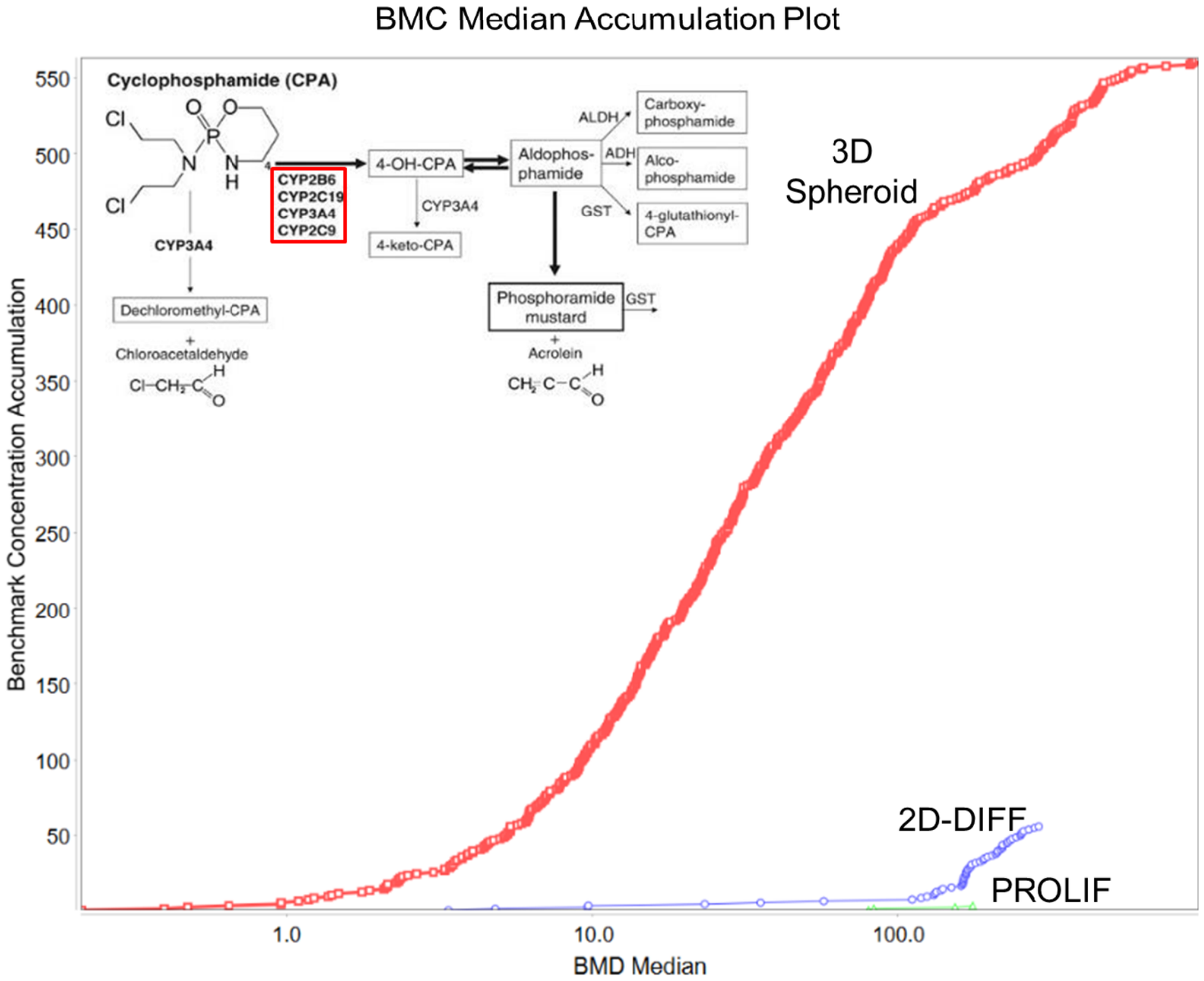
# Cyclophosphamide

(2-fold filter)

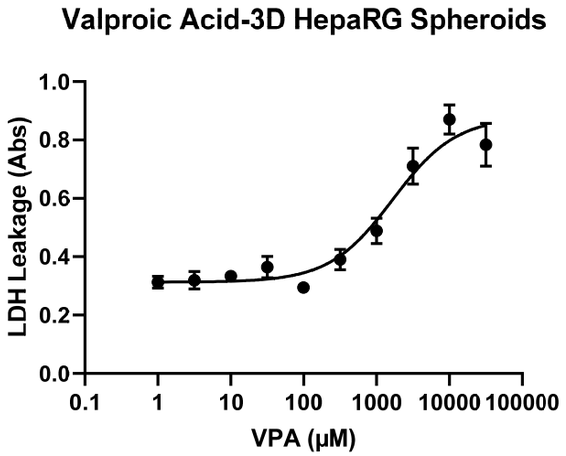
- 3D HepaRG Spheroids
- 2D-DIFF HepaRG
- PROLIF HepaRG

- Plausibly-relevant response pathways including:
  - Lipid hydroxylation
  - P450 metabolism
  - Cell cycle
  - ROS
  - DNA damage
  - Hypoxia

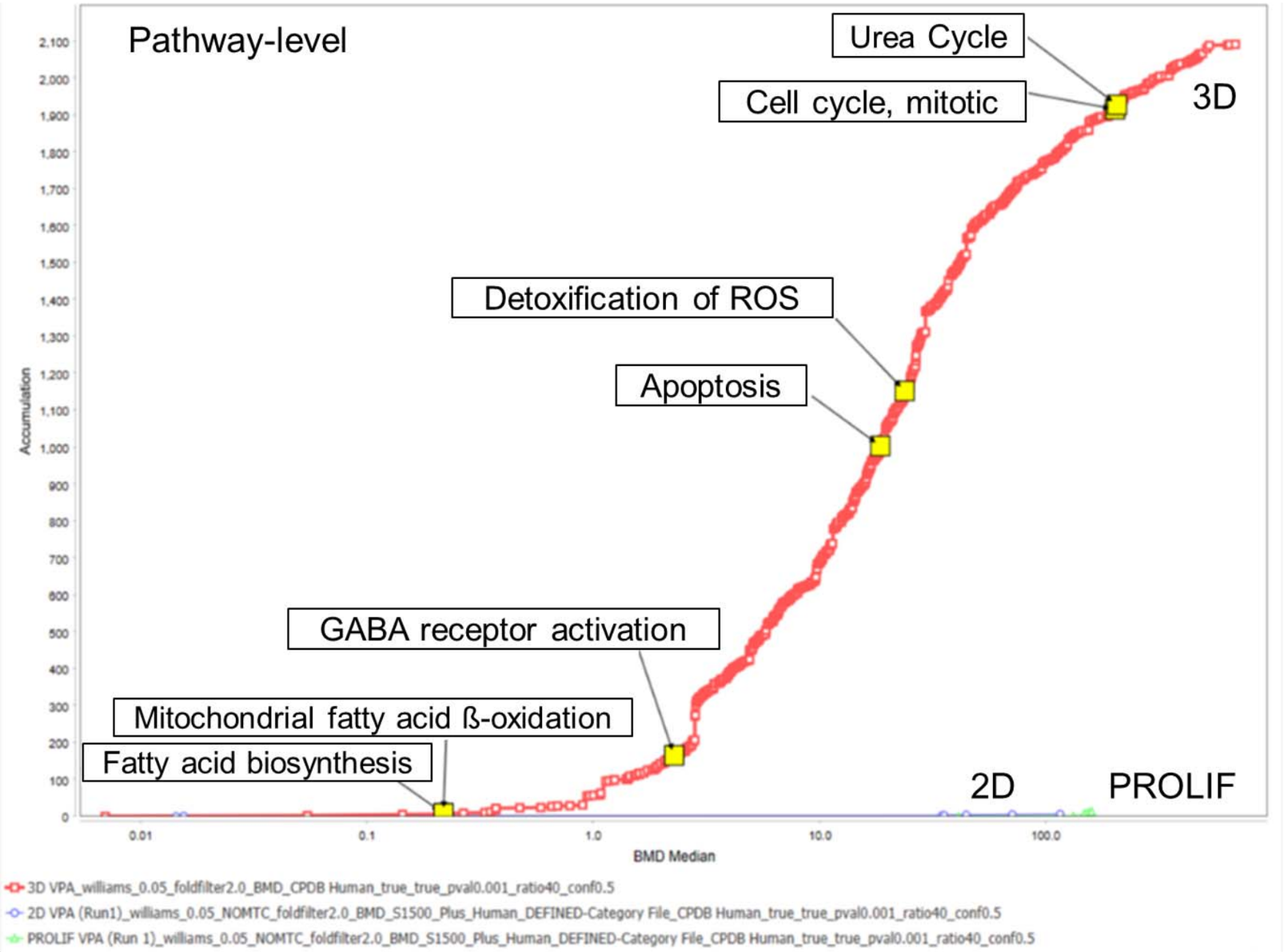
Unpublished Data



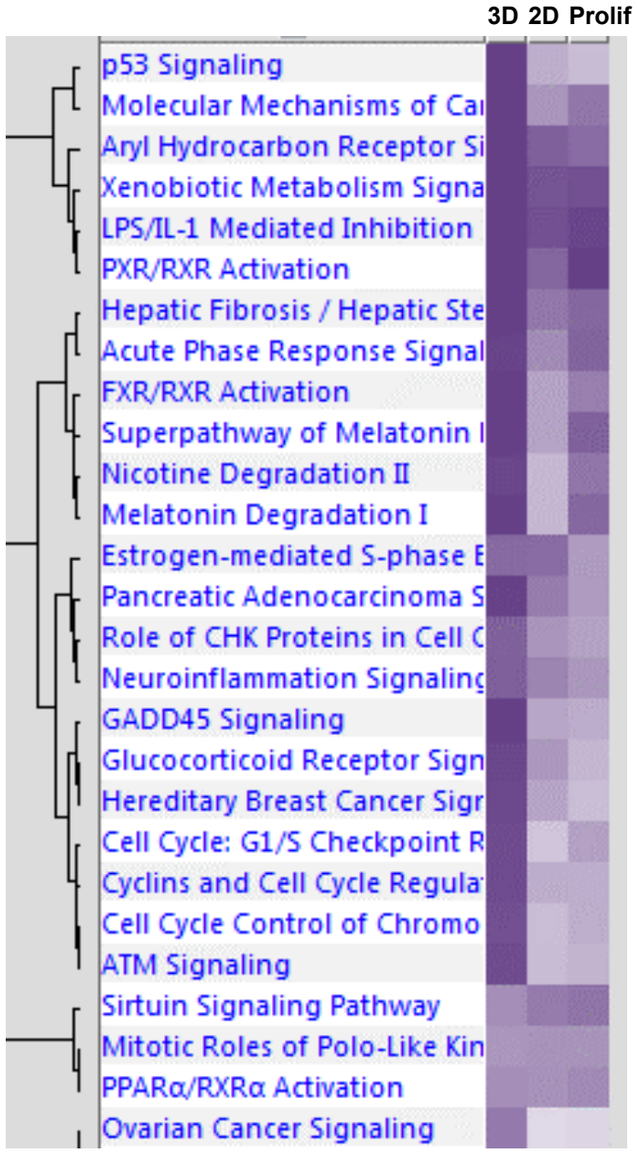
# Valproic Acid HTT in 3D HepaRG Spheroids




- $C_{max}$ ~240 µM (human plasma)
- Extensively metabolized (P450s)
- Cytotoxicity @ 1000 µM (3D only)
- Alters lipid & fatty acids levels
- Therapeutic target GABAergic receptor
- Hepatic mitochondrial toxicity & hyperammonemia
- Idiosyncratic liver injury compound



# 3D Spheroids & Biological Pathway Enrichment




Benzo(a)pyrene (Group 1 carcinogen (IARC)) exposure on HepaRG cell culture models

| | Significantly changed genes | | |
|---|---|---|---|
| Canonical Pathways | 3D_3 µM | 2D_DIFF _3 µM | 2D_PROLIF _3 µM |
| P53 Signaling | 39 | 14 | 14 |
| Molecular Mechanisms of Cancer | 71 | 32 | 40 |
| AhR Signaling | 38 | 23 | 23 |
| Xenobiotic Metabolism Signaling | 52 | 35 | 39 |
| PXR/RXR Activation | 27 | 16 | 20 |
| Hepatic Fibrosis | 38 | 24 | 28 |
| Acute Phase Response Signaling | 36 | 22 | 28 |
| Pancreatic Adenocarcinoma | 30 | 19 | 18 |
| GADD45 Signaling | 12 | 7 | 7 |
| ATM Signaling | 26 | 12 | 14 |

**Dietary Intake of Heterocyclic Amines and Benzo(*a*)Pyrene: Associations with Pancreatic Cancer**

Kristin E. Anderson,[1] Fred F. Kadlubar,[2] Martin Kulldorff,[3] Lisa Harnack,[1] Myron Gross,[1] Nicholas P. Lang,[4] Cheryl Barber,[1] Nat Rothman,[5] and Rashmi Sinha[5]

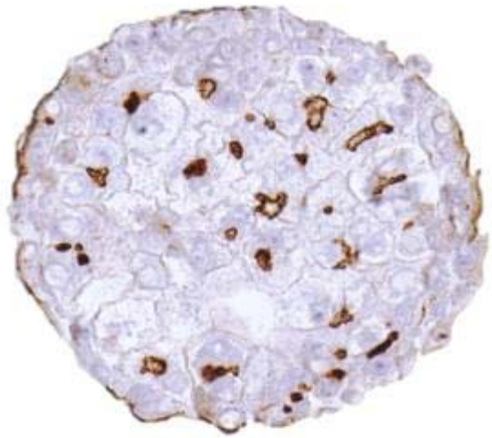
[1]School of Public Health, University of Minnesota, Minneapolis, Minnesota; [2]National Center for Toxicological Research, Jefferson; [3]Department of Ambulatory Care and Prevention, Harvard Medical School and Harvard Pilgrim Health Care, Boston, Massachusetts; [4]Central Arkansas Veterans Healthcare System and Department of Surgery, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas; and [5]National Cancer Institute, Rockville, Maryland

Cancer Epidemiol Biomarkers Prev. 2005 Sep;14(9):2261-5.

# Opportunities & Challenges with 3D Hepatocyte Spheroids

## Opportunities

- Enhanced hepatocyte functionality & population of transcriptomic pathways
- Long-term differentiation for repeated exposure, time-course, & reversibility
- Simple model system readily compatible with most cell culture labs
- Efficient use of hepatocytes
- In vitro pathology to image microtissues for toxicological outcomes (e.g., steatosis, cholestasis, fibrosis)
- Explore potential mechanisms linked with in vitro pathology characteristics



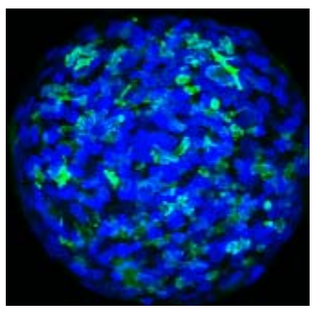
**Biliary Efflux Transporter MRP-2 Immunostaining of HepaRG Spheroids (21d)**

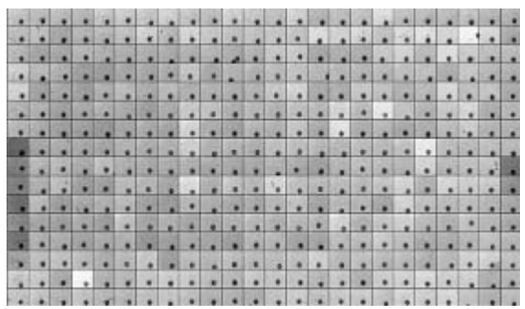
## Challenges

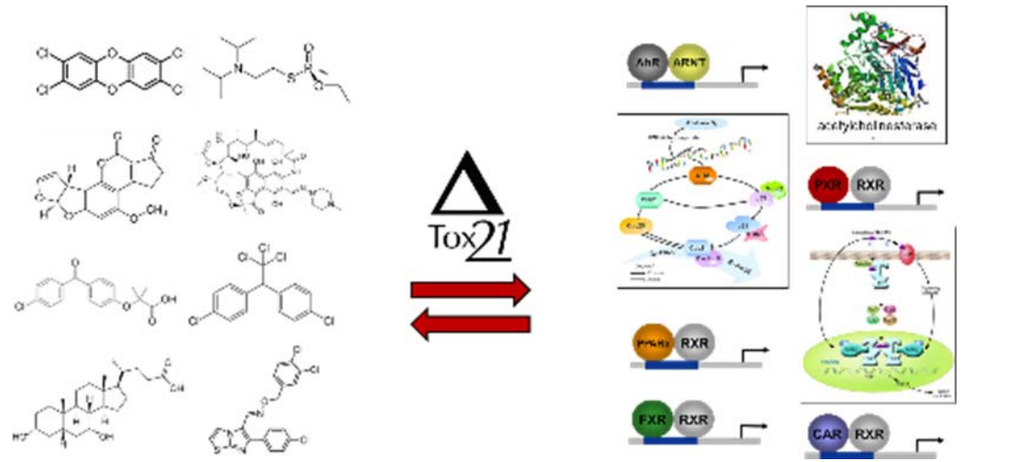
- Changing culture media without liquid handling, aspirating floating spheroids
- High throughput imaging of 3D spheroids
- Recent plate manufacturing issues
- Insufficient knowledge of spheroid maturation & context of use (e.g., phenotypes & outcomes)
- Allometric scaling & biomass challenges (e.g., metabolite profiles over time)
- Inadequate optimization of cell culture media largely adopted from 2D (e.g., DMSO, hydrocortisone)

# Upcoming Research with 3D HepaRG Spheroids

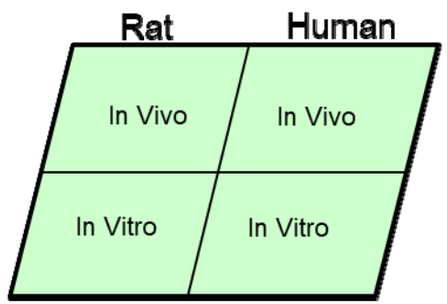
- Tox21 Cross-partner Project #5
  - Develop robust chemical reference dataset for interpretation of high-throughput transcriptomic data: 3D HepaRG spheroids (NTP), MCF-7 (EPA)
  - Exposures to >300 pharmaceuticals & chemicals with established high affinity linkages to specific molecular targets (e.g., agonist, antagonist, inhibitor)
  - Define signatures of transcriptomic response to reference chemicals to contextualize environmental chemicals

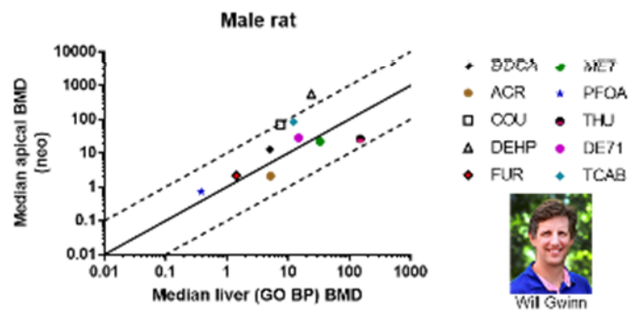







- NTP interspecies parallelogram evaluation of 20 historical chemicals
  - Chronic in vivo bioassay results (e.g., 2-year carcinogenicity)
  - 5-day in vivo rat liver/kidney transcriptomics
  - 3D in vitro rat hepatocyte spheroids
  - 3D in vitro human HepaRG spheroids

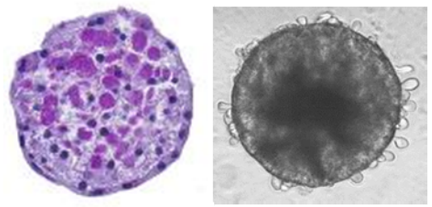






- Histo- and clinical-pathology modeling of liver and renal proximal tubule

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