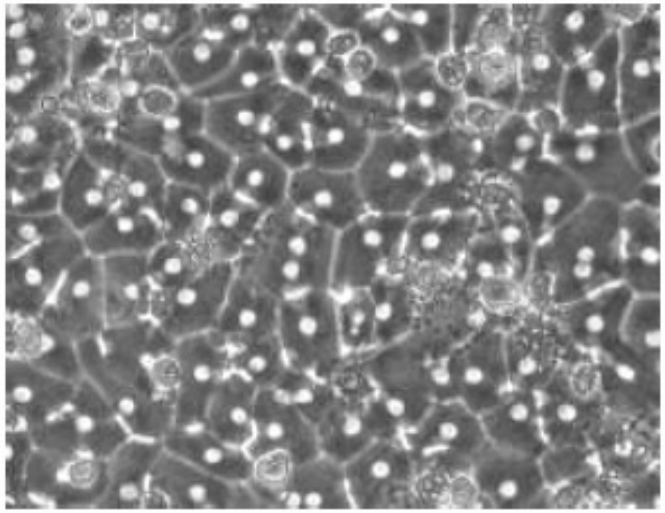

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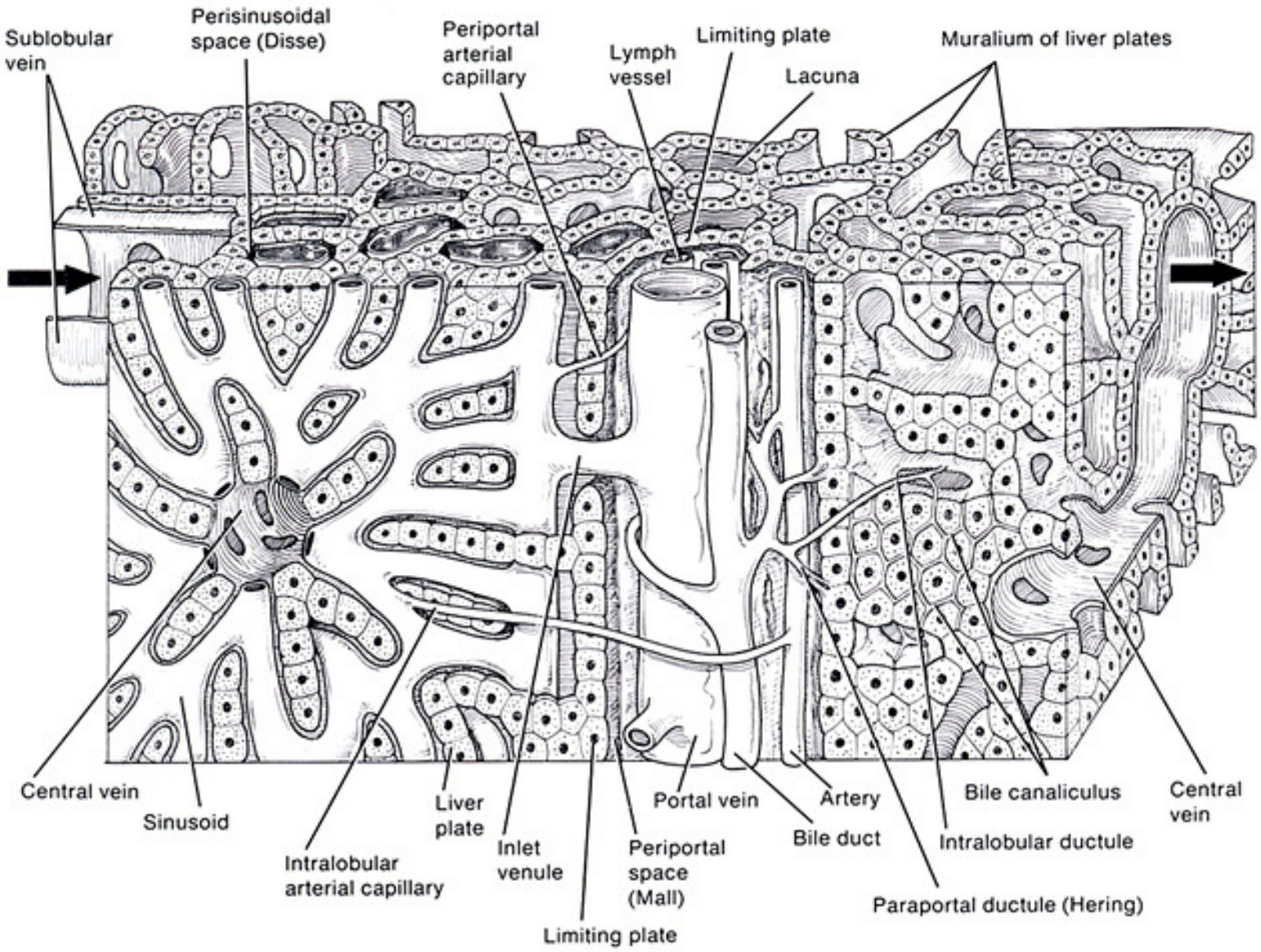
The ToxCast 320 Chemical Library in Cultures of Primary Human Hepatocytes:

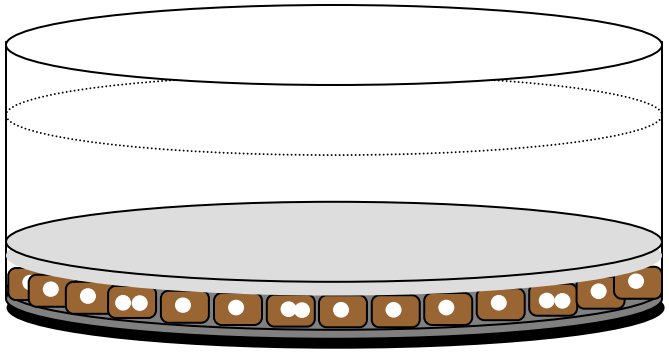
qNPAs as Windows into Chemical-Induced Hepatocyte Biology

Stephen S. Ferguson, Ph.D.

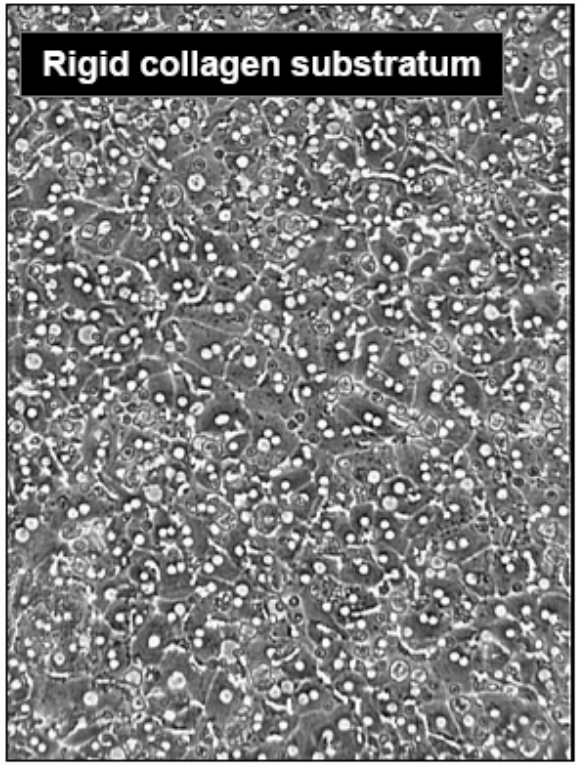


Tissues and Organs: a text of scanning electron microscopy, Kessel, RG and Kardon,RH, 1979

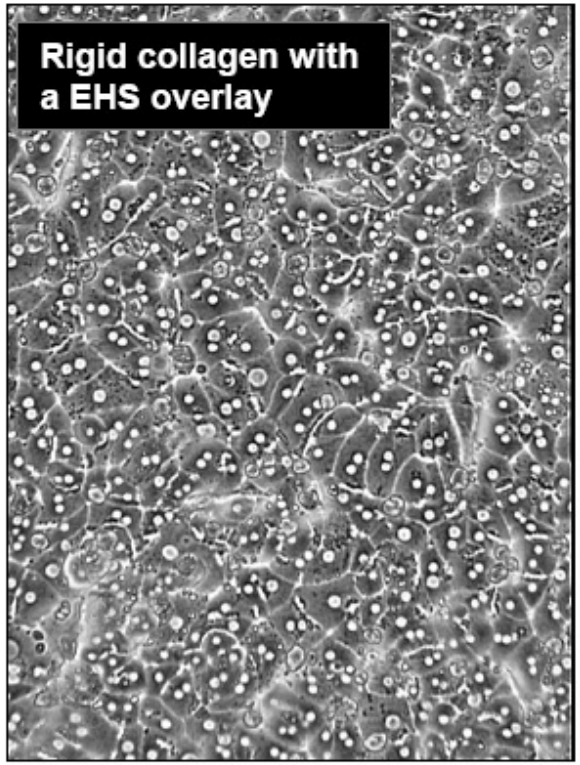
Primary Hepatocyte Culture Systems



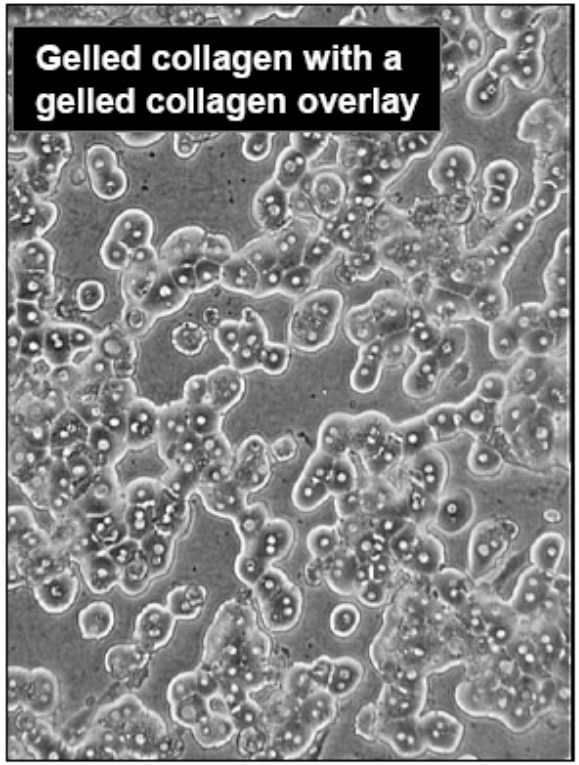
Culture Media
Overlay (e.g. Geltrex®)
Primary Hepatocytes
Basement Matrix (e.g. collagen)



Conventional monolayer



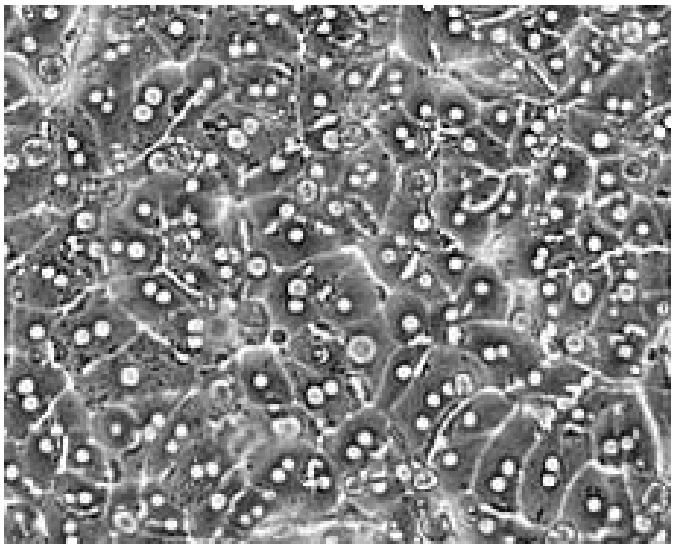
Sandwich culture 1



Sandwich culture 2

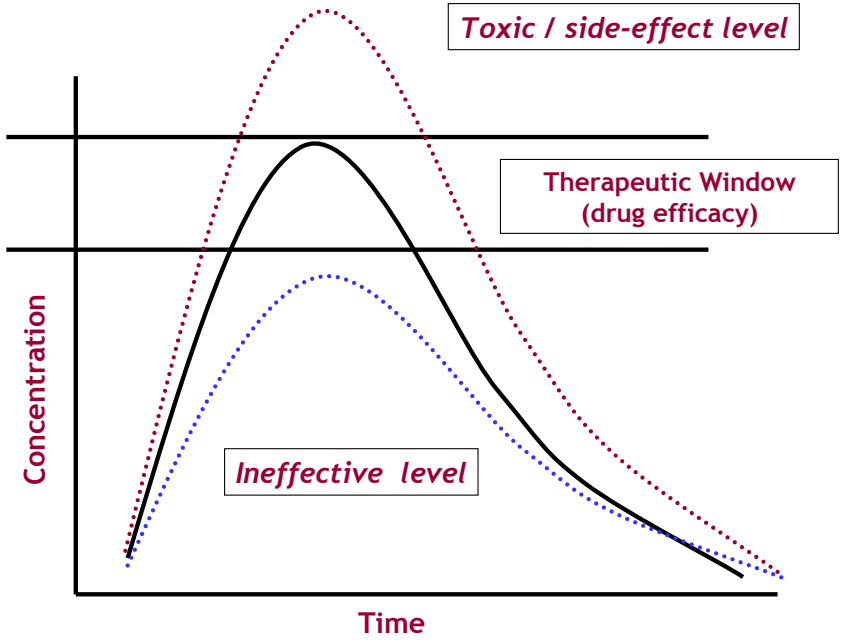
Primary Cultures of Human Hepatocytes: Effective Models of the Liver

- Gold Standard in vitro model system for human liver
- Metabolically competent model system (metabolite effects)
- Effective models of hepatic metabolism (e.g. Phase I, Phase II, CL_{INT})
- Hepatic transporter function modeled (uptake and efflux) to assess exposure
- Long-term (days-weeks) culture can model chronic exposure endpoints
- Multi-donor studies possible to assess interindividual variability and correlate genetic polymorphism effects
- Induction/suppression: Retain liver-like cell signaling (e.g. nuclear receptor expression) to model species-specific induction



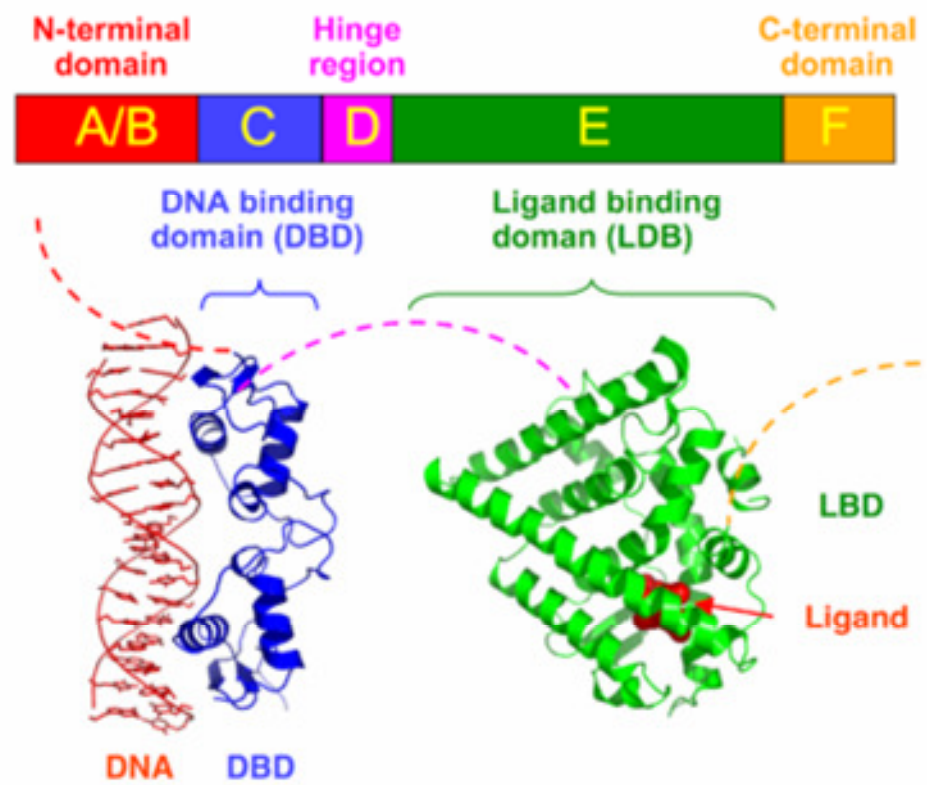
Liver Function: Why Is It Important to Assess Induction-Adaptive response?

- Chronic receptor activation (e.g. CAR) linked with cancer
- Xenobiotic toxicity potentiated by induced/suppressed metabolism
- Induction relevance to cytotoxicity (cause or biomarker)
- Perturbation of endogenous chemical (e.g. steroid) homeostasis
- Failure of therapeutic drugs (e.g. OC's, epilepsy, HIV)
- Altered cellular exposure over time (altered transport and/or metabolism)



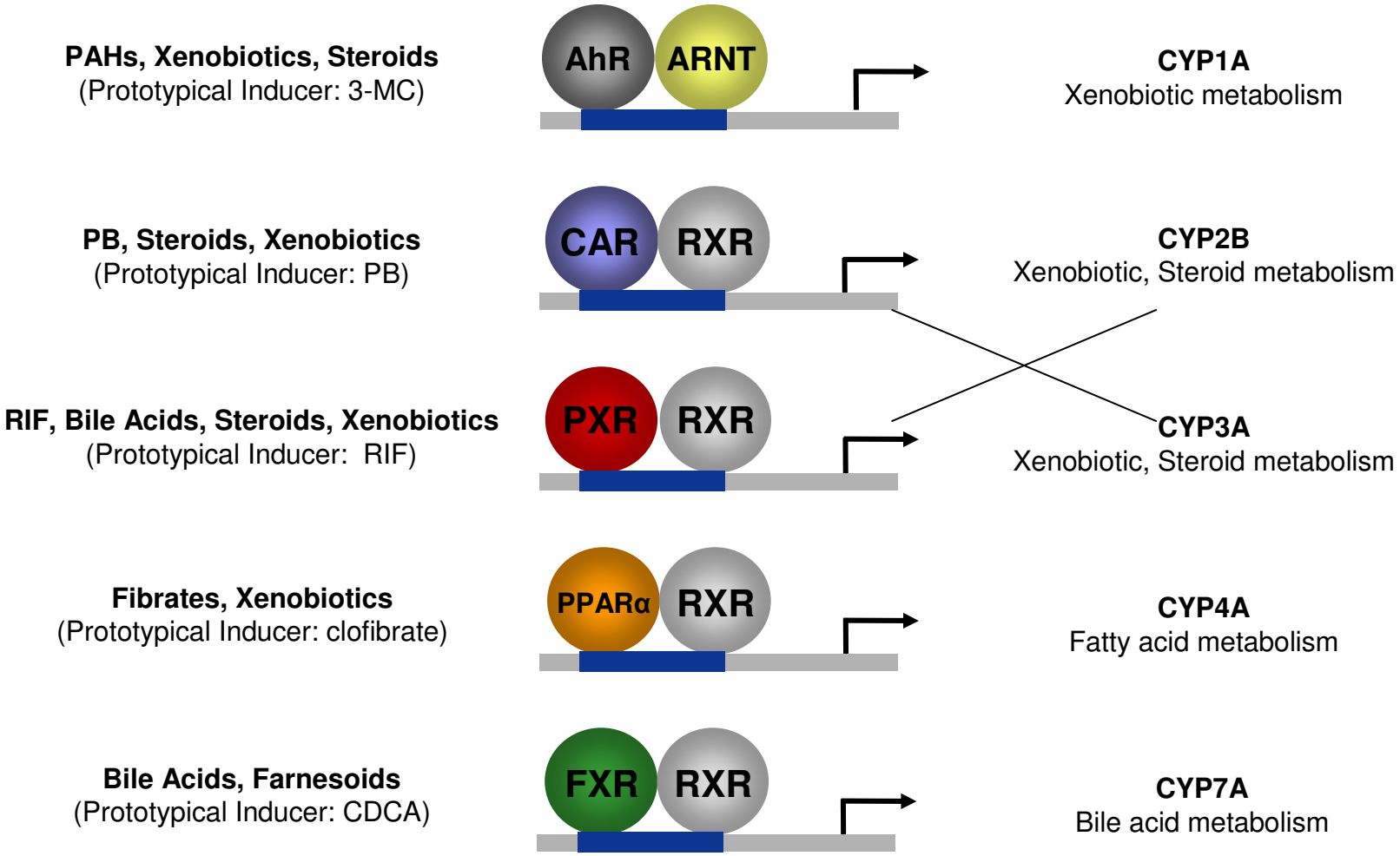
General Mechanisms of P450 Induction

- mRNA stabilization
- Protein stabilization
- Receptor-mediated transcriptional activation



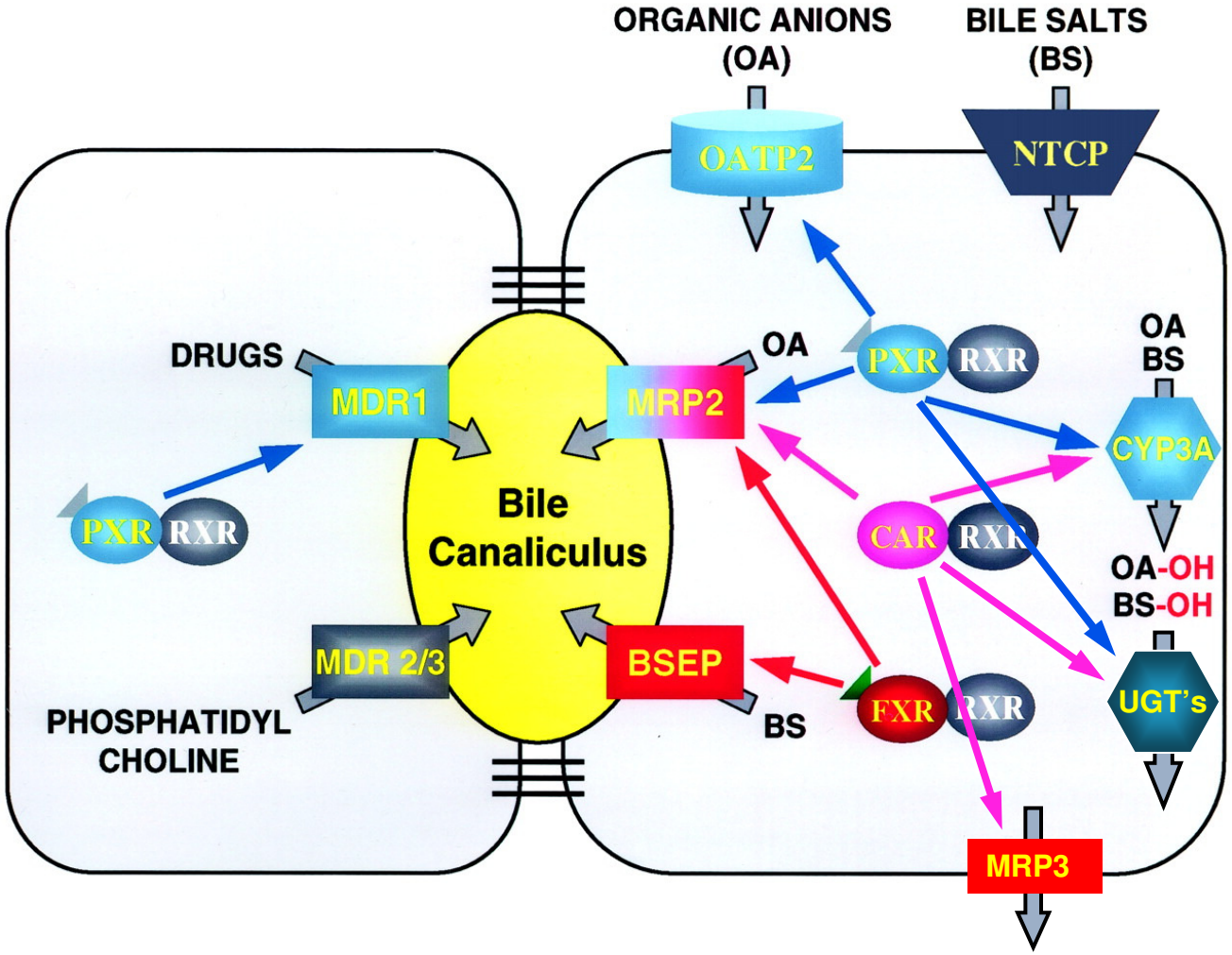
Coordinates: Kumar R, Thompson EB (1999). "The structure of the nuclear hormone receptors". Steroids 64 (5): 310–9

Important Hepatic Receptor Pathways



Others: LXR, PPARγ, VDR, ER, TR, NRF-2…

Induction and Receptor Regulation of Hepatic Transport as well as Phase I and II metabolism



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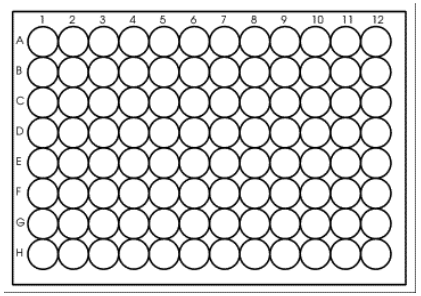
Questions

- How do we assess a chemical's potential to modulate these hepatic pathways?
  - mRNA
  - Protein
  - Functional activity
- What receptor pathways appear to be affected, and how do we study in a metabolically-competent, liver-like system
  - Sentinel gene targets and selective PC inducers
  - Receptor antagonist studies
  - Knock-down of receptors
  - Transfection of receptor reporters into hepatocytes
- How do we assess thousands of chemicals
  - high throughput (≥96-well) cultures of primary hepatocytes
  - Multiplexed, high content, and complementary endpoints
- What are the concentration-response and time-response profiles of these effects?
- How do we use time as a tool to characterize the adaptive responses (e.g. cause/effect) and probe the potential for human liver interactions?

Experimental Study Design

- Cultures of primary human hepatocytes
- 96-well format
- 6 reference chemicals (known receptor activators)
- ToxCast 320 chemical library
- Endpoints
  - qNPA
    - 14 gene targets important for liver biology
      - 6 P450 genes
      - 4 transporter genes
      - 3 phase II metabolism genes
      - 1 cholesterol synthesis gene
      - 2 endogenous control genes
  - CYP1A Enzymatic Activity (EROD)
  - Cell morphology (Cell Health)
- Dose-response curves (≥ 5 concentrations)
- 4 time points (6, 24, and 48 hours)



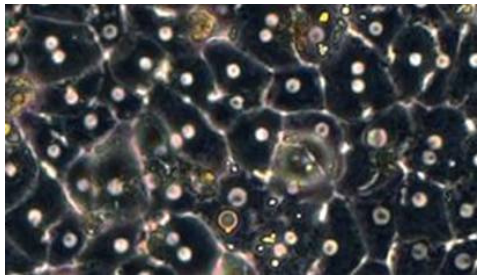


Image cultures

EROD Assays

Harvest qNPA

qNPA detection

Data Analysis

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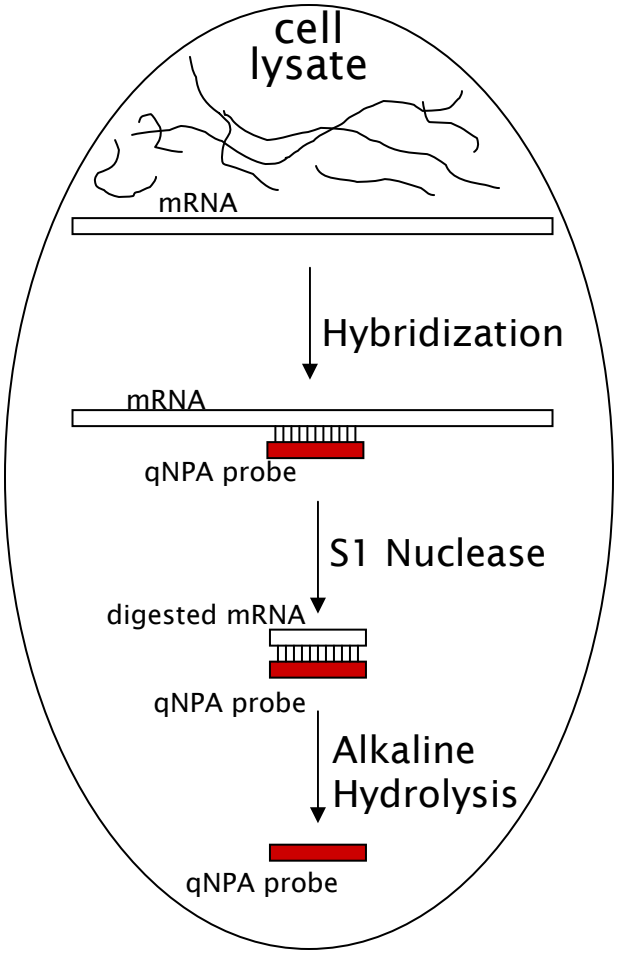
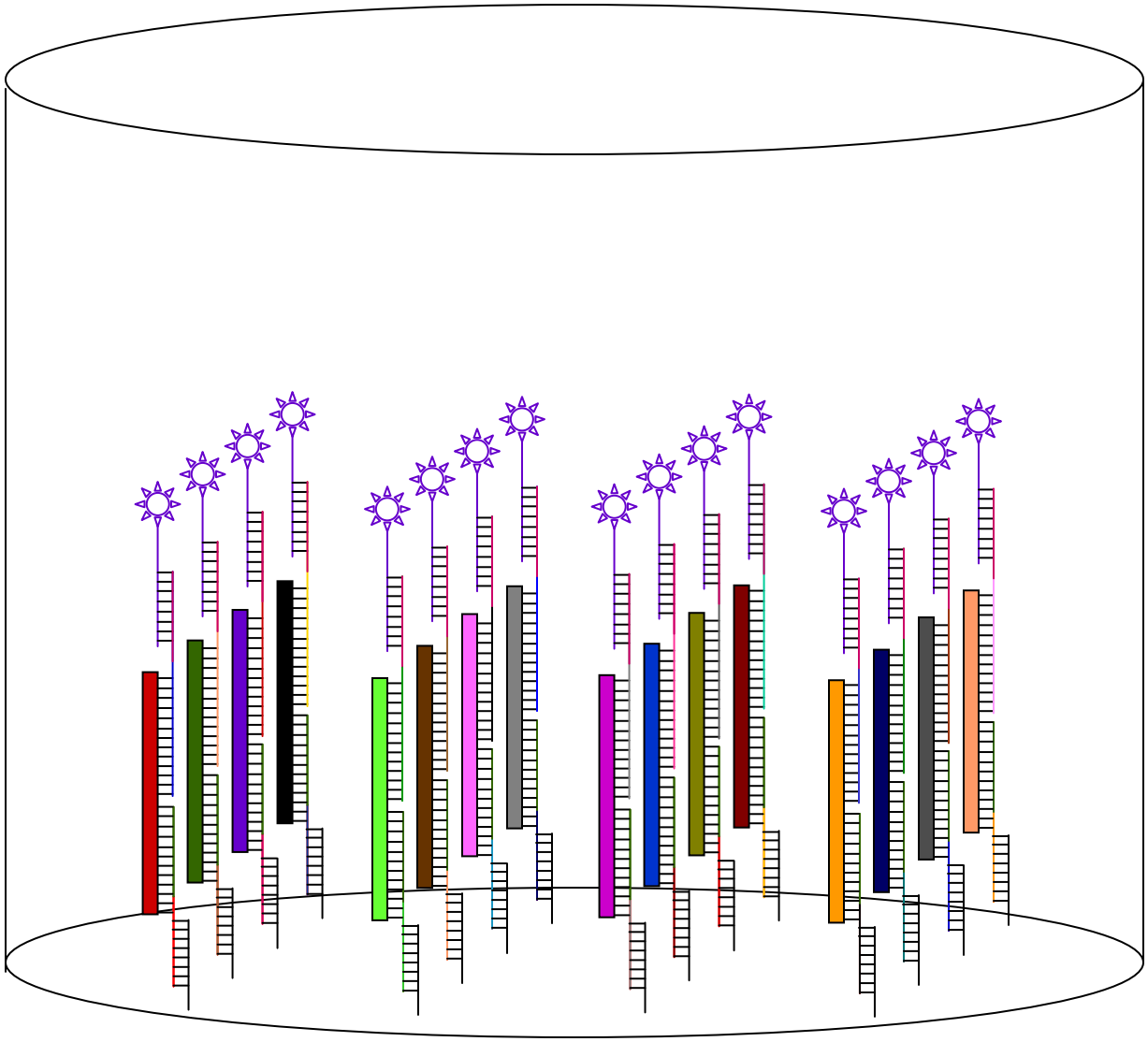
How the qNPA assay works…





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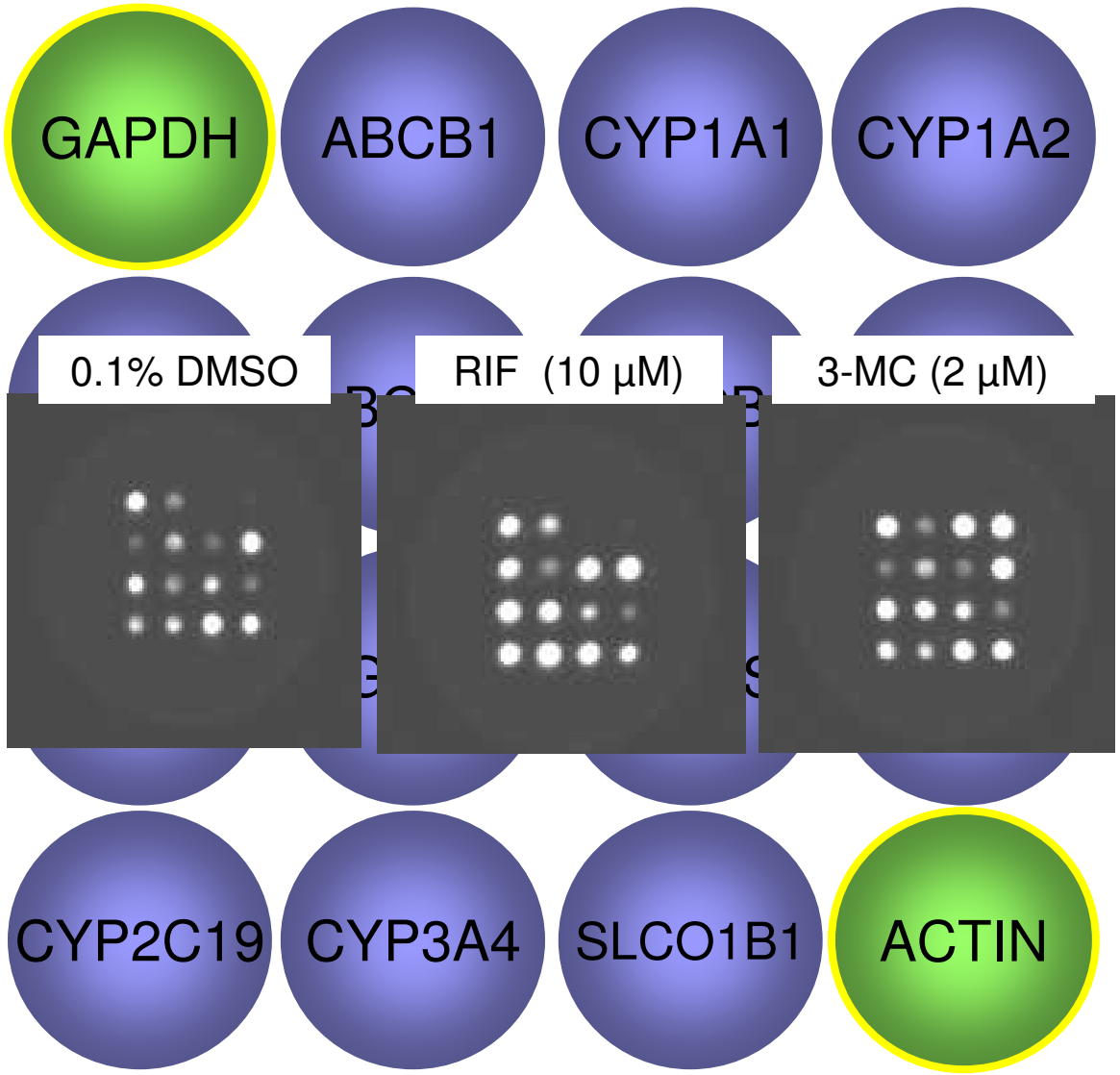
Hepatic Expression Systems (HES): High Throughput RNAse Protection Assay (Human)



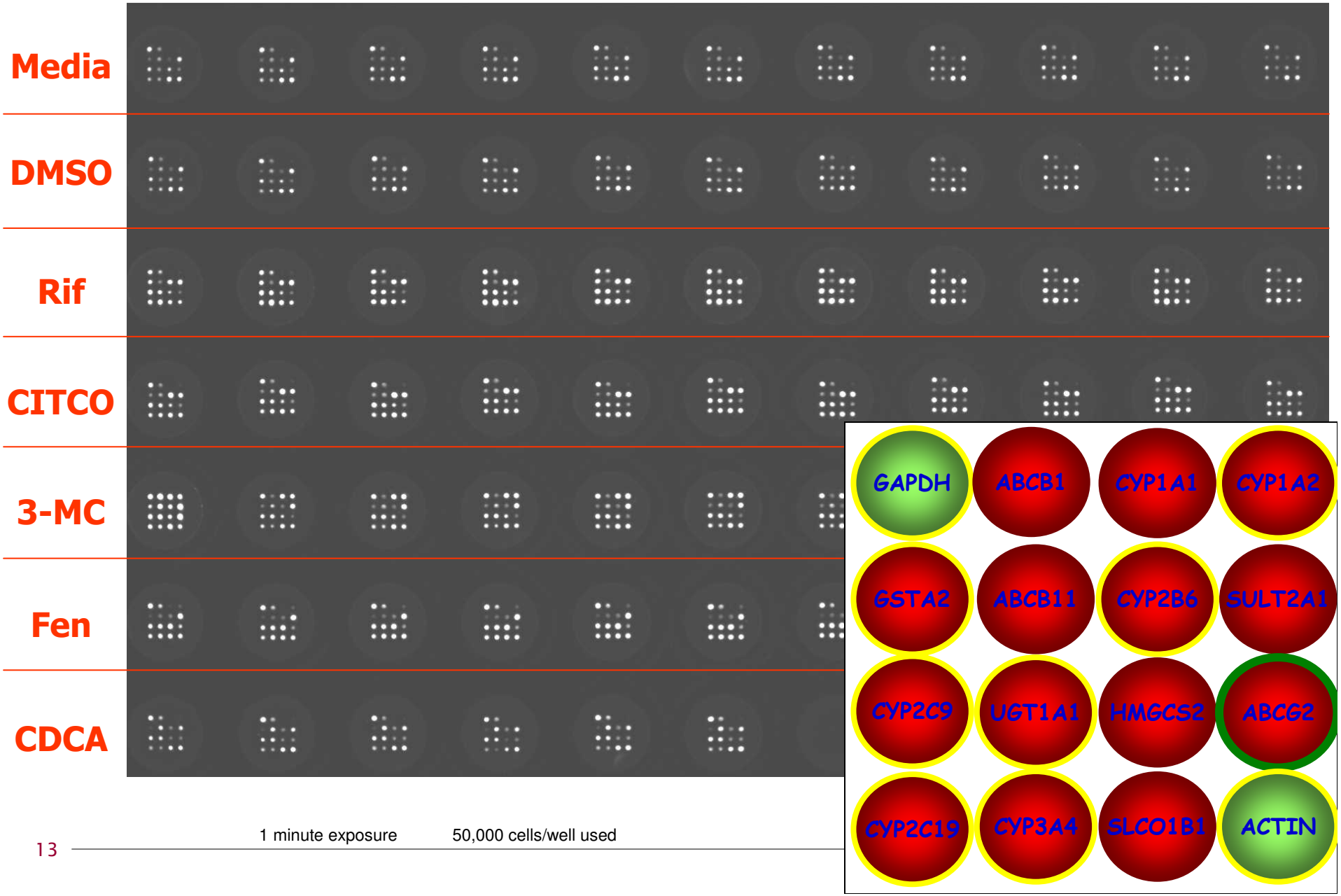
Target Genes
Receptor Pathways

AhR
CAR
PXR
PPARα
FXR

Target Genes Category

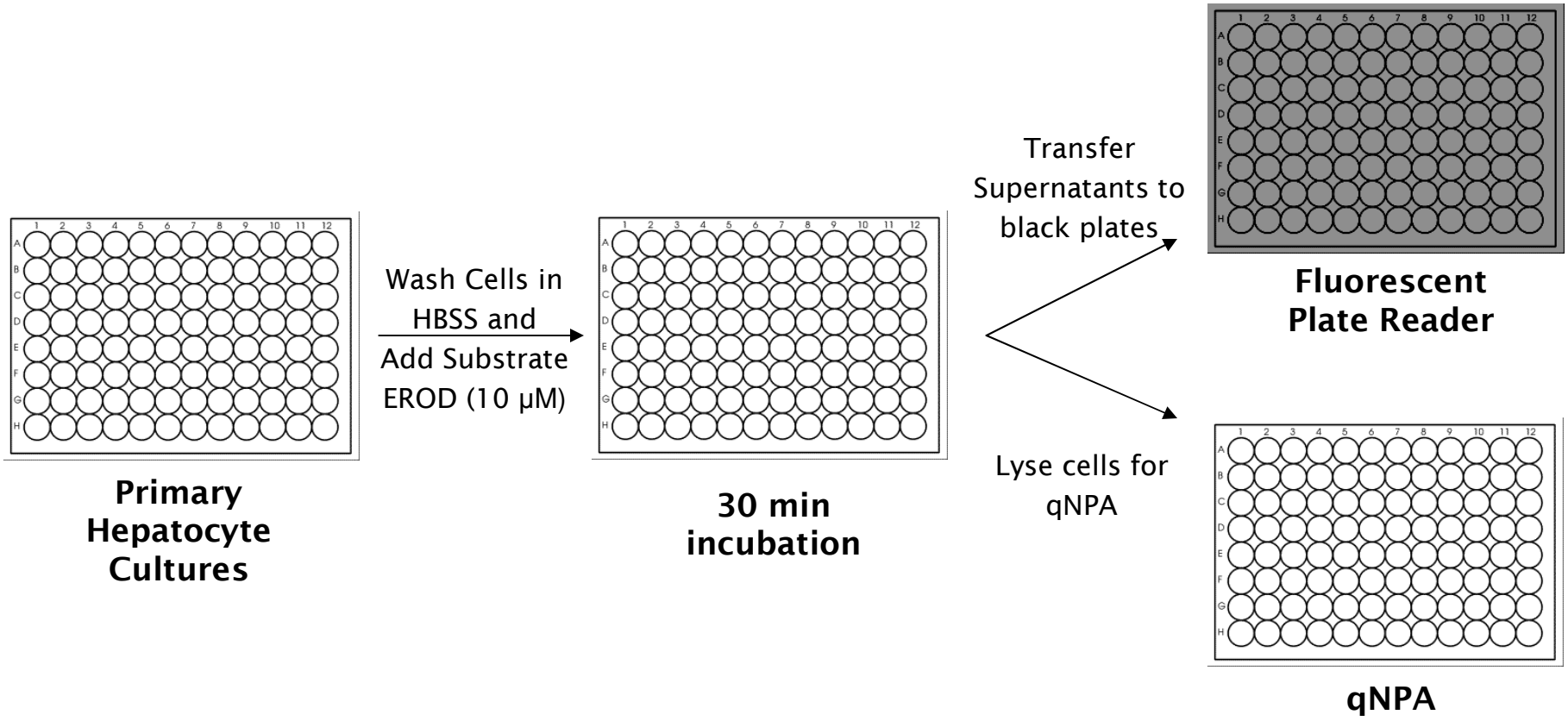
CYP450 (6)
Transporter (4)
Phase II Metabolism (3)
Endogenous Metabolism (7)
'Housekeeping' gene (2)

Plate Image

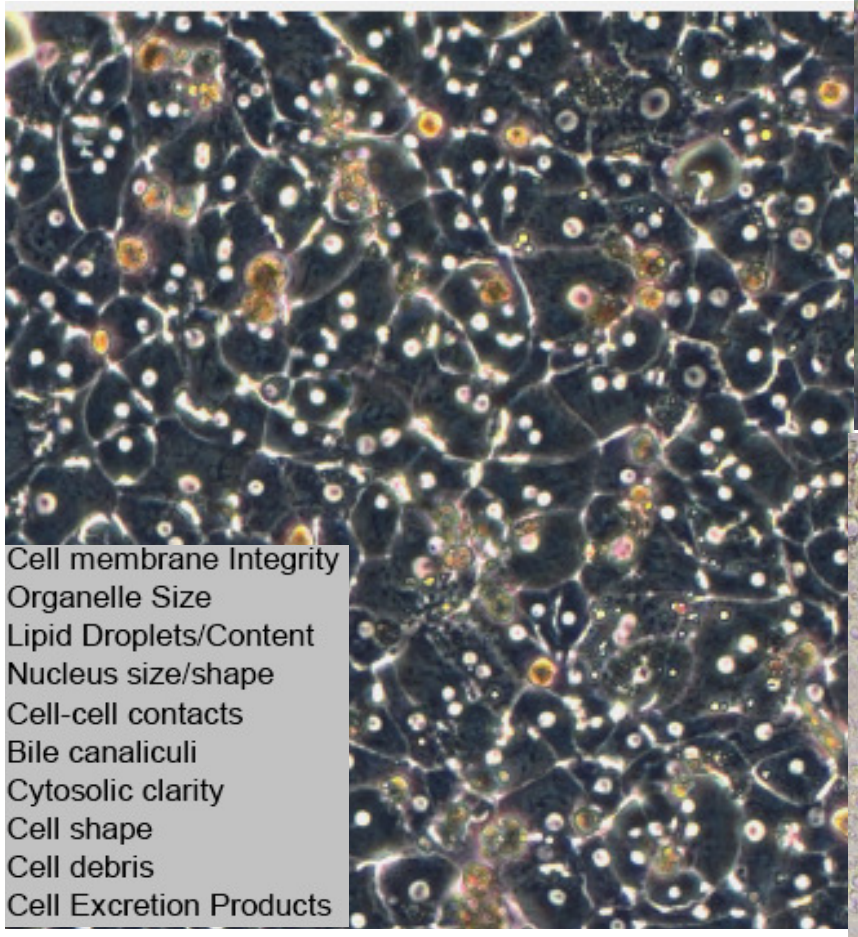


1 minute exposure 50,000 cells/well used

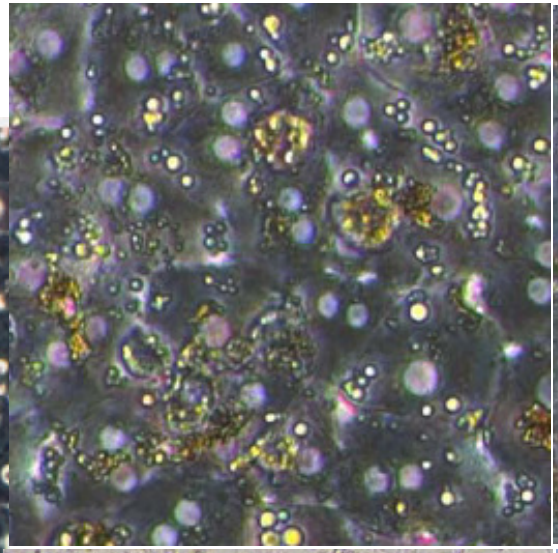
EROD assay: CYP1A Enzymatic Activity

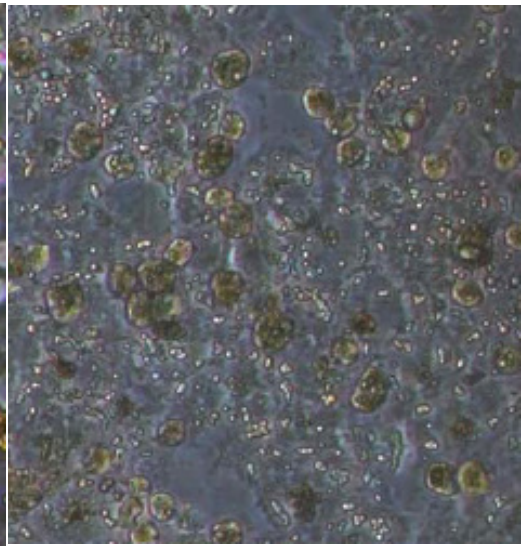


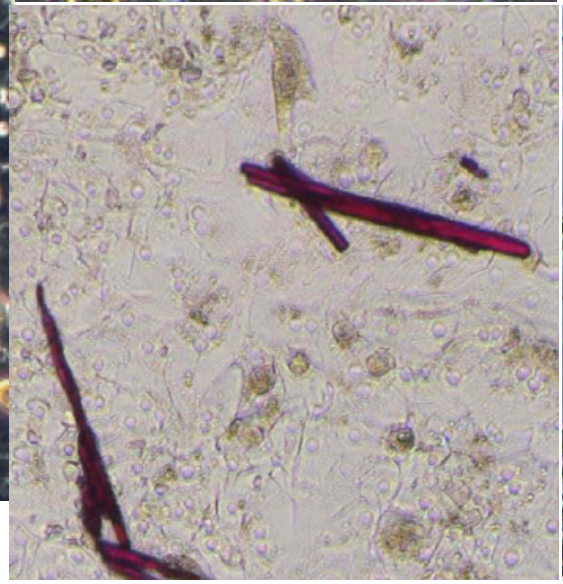
Cell Morphology Assessments

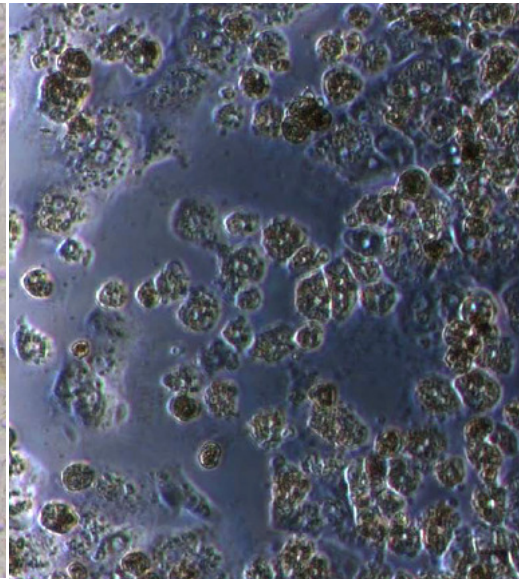


Cell membrane Integrity
Organelle Size
Lipid Droplets/Content
Nucleus size/shape
Cell-cell contacts
Bile canaliculi
Cytosolic clarity
Cell shape
Cell debris
Cell Excretion Products









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Data Analysis

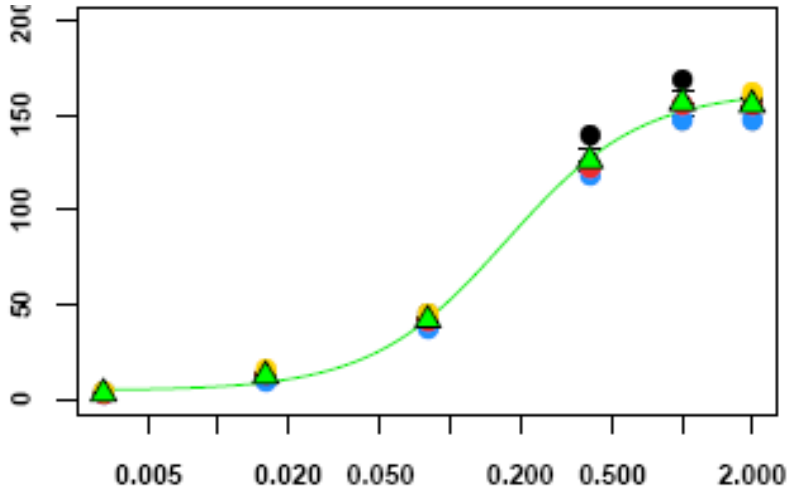
- Compiling >300,000 data points into viewable database
- Developed data analysis tools to cluster data across both concentration- and time-response (k-means) in 210-dimensional space
- Curve fitting across concentration-response
  - EC_{50}
  - E_{max}
  - E_{min}
  - Hill slope
- Statistically Significant Responses (f-test)
- Choice of Reference Chemicals (Z-factor)
- Correlation Analyses (e.g. EROD, gene-to-gene, etc…)
- Time Effects: Ongoing

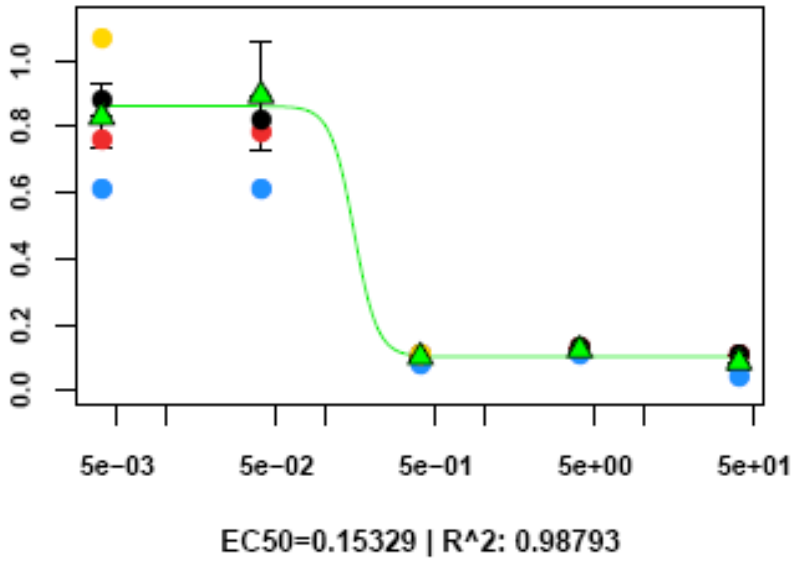
Curve Fitting Approach

- Four parameters that define the curve
  - E_{max}
  - E_{min}
  - EC_{50}
  - Hill slope
- Equation:

$$E_{MAX} - \left[\frac{(E_{MAX} - E_{MIN})}{1 + \left(\frac{x}{EC_{50}} \right)^{hillslope}} \right]$$

- Fit using non-linear least squares in the R statistical language





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Reference Chemical Selection

- Why do this:
  - Choice based on lit vs. data here
  - Genes not well characterized with prototypical inducers accounted for through study data
- Z-factor[1]: $1-\left[\frac{3*(\sigma_p+\sigma_n)}{|\mu_p-\mu_n|}\right]$
  - σ_p , σ_n : Positive and Negative control standard deviations
  - μ_p , μ_n : Positive and Negative control E_{max} averages

Z-factor	Evaluation
1.0	Ideal. Large dynamic range and tiny standard deviation. A Z-factor can not ever actually equal 1 and is a theoretical Limit
0.5 < Z-factor < 1	Excellent Assay.
0 < Z-factor <0.5	Marginal Assay
Z-factor < 0	Positive and Negative controls overlap.

[1]Zhang JH, Chung TD, Oldenburg KR, "A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays." *J Biomol Screen.* **1999**;4(2):67-73

PAHs, Xenobiotics
(Reference Chemical: 3-MC)

CYP1A1/2
Xenobiotic metabolism

PB, Steroids, Xenobiotics
(Reference Chemical: PB)

CYP2B6
Xenobiotic, Steroid metabolism

RIF, Bile Acids, Steroids, Xenobiotics
(Reference Chemical: RIF)

CYP3A4
Xenobiotic, Steroid metabolism

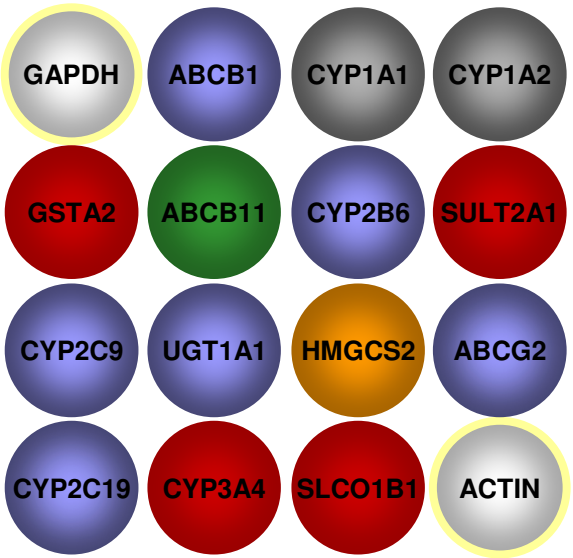
Fibrates, Xenobiotics
(Reference Chemical: Fenofibric Acid)

HMGCS2
Fatty acid metabolism

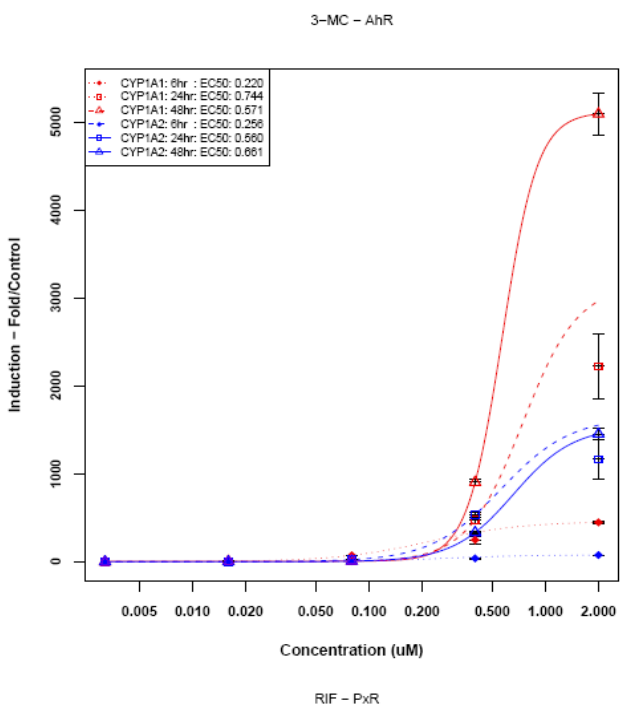
Bile Acids, Farnesoids
(Reference Chemical: CDCA)

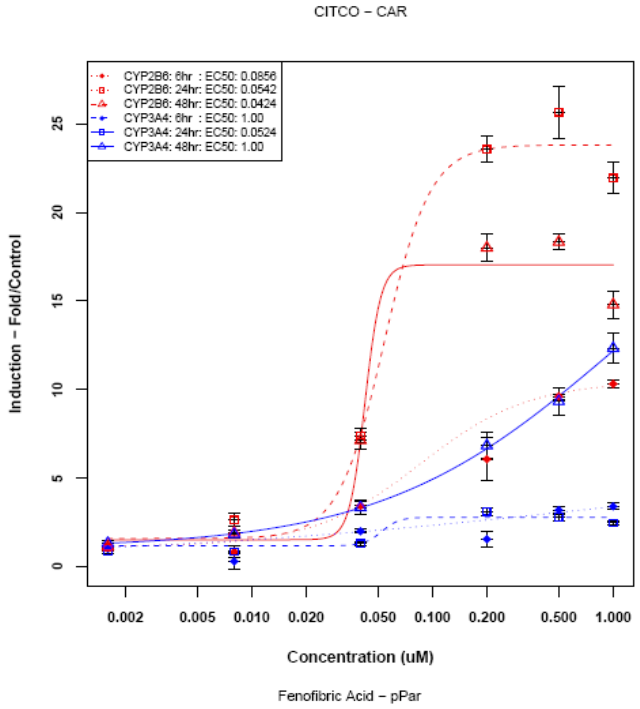
ABCB11 (BSEP)
Bile acid metabolism and transport

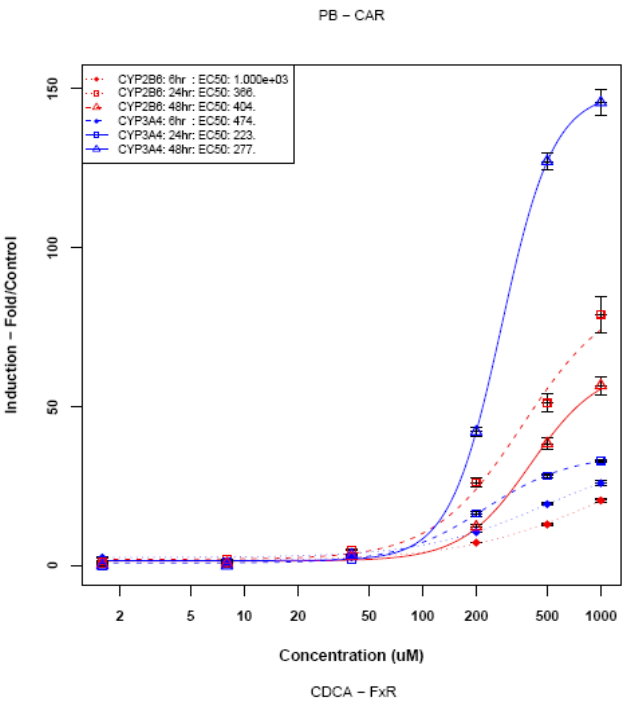
Reference Chemical Results

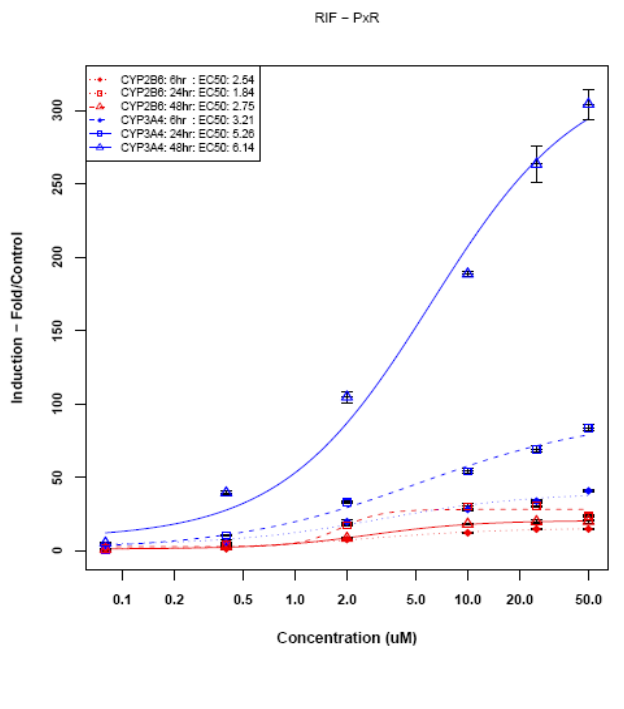


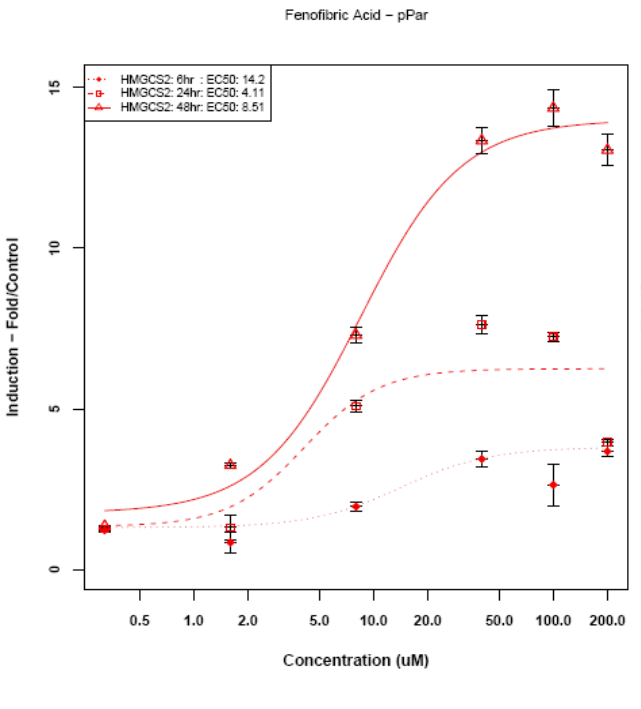
Reference Chemical Concentration- and Time-Response Curves

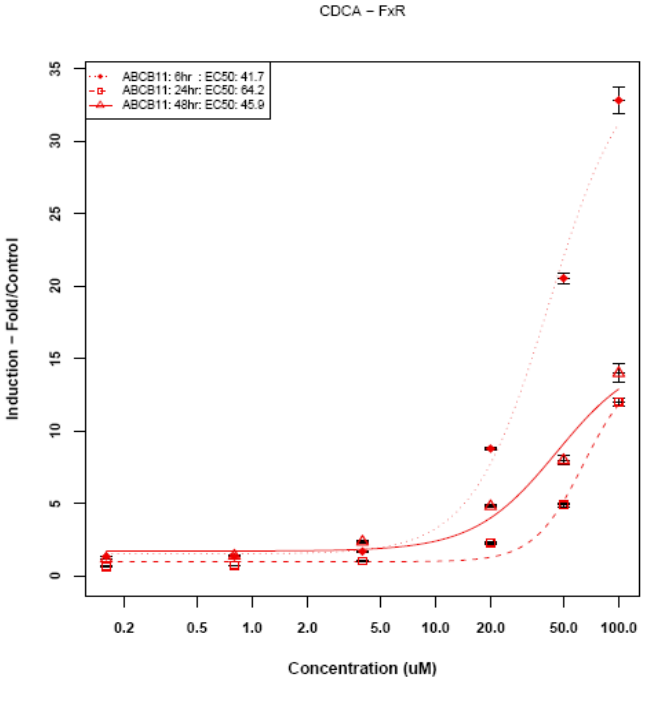


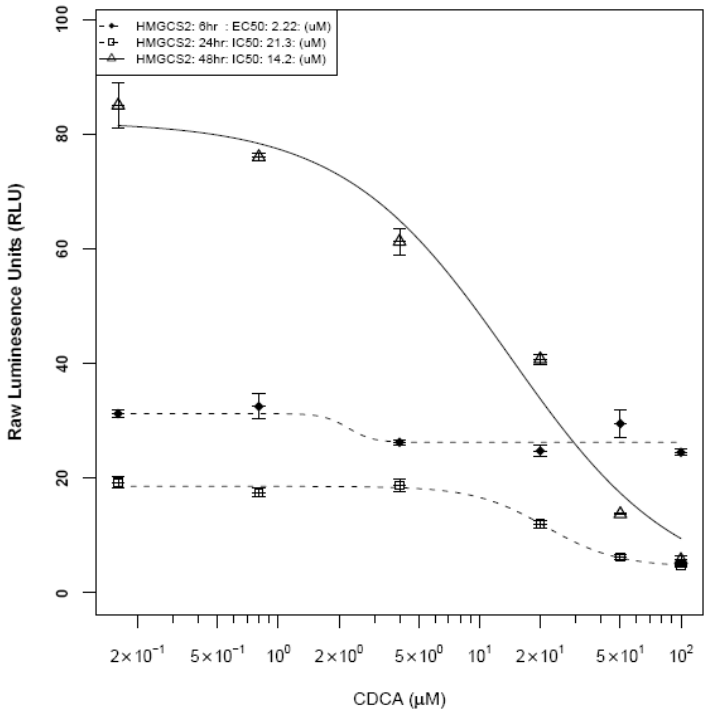


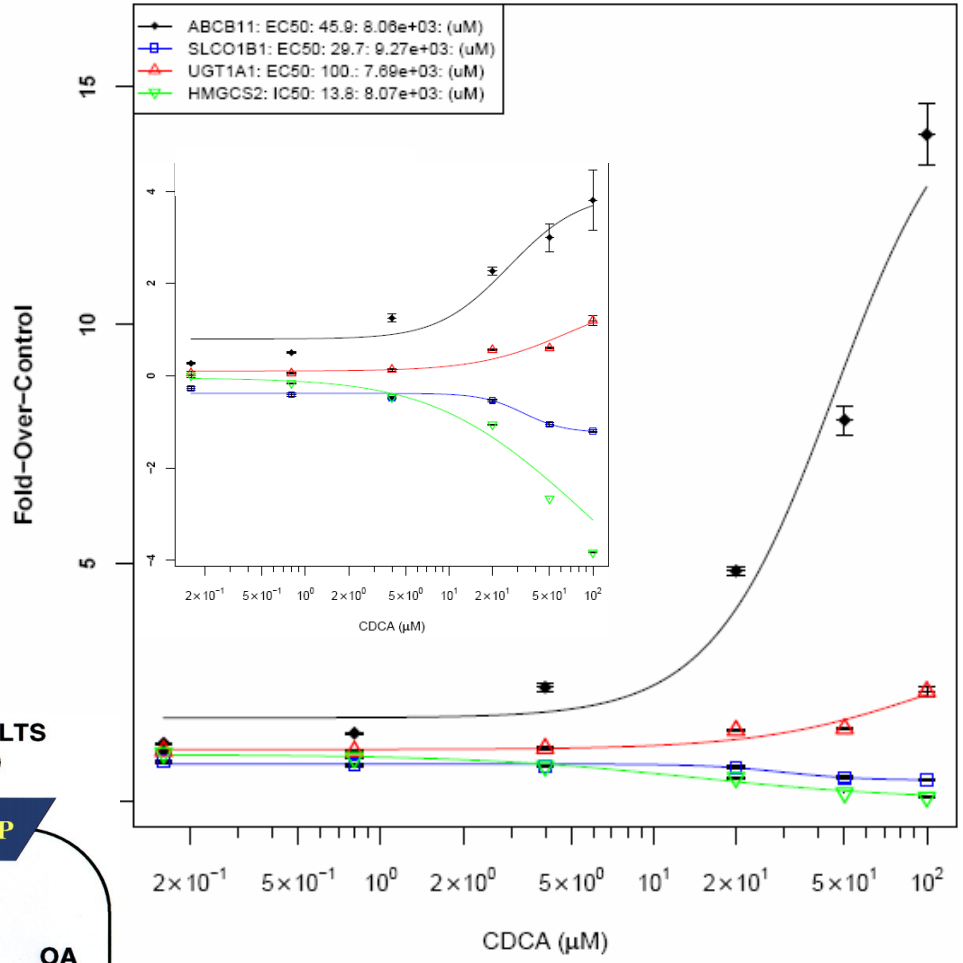


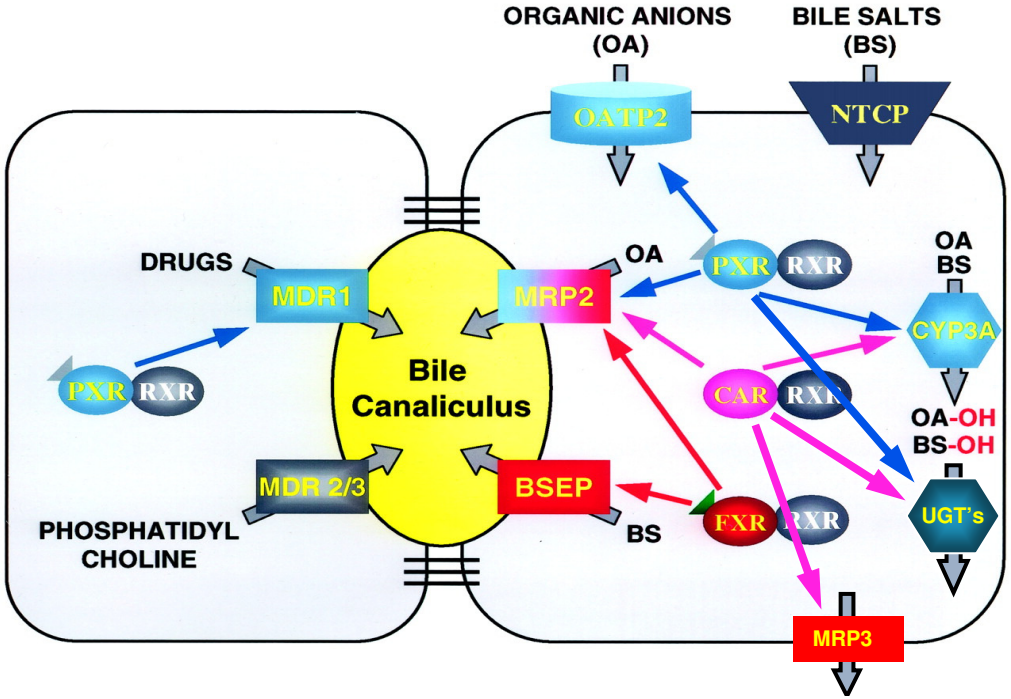




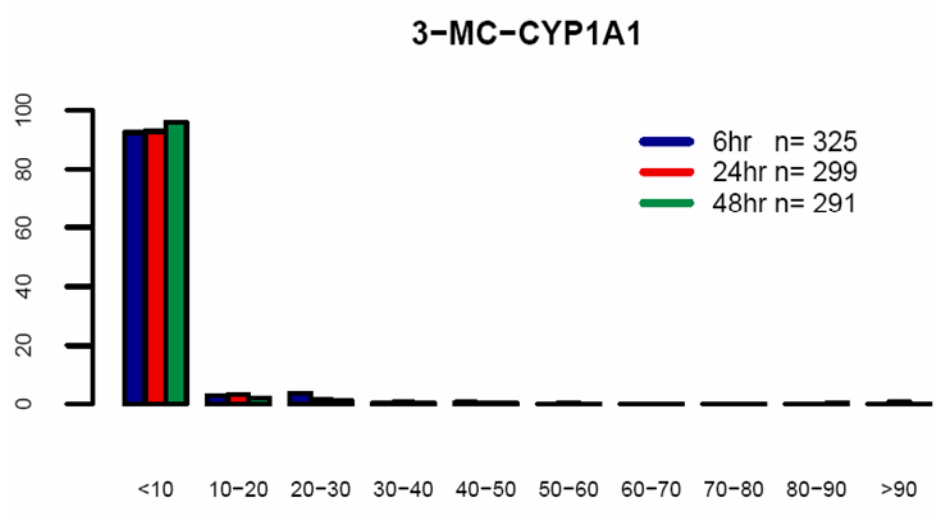


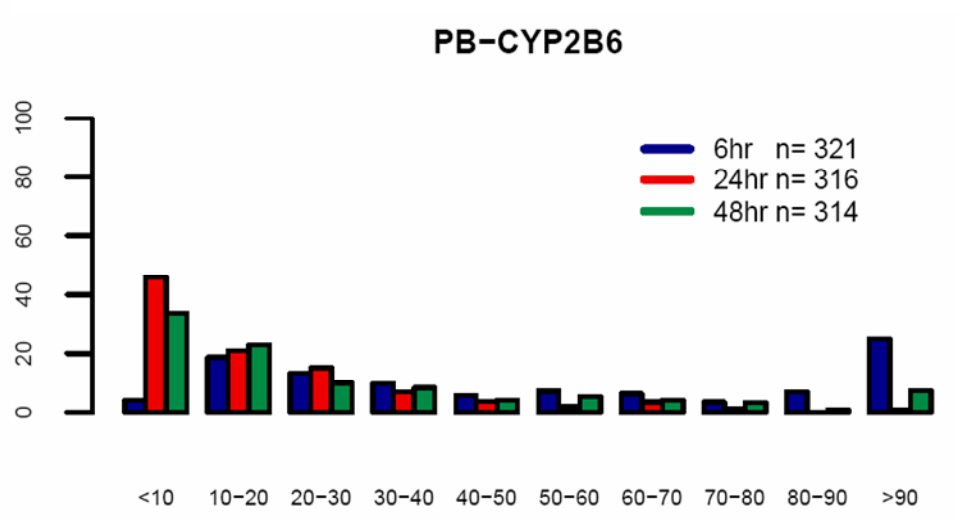

HMGCS2: 6hr : EC50: 2.22: (uM)
HMGCS2: 24hr: IC50: 21.3: (uM)
HMGCS2: 48hr: IC50: 14.2: (uM)
Raw Luminescence Units (RLU)
CDCA (µM)

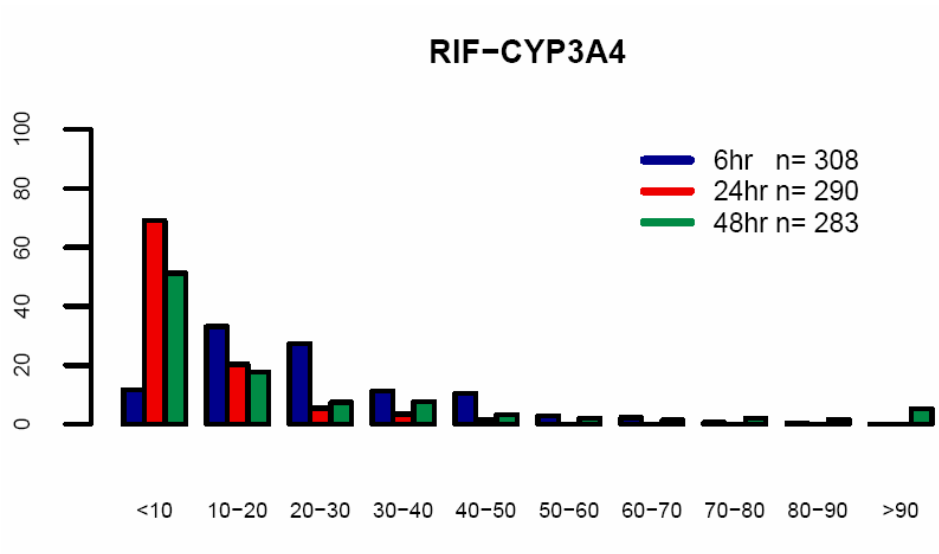

ABCB11: EC50: 45.9: 8.06e+03: (uM)
SLCO1B1: EC50: 29.7: 9.27e+03: (uM)
UGT1A1: EC50: 100.: 7.69e+03: (uM)
HMGCS2: IC50: 13.8: 8.07e+03: (uM)
Fold-Over-Control
CDCA (µM)

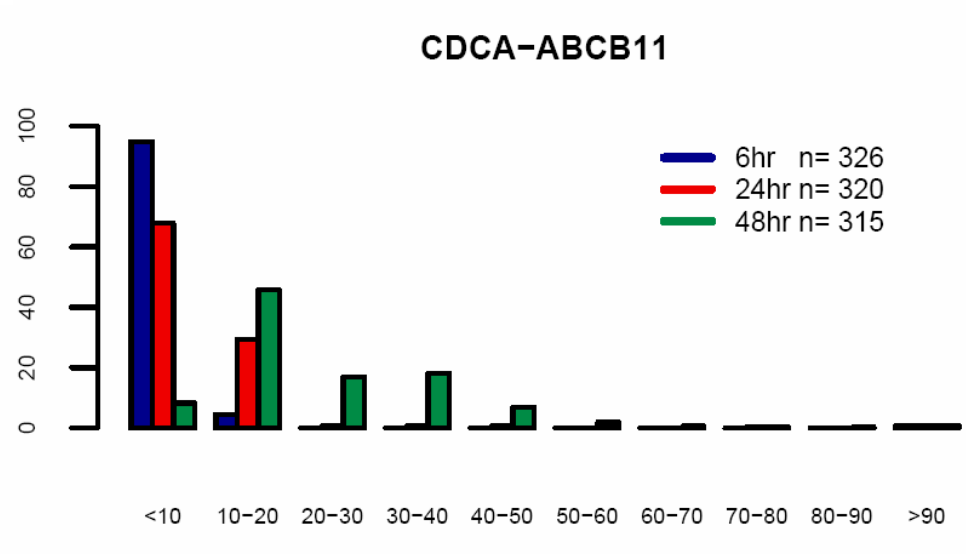

ORGANIC ANIONS (OA)
BILE SALTS (BS)
OATP2
NTCP
DRUGS
MDR1
MRP2
OA
PXR
RXR
OA
BS
CYP3A
Bile Canaliculus
CAR
OA-OH
BS-OH
UGT's
MDR 2/3
BSEP
PHOSPHATIDYL CHOLINE
BS
FXR
MRP3

Histograms: Percent of Chemicals vs. % of Reference Chemical Response









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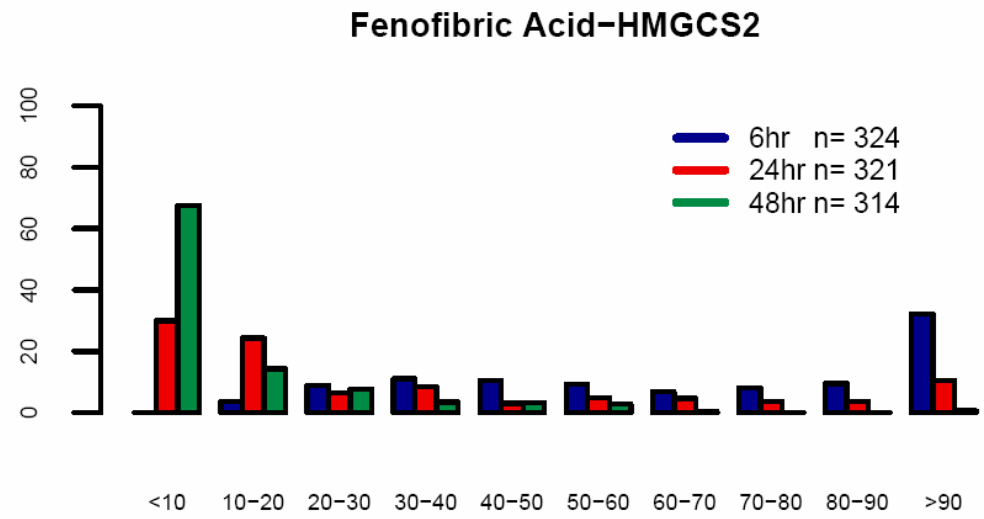
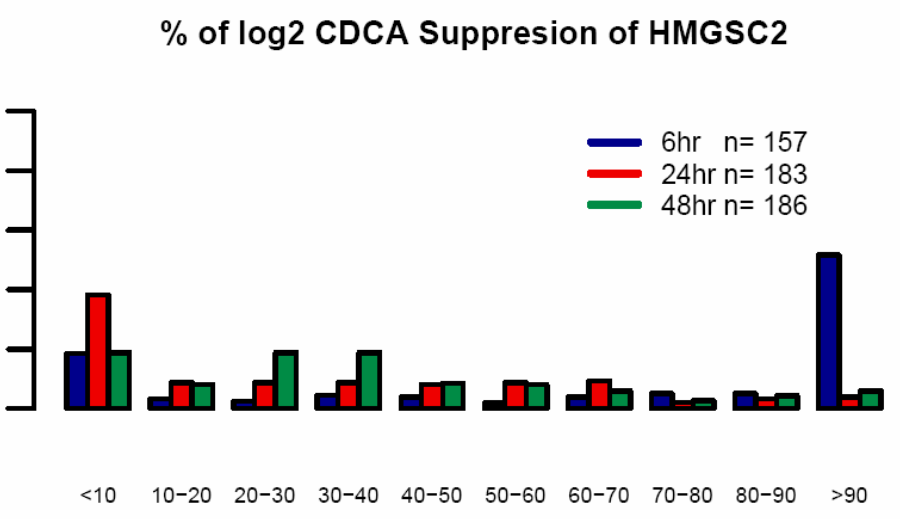
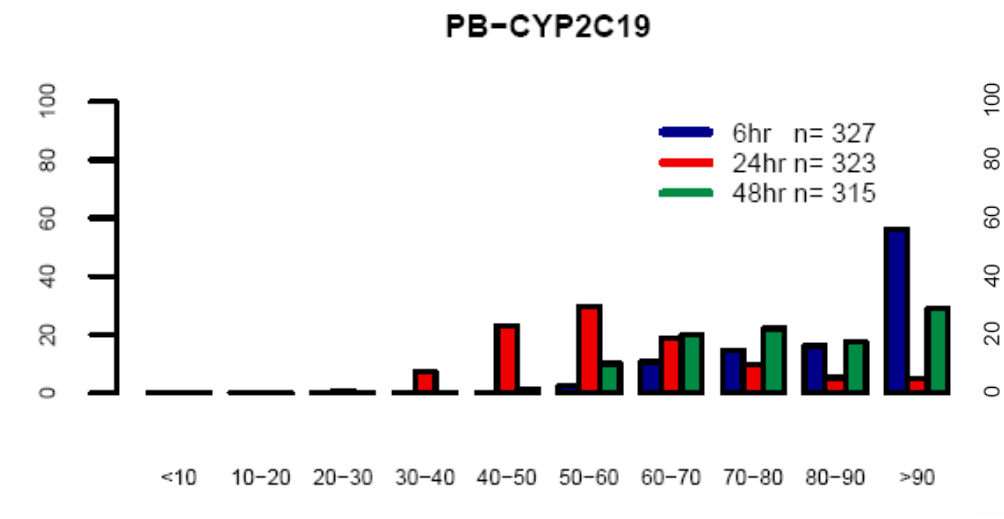
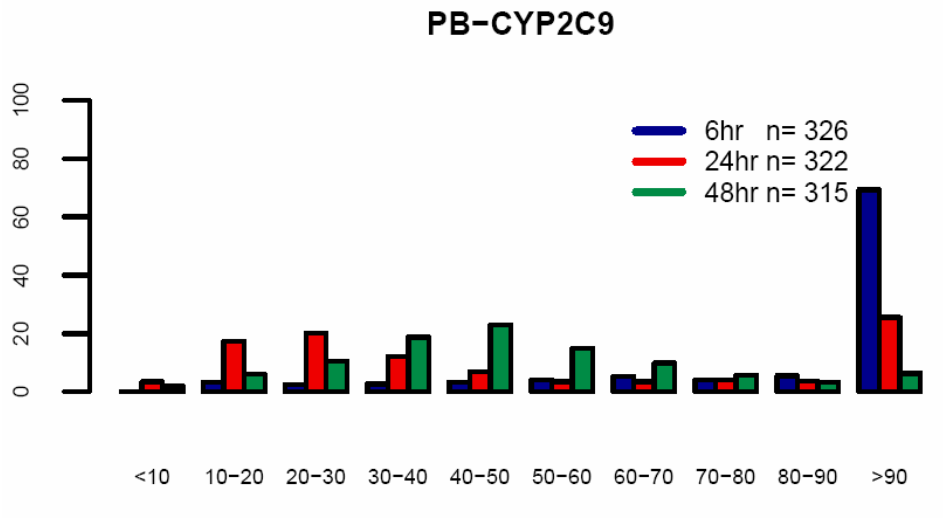
Histograms: Percent of Chemicals vs. % of Reference Chemical Response









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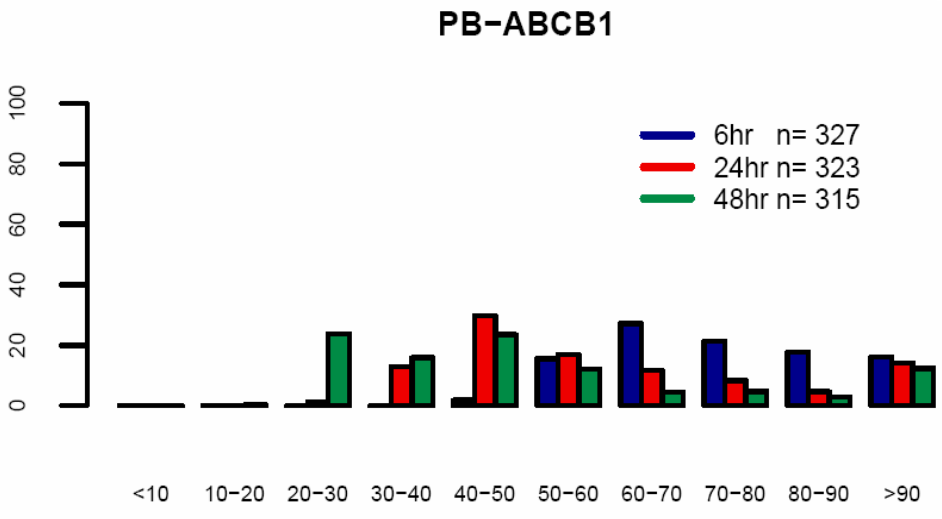
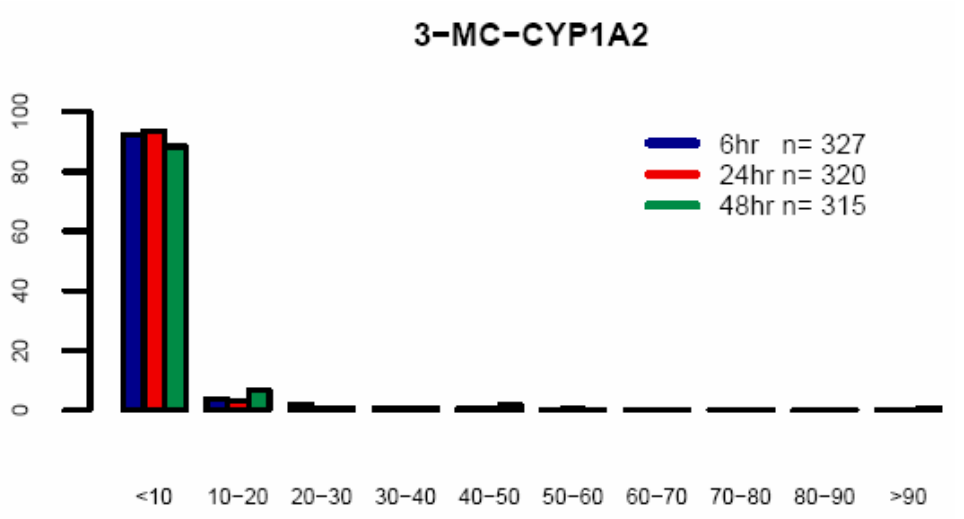
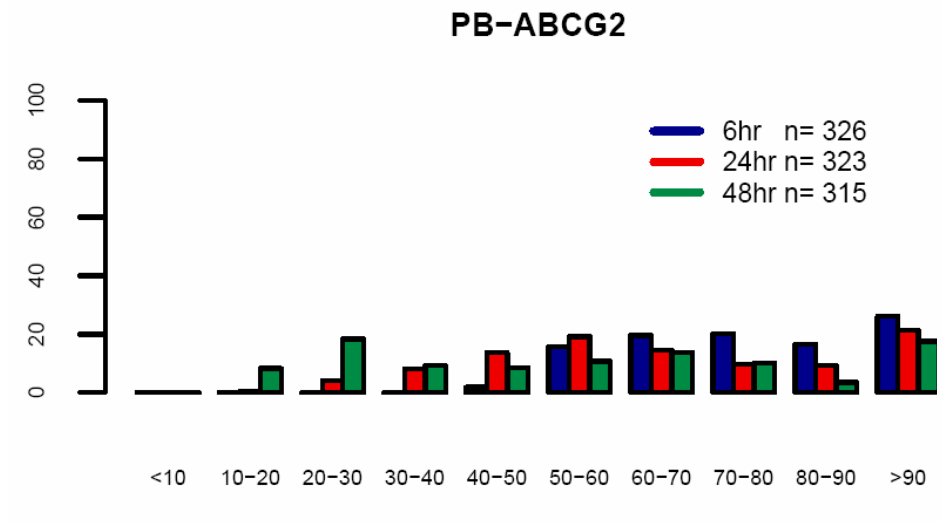
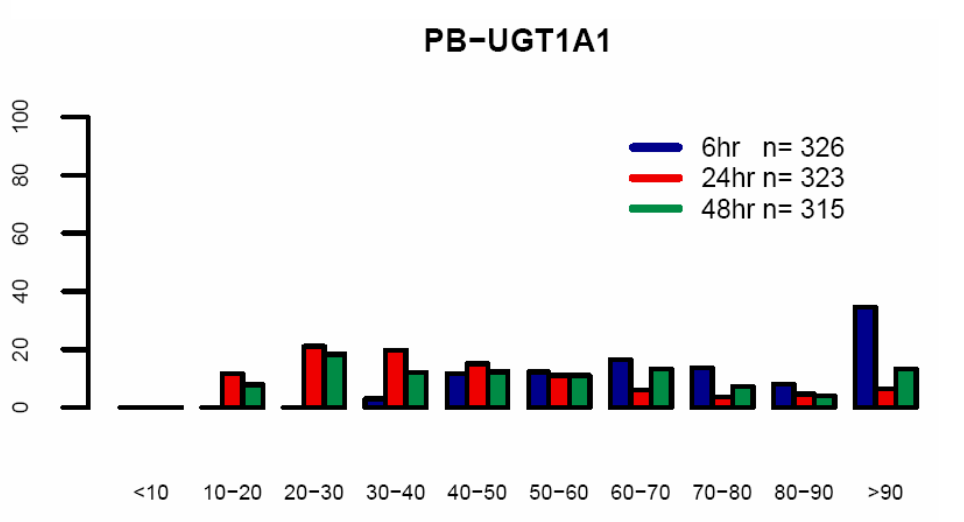
Histograms: Percent of Chemicals vs. % of Reference Chemical Response









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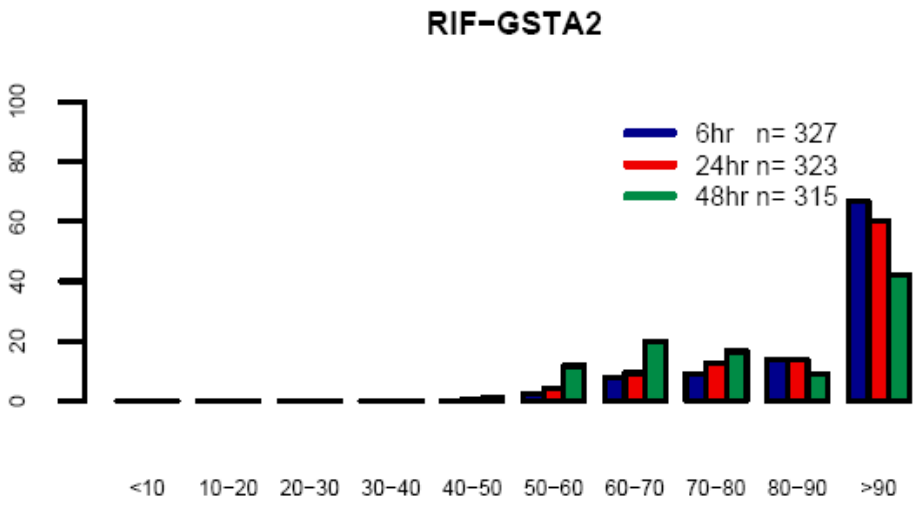
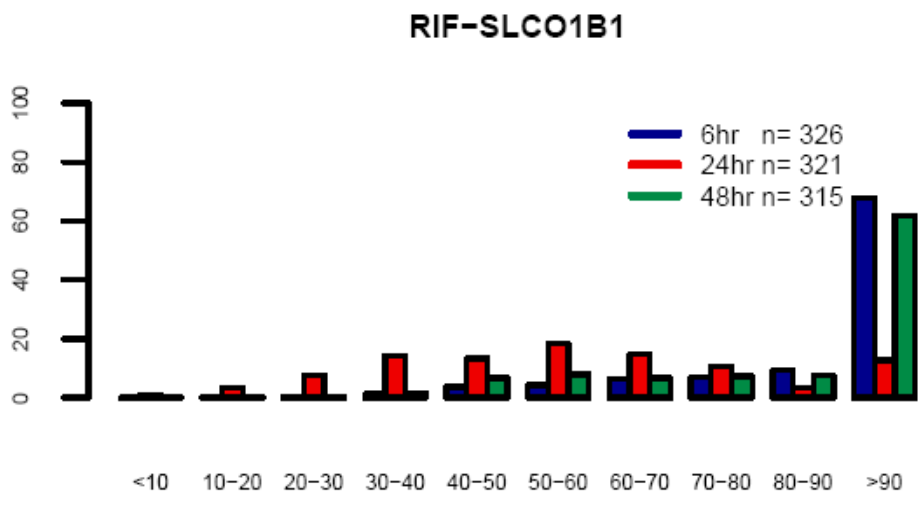
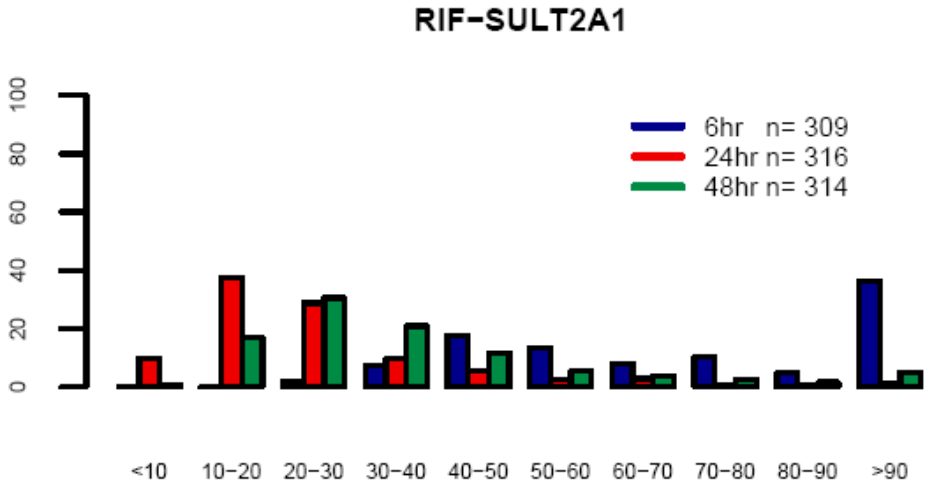
Histograms: Percent of Chemicals vs. % of Reference Chemical Response







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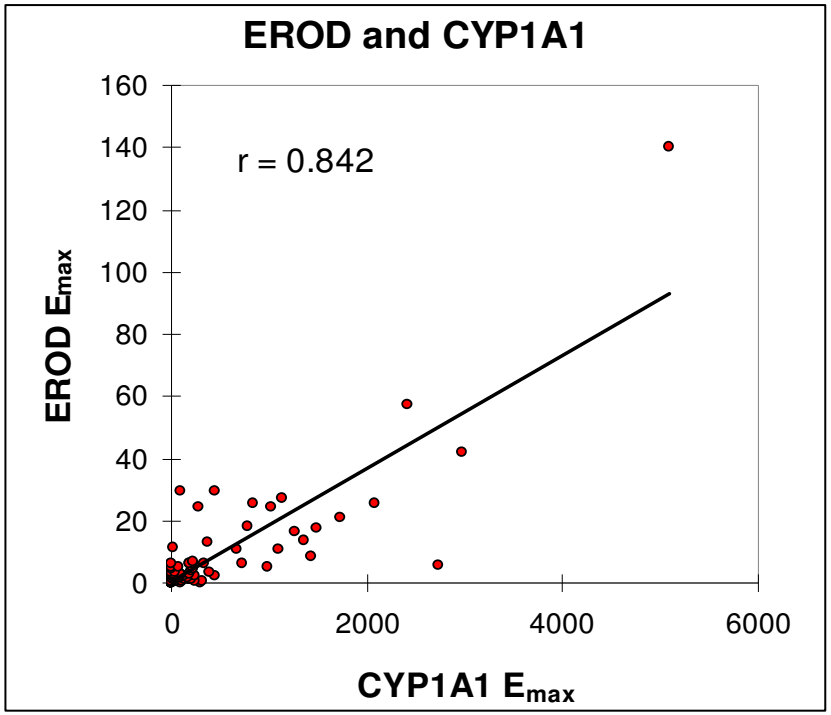
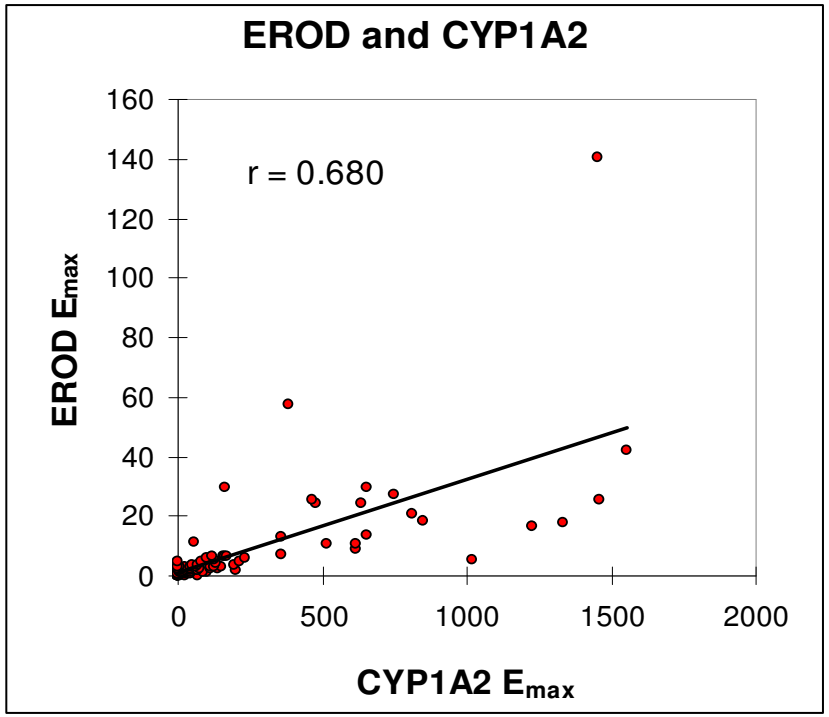
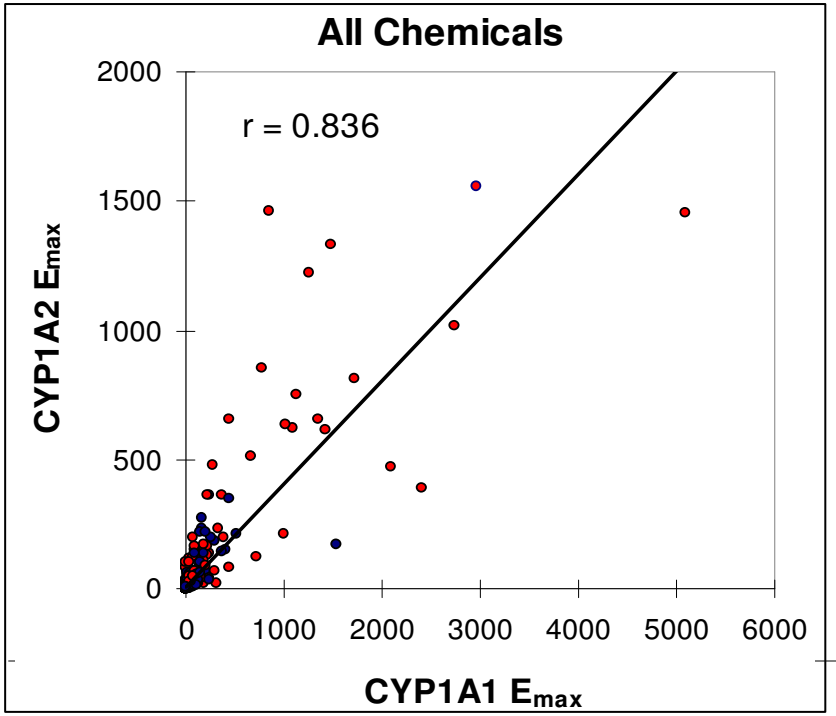
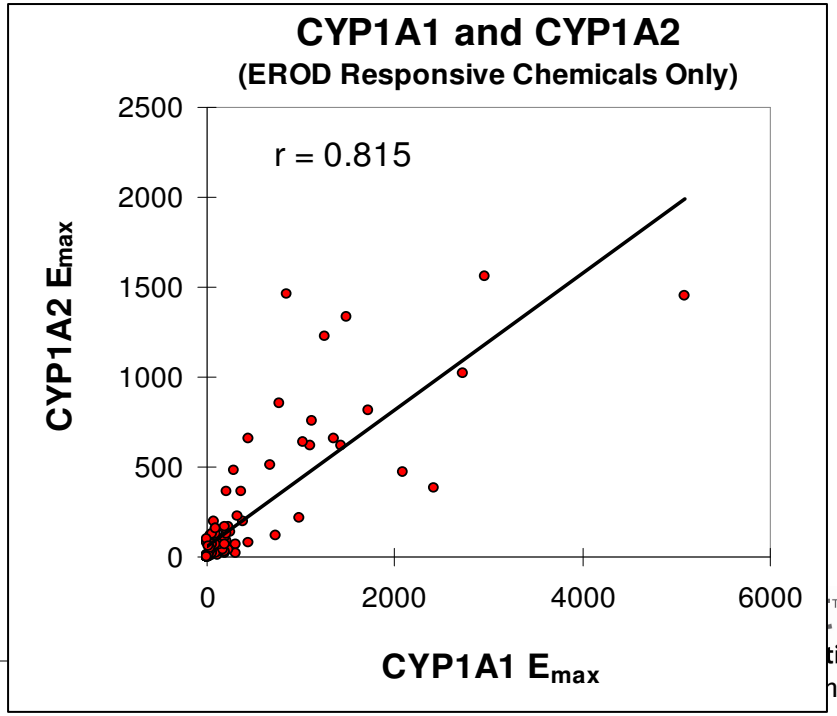

EROD and CYP1A1
r = 0.842
EROD Emax
CYP1A1 Emax
0
20
40
60
80
100
120
140
160
0
2000
4000
6000


EROD and CYP1A2
r = 0.680
EROD Emax
CYP1A2 Emax
0
20
40
60
80
100
120
140
160
0
500
1000
1500
2000


All Chemicals
r = 0.836
CYP1A2 Emax
CYP1A1 Emax
0
500
1000
1500
2000
0
1000
2000
3000
4000
5000
6000


CYP1A1 and CYP1A2
(EROD Responsive Chemicals Only)
r = 0.815
CYP1A2 Emax
CYP1A1 Emax
0
500
1000
1500
2000
2500
0
2000
4000
6000

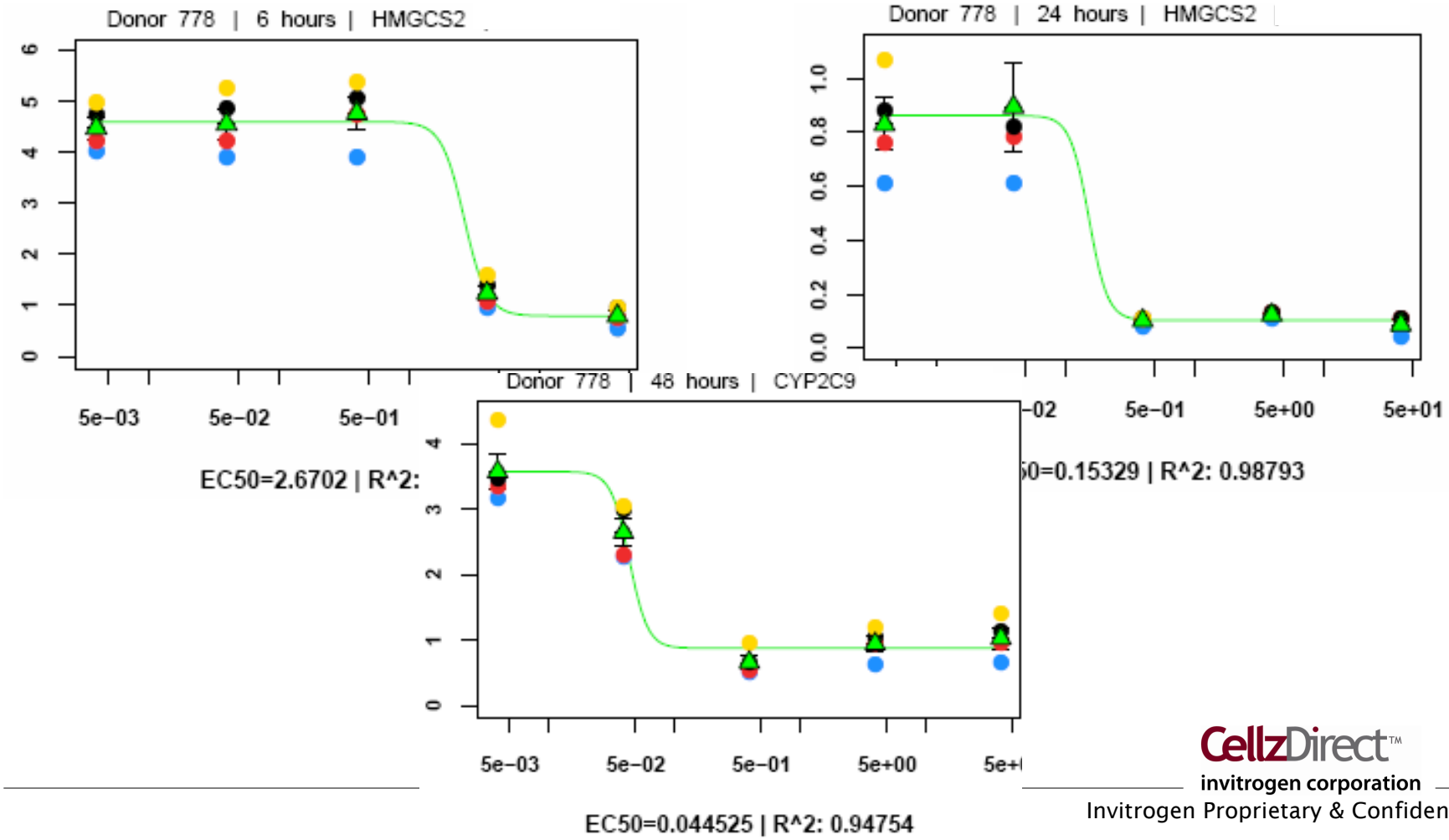
Highlight Table

Gene 1 (time)	r	Gene 2 (time)
CYP1A1 (24)	0.9445	CYP1A2 (24)
ABCB11 (48)	0.9053	SLCO1B1 (48)
ABCB11 (48)	0.8859	ABCG2 (48)
ABCG2 (48)	0.8645	SLCO1B1 (48)
CYP1A1 (24)	0.8610	CYP1A2 (48)
ABCB1 (48)	0.8515	ABCG2 (48)
CYP1A1 (6)	0.8312	CYP1A2 (24)
CYP1A2 (24)	0.8220	CYP1A2 (48)
CYP1A1 (24)	0.8159	CYP1A1 (48)
CYP1A1 (6)	0.8009	CYP1A1 (24)
CYP1A2 (24)	0.7963	CYP1A1 (48)
CYP1A1 (6)	0.7940	CYP1A1 (48)
SLCO1B1 (48)	0.7801	SULT2A1 (48)
ABCB11 (48)	0.7660	SULT2A1 (48)
CYP1A1 (48)	0.7613	CYP1A2 (48)
ABCG2 (48)	0.7555	SULT2A1 (48)
CYP1A1 (6)	0.7519	CYP1A2 (48)
CYP2B6 (48)	0.6415	UGT1A1 (48)
CYP2B6 (6)	0.6171	CYP3A4 (6)
ABCB1 (48)	0.6156	CYP3A4 (48)
CYP2B6 (24)	0.5924	CYP3A4 (24)
CYP2C9 (48)	0.5896	CYP3A4 (48)
CYP2B6 (24)	0.5522	CYP2B6 (48)
CYP2C9 (24)	-0.2103	HMGCS2 (6)

Note time similarities are pronounced

Suppression

- Only ~550 suppression responses relative to thousands of observed induction responses at various time points and doses
- HMGCS2 gene was the most frequently suppressed gene followed by others including CYP2C9



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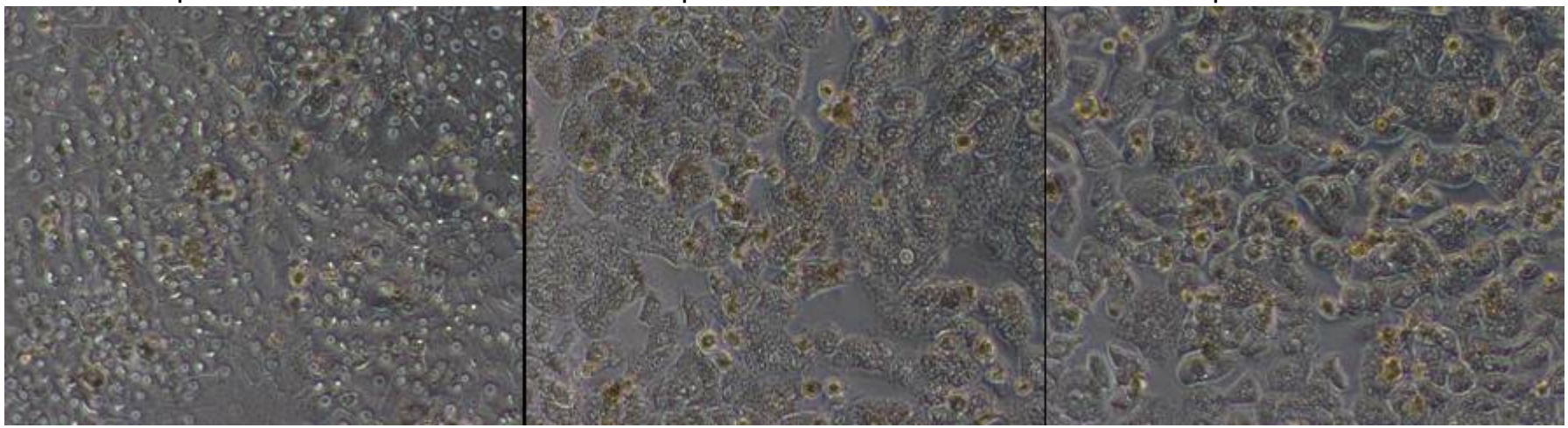
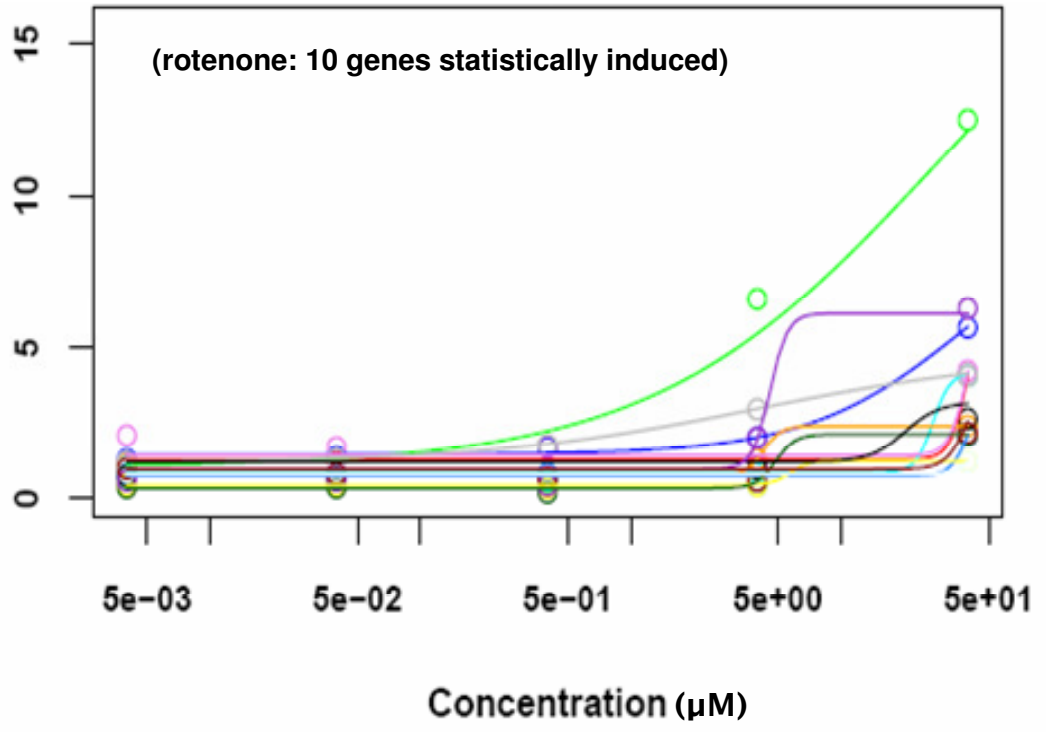
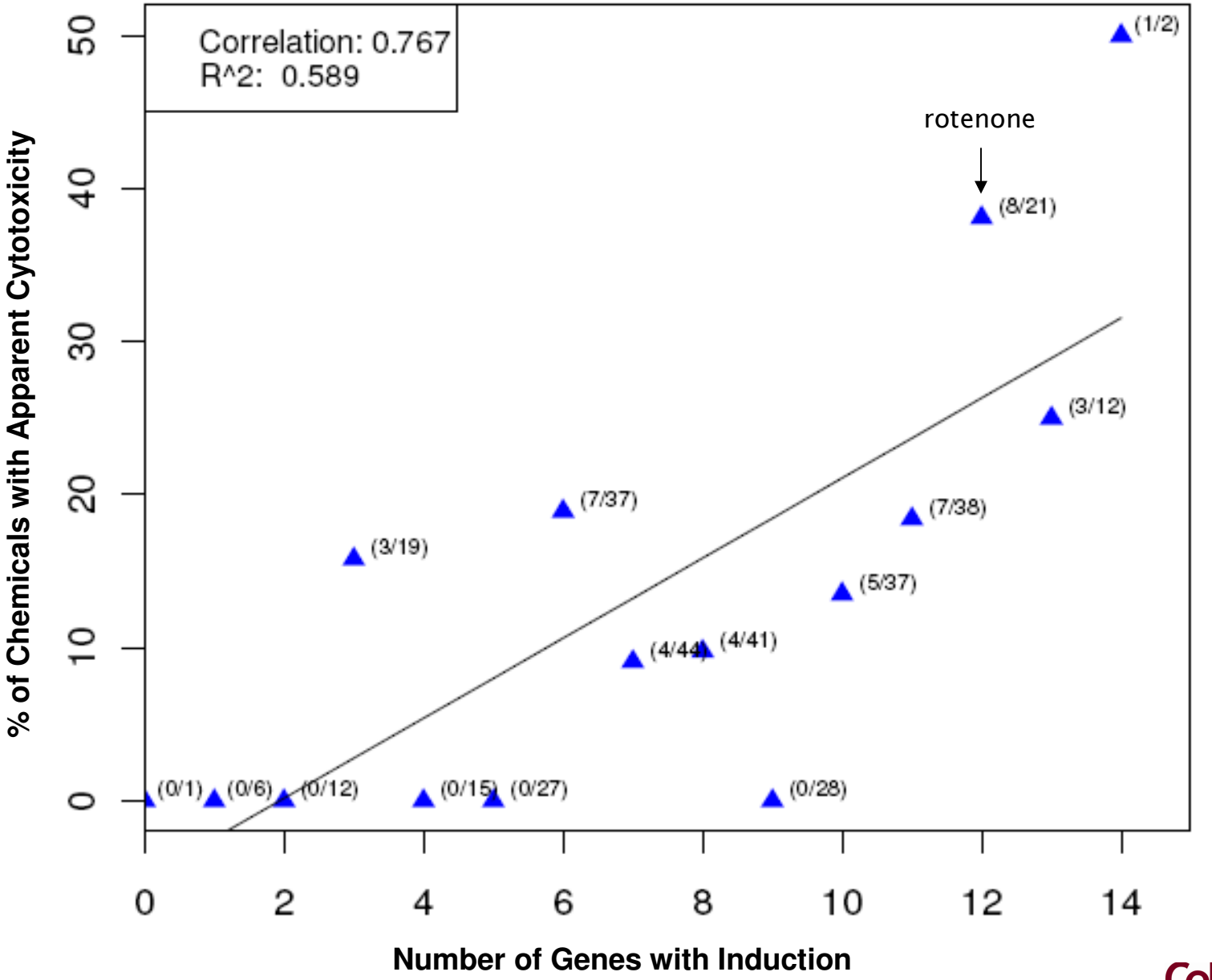
Changes in Cell Morphology in Response to Chemical Exposure

0.4 µM rotenone | 4 µM rotenone | 40 µM rotenone



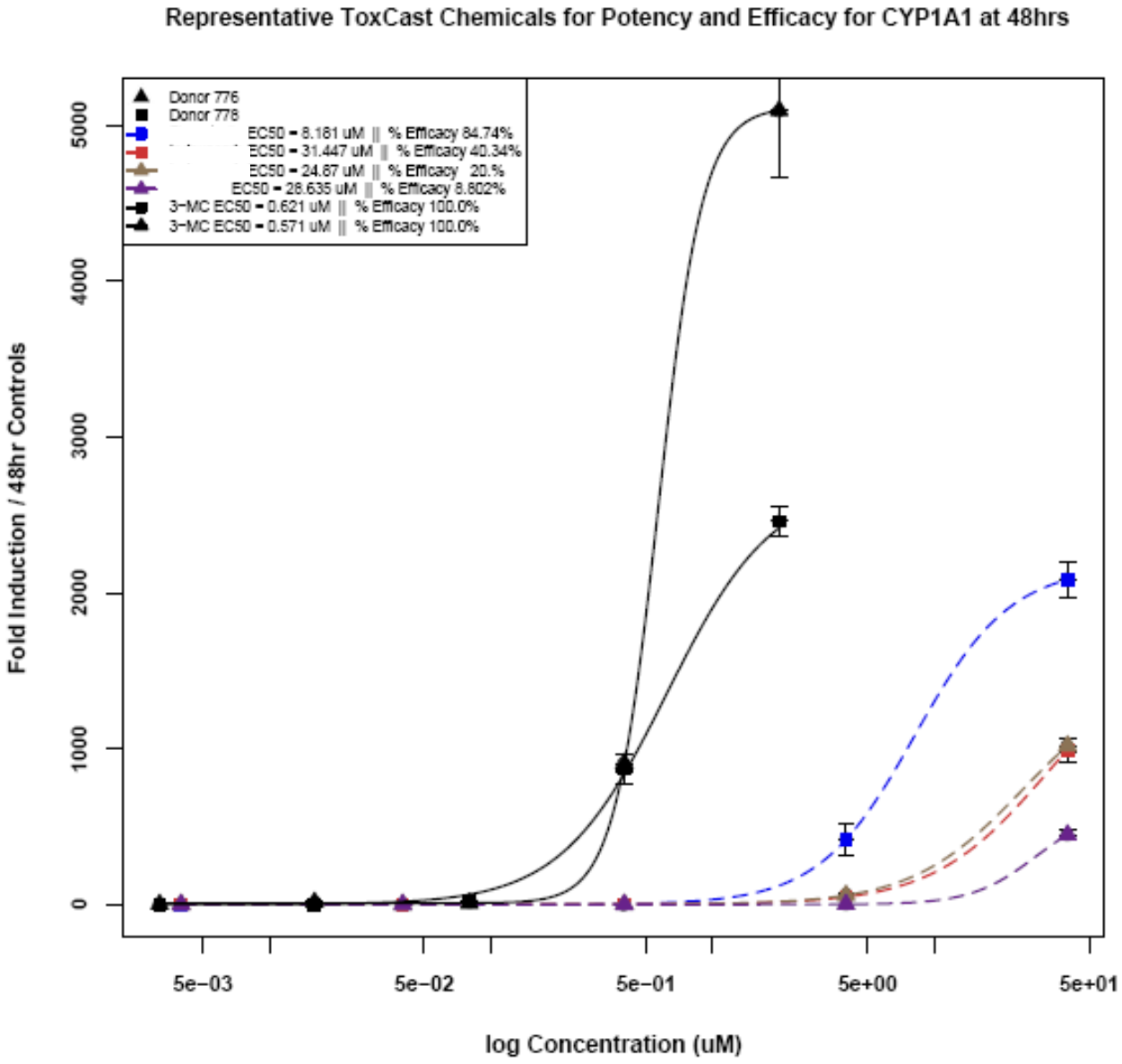


Induction vs. % Chemicals with Apparent Cytotoxicity



Preliminary Data

Representative Efficacious ToxCast 320 CYP1A1 Inducers



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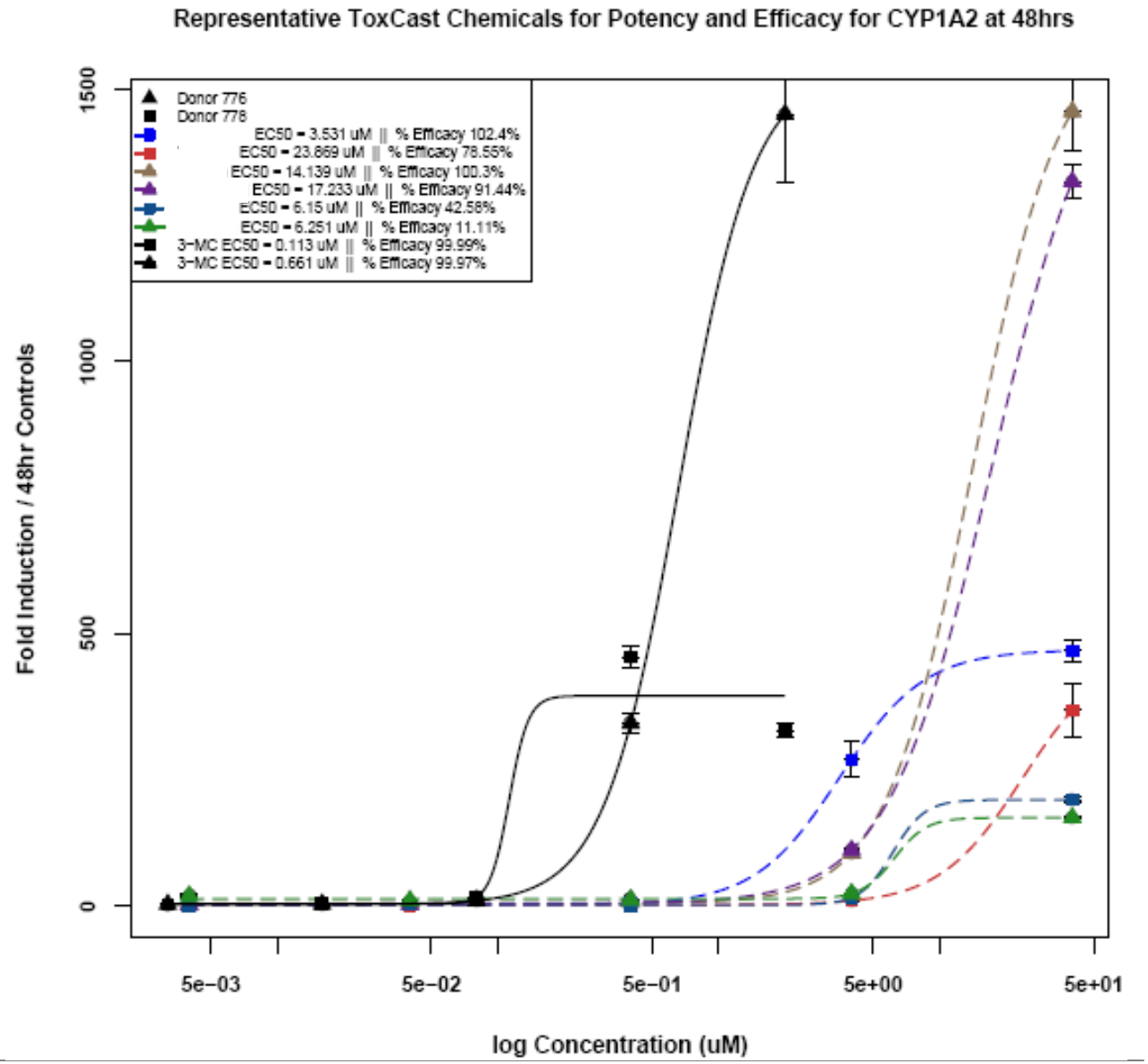
Representative Efficacious ToxCast 320 CYP1A2 Inducers



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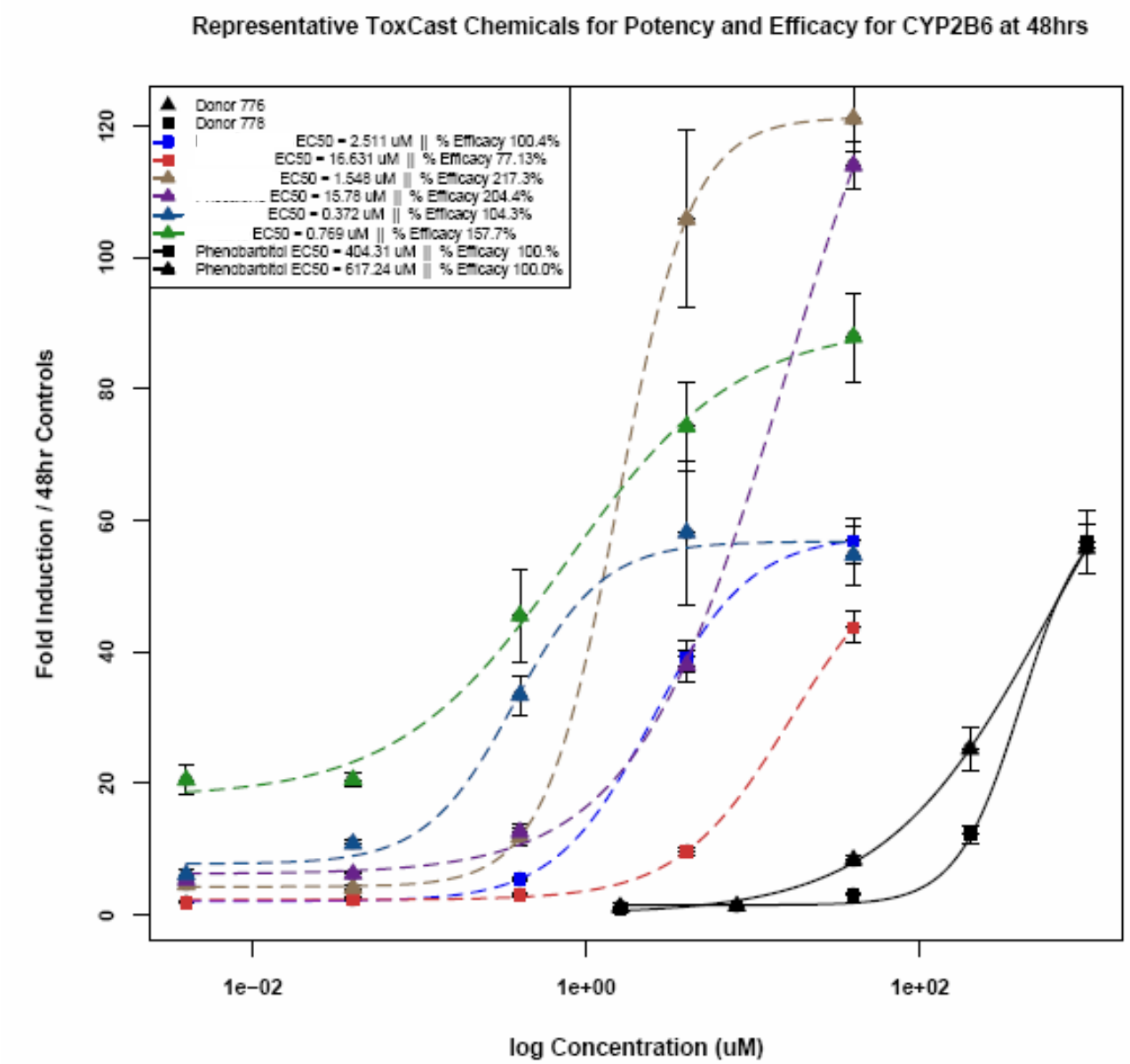
Representative Efficacious ToxCast 320 CYP2B6 Inducers



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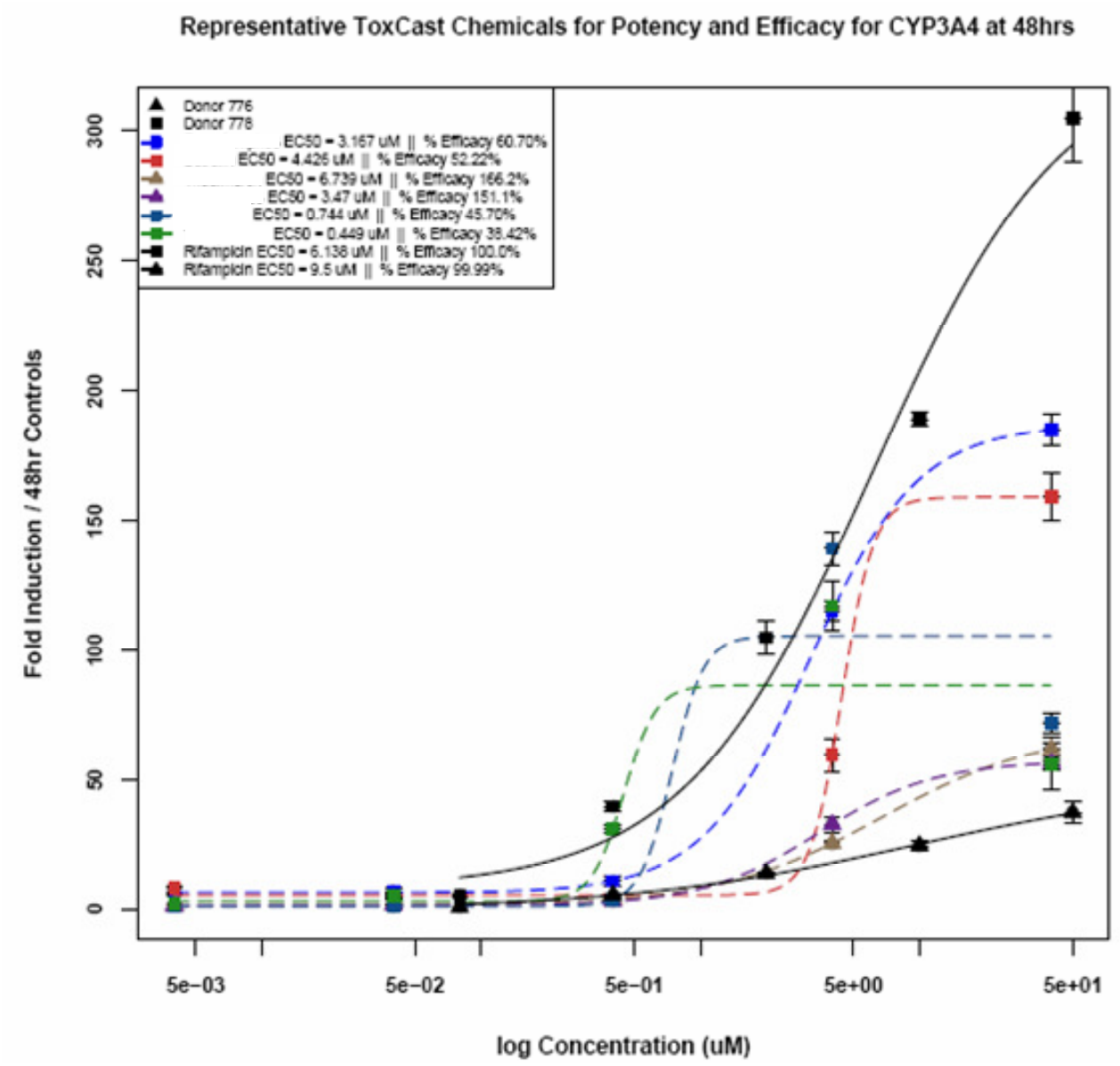
Representative Efficacious ToxCast 320 CYP3A4 Inducers



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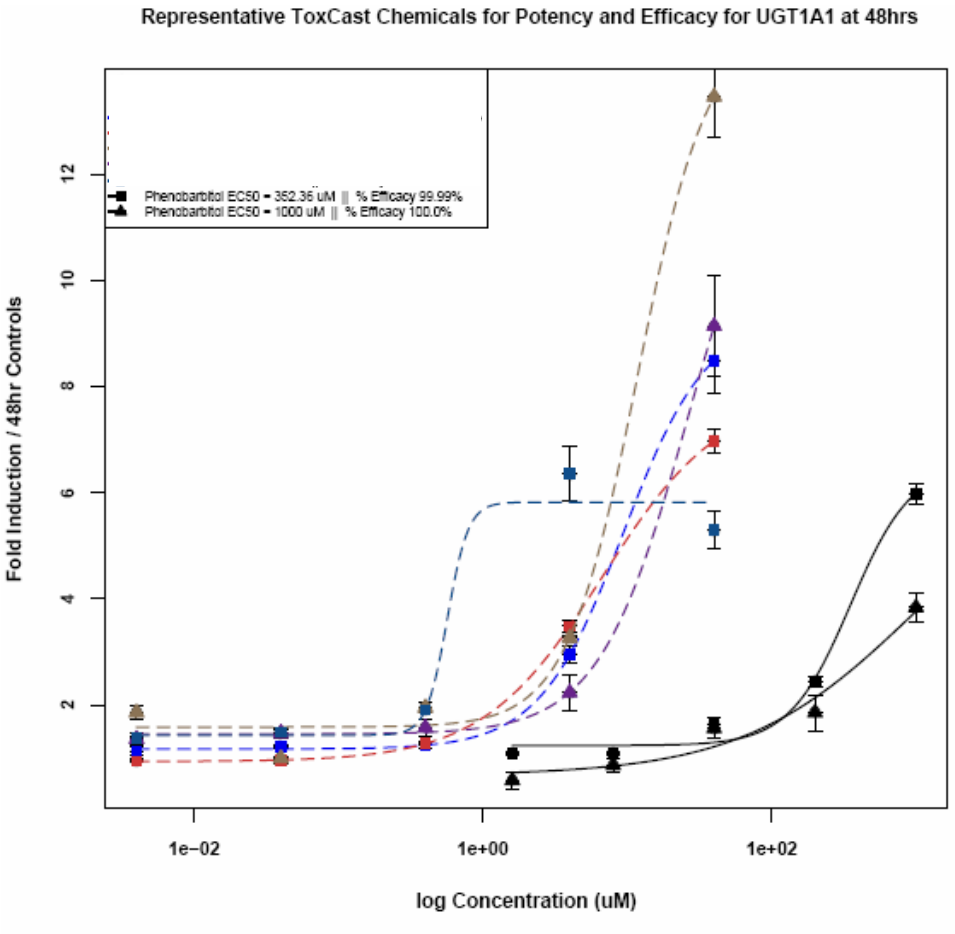
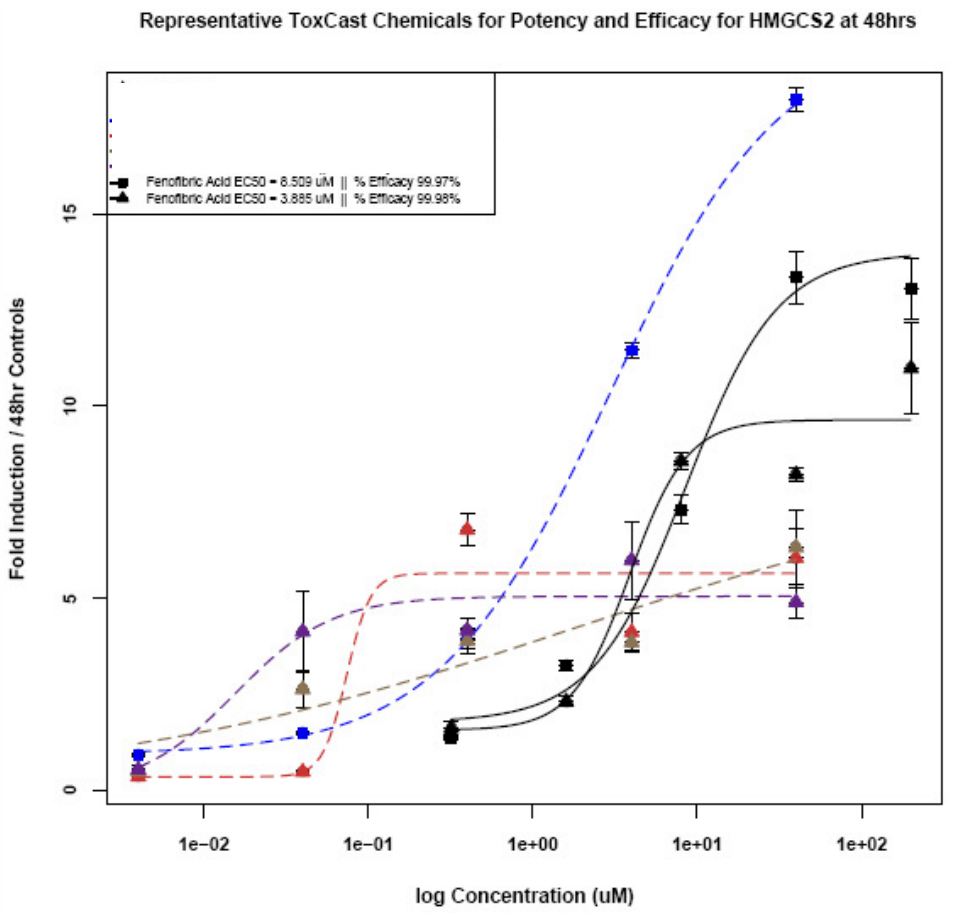
Representative ToxCast 320 UGT1A1 & HMGCS2 Inducers





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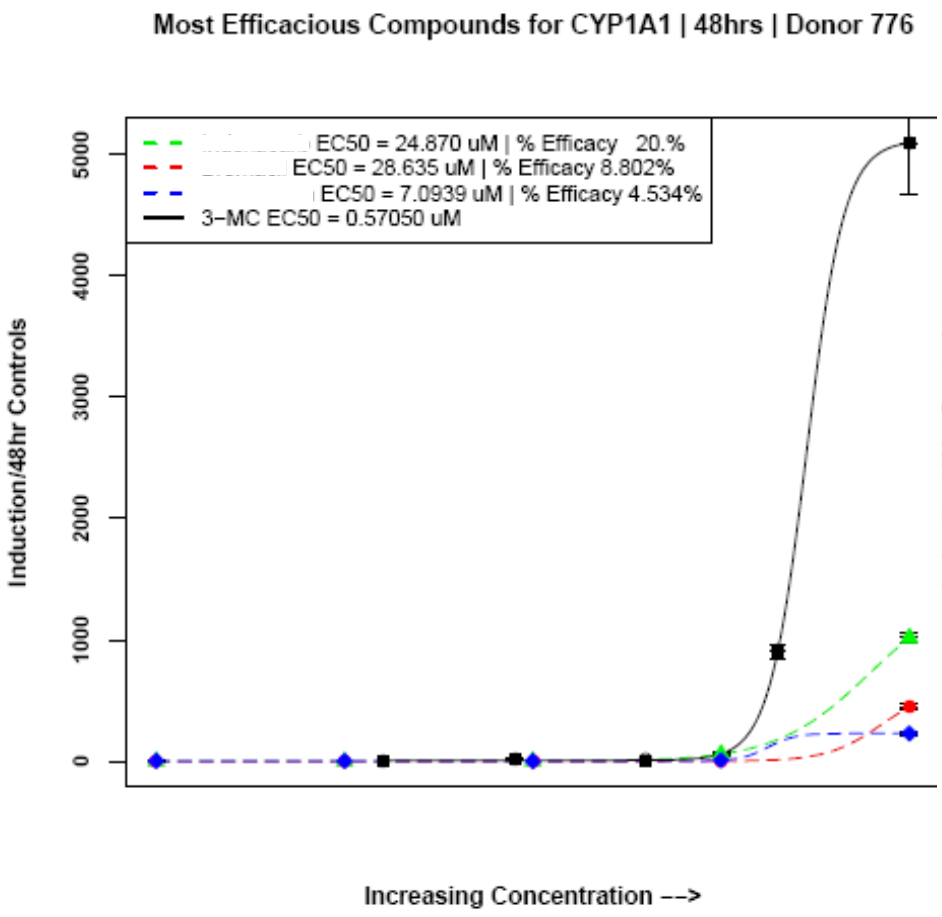
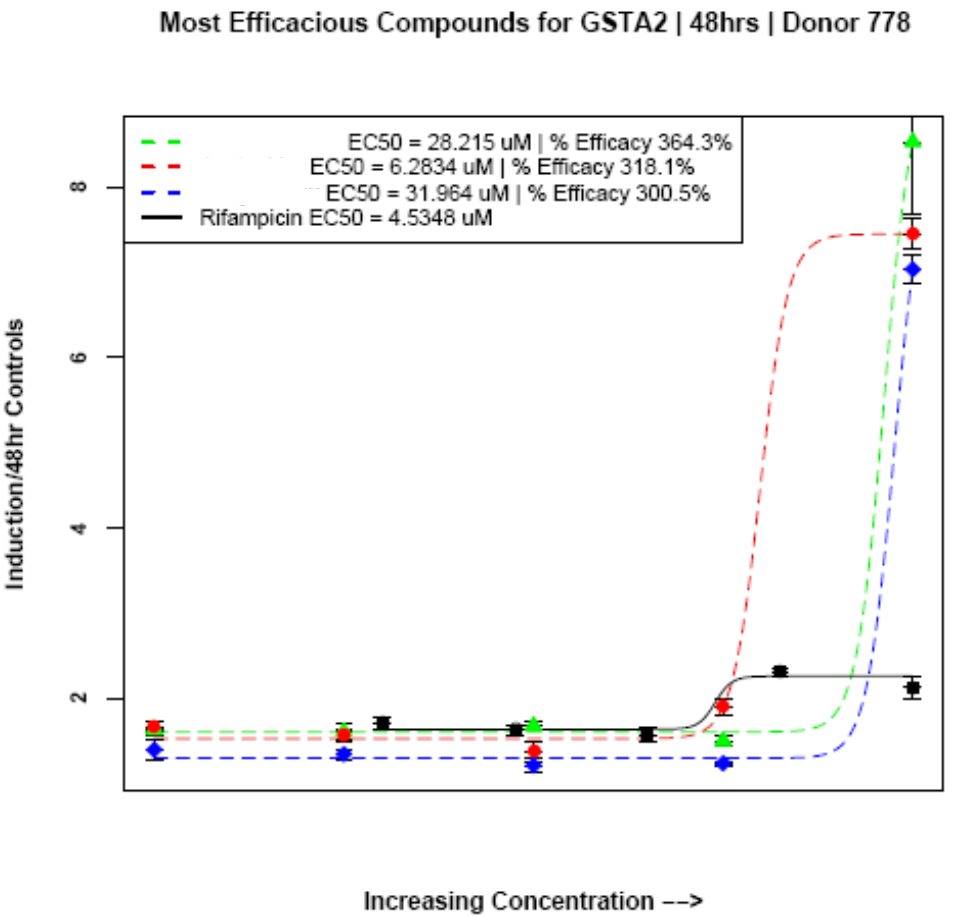
Efficacious ToxCast 320 Responses





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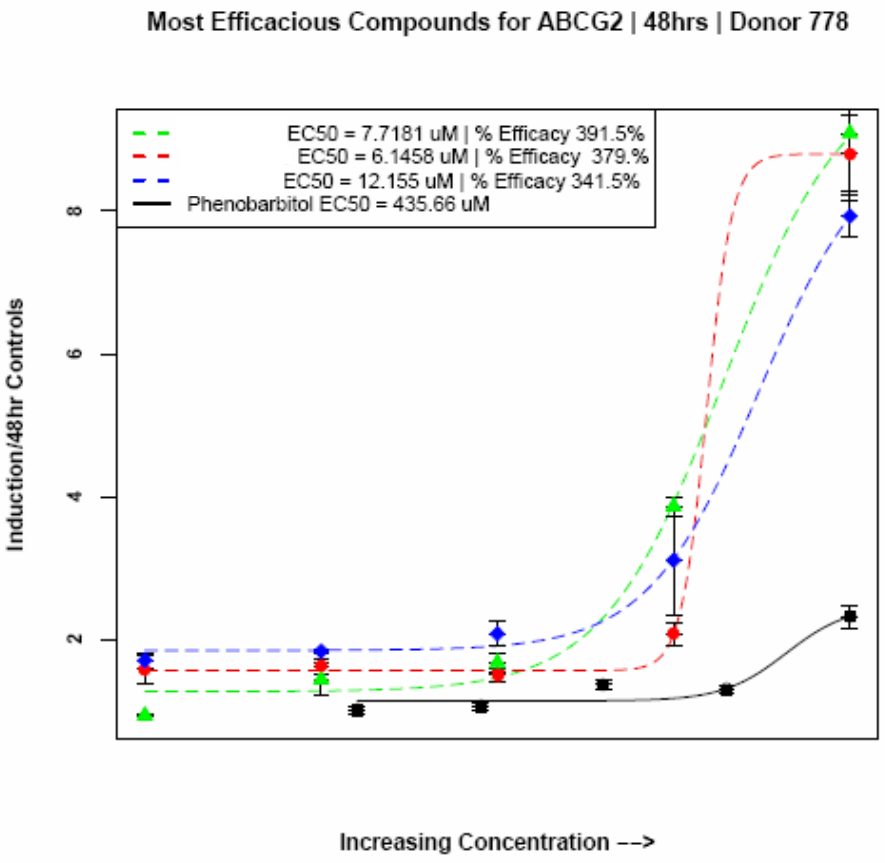
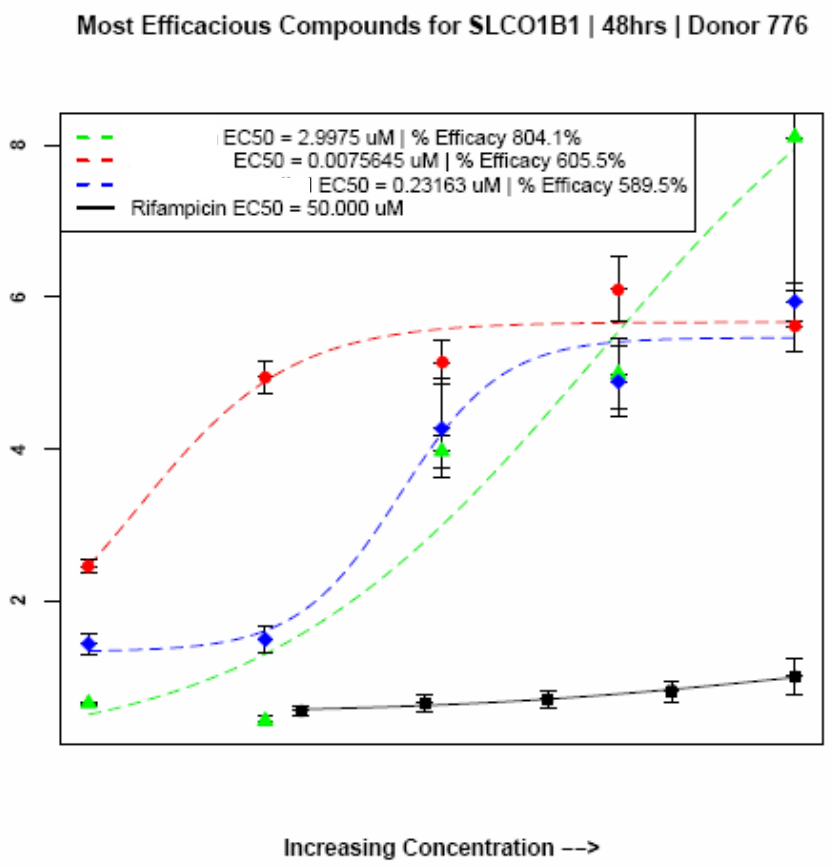
Representative dose-response curves





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Summary & Next Steps

- Effectively cultured primary human hepatocytes on 218 plates in 96-well format across 5 chemical concentrations and 4 time points within 2 week period

- Assessed endpoints of cell morphology, EROD, and multiplexed qNPA for 14 gene targets and completed experiments within 6 weeks to generate over 300,000 publication-quality data points

- Profiled and clustered the ToxCast 320 chemicals' relative receptor activators for the AhR, CAR, PXR, FXR, and PPARα pathways

- Fit over 14,000 dose response curves to generate EC_{50}, E_{max}, E_{min}, and Hill slopes for qNPA and EROD data to explore and classify chemical responses

- Most chemicals have relatively low efficacy effects (<40% relative to reference chemicals) on well-characterized gene targets (e.g. CYP1A) based on histograms

- The large efficacious inductions observed with select ToxCast 320 chemicals (relative to reference chemicals) for gene targets such as GSTA2, SLC01B1, ABCG2, and ABCB1 indicate further studies are needed to explore their gene regulation

- Data demonstrate a clear suppressive effect of bile acid CDCA on cholesterol synthesis gene and PPARα induction target gene HMGCS2 that is not a hallmark of other FXR-like inducers based on these data

Summary & Next Steps Continued

- 'Bursts' of induction across multiple gene targets simultaneously appears to be related to the observation of chemical-related hepatocyte cellular stress
- Correlations confirm EROD and CYP1A1 relationship and suggest that CYP1A2 is similar to CYP1A1 but not well correlated with EROD activity
- Correlations demonstrate new insights into transporter gene regulation and support current thoughts regarding CYP2C9 induction
- Data demonstrate that suppression is a relatively rare event compared with induction for the selected gene targets
- The apparent correlation between diverse transporter gene targets suggests an adaptive response that is not well understood at the molecular level
- CYP2B6 appeared to be the most frequently induced gene target suggesting an important role for CAR (and PXR) in hepatocyte response to xenobiotics
- Ongoing analyses
  - Correlations of In vivo/in vitro data
    - Relative risk factor
    - Clustering analysis
  - Time effects: continue to explore cause/effect responses across multiple endpoints (e.g. apparent relationship between induction and cytotoxicity)

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