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Retrofitting High-Throughput In Vitro Assays for Metabolic Competence

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Office of Research and Development
National Center for Computational Toxicology

EDSP Workgroup
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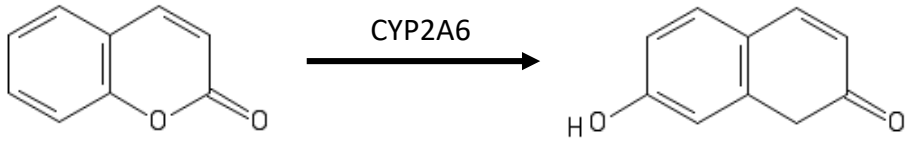
Why is Metabolic Competence Important for *in vitro* Assays?

Existing *in vitro* assays have limited or no metabolic capacity. This leads to two problems:

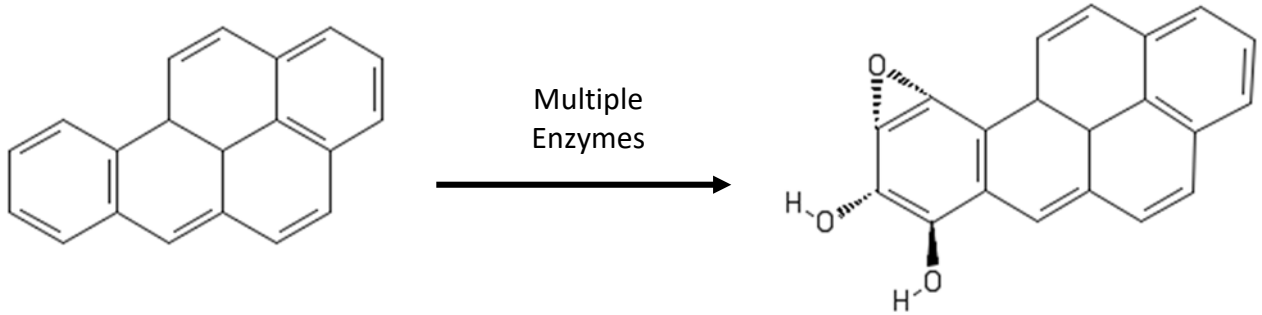
1. Overestimation of chemical hazard *in vitro* if the parent compound is detoxified to a less toxic or non-toxic metabolite *in vivo*

Example: Coumarin



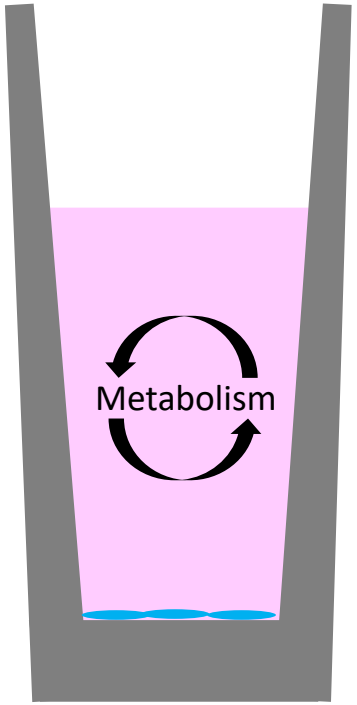
2. Underestimation of chemical hazard *in vitro* if the parent compound is activated to a more toxic metabolite *in vivo*

Example: Benzo[a]pyrene



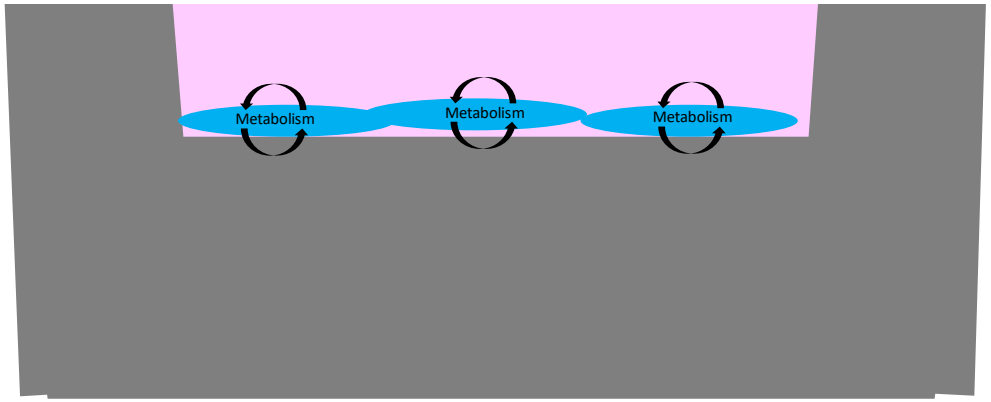
Two Scenarios- Two Strategies for Retrofitting

"Extracellular" Strategy



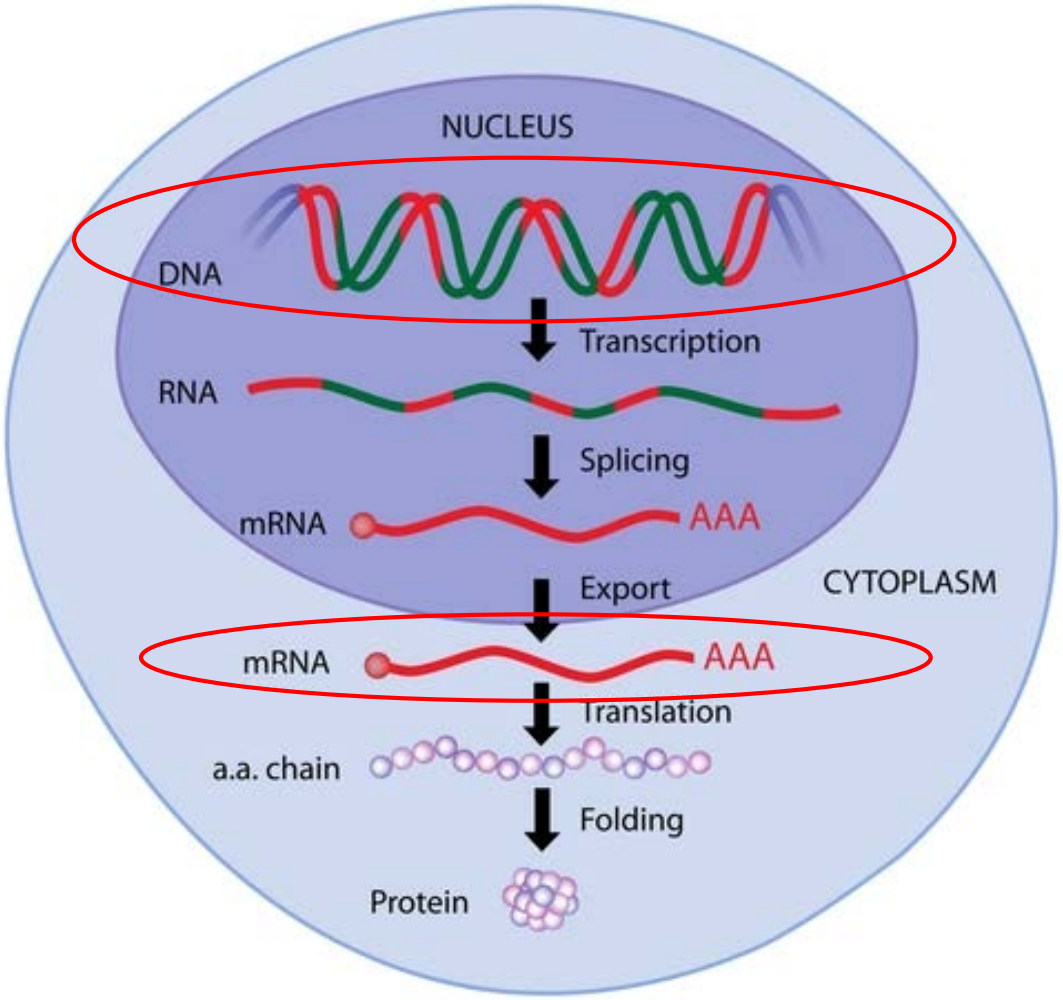
- Capable of metabolizing chemicals in the media or buffer of cell-based and cell-free assays
- More closely models hepatic metabolism and effects of circulating metabolites

"Intracellular" Strategy



- Capable of metabolizing chemicals inside the cell for cell-based assays
- More closely models effects of direct-acting metabolites

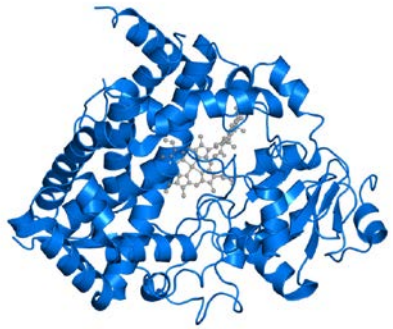
Intracellular Metabolism



- Introducing xenobiotic-metabolizing enzyme (XME)-encoding genes back into cells with low/no expression is not a new idea
- Plasmid transfection, electroporation, and various viral vectors introduce XME-encoding genes (DNA) back into cells under control of gene promoters that drive strong expression (transcription)
- Transcription levels vary greatly between cell types and tightly controlled co-expression genes is difficult

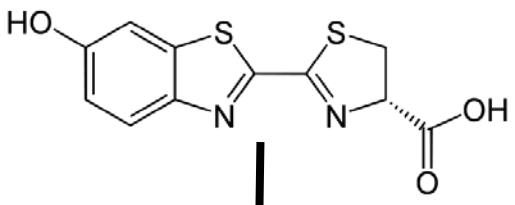
- Transfection of XME-encoding mRNAs is a novel approach that bypasses cellular transcription
- Chemically-modified nucleotides and cap eliminate the toxicity traditionally seen with RNA transfection
- Rapid XME expression and permits user to define composition and ratios of input mRNAs
- Method development focused on cytochrome P450 (CYP) enzymes, responsible for Phase I metabolism

P450-Glo Assay Principle

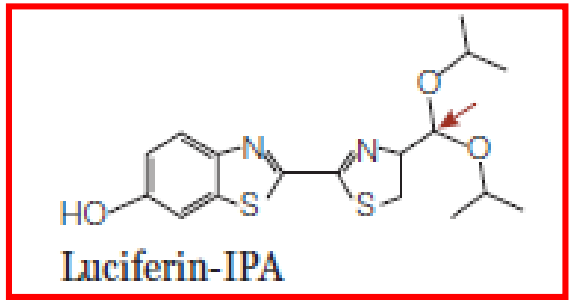


P450-Glo™ Substrate
(pro-luciferin)

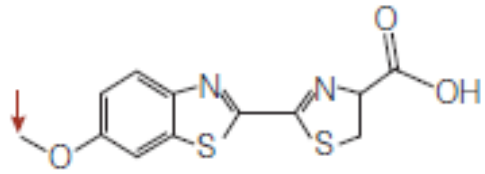
CYP
Enzyme



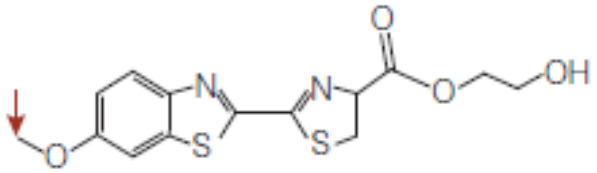
Light
Signal



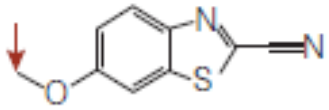
Luciferin-IPA



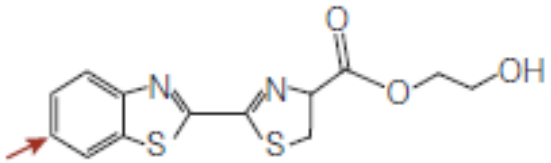
Luciferin-ME



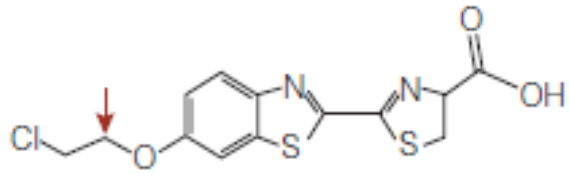
Luciferin-ME EGE



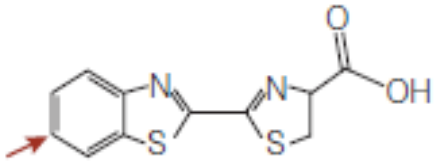
Luciferin-1A2[3]



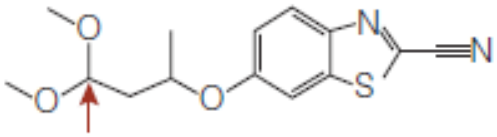
Luciferin-H EGE



Luciferin-CEE

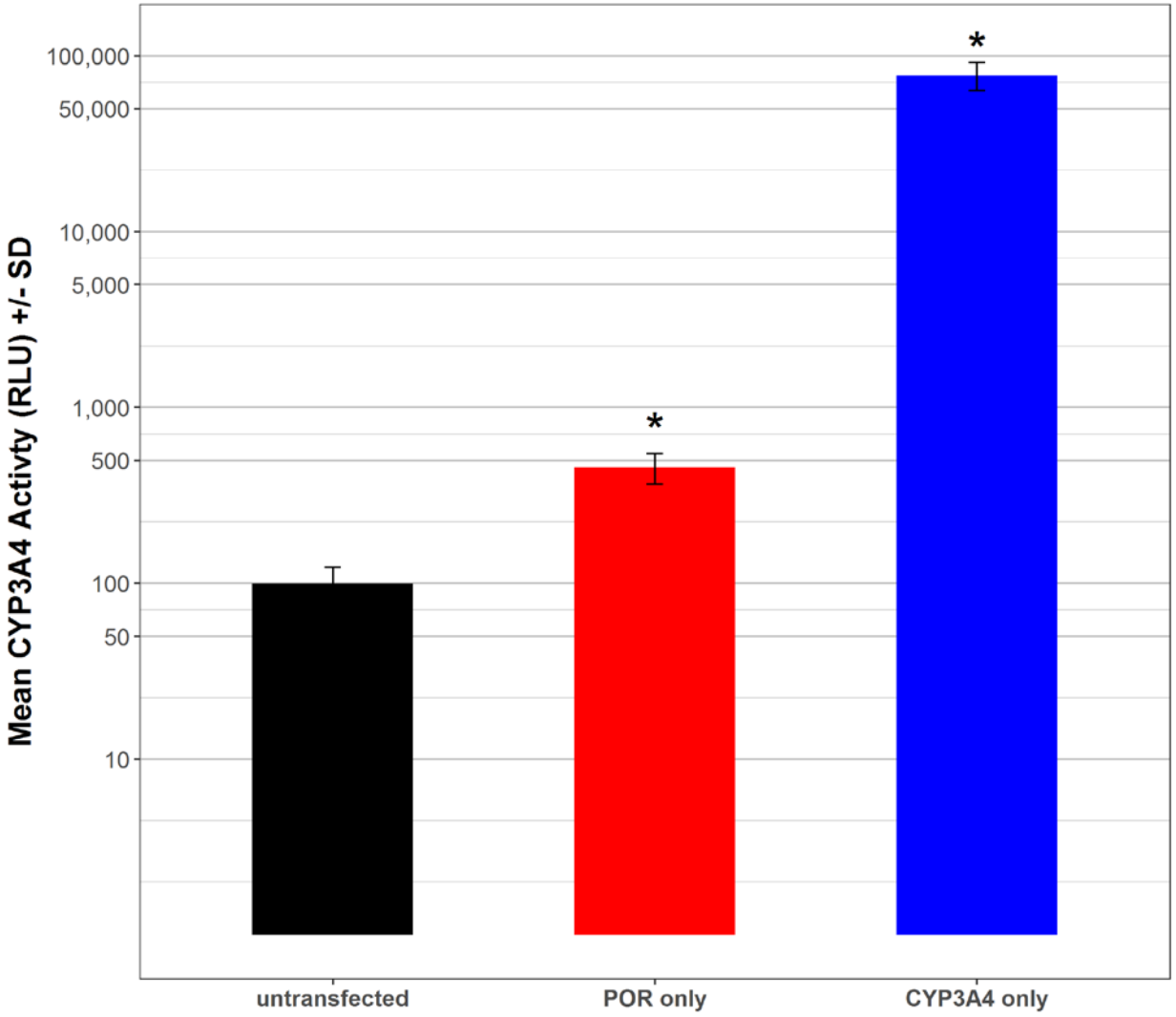


Luciferin-H



Luciferin-2B6[3]

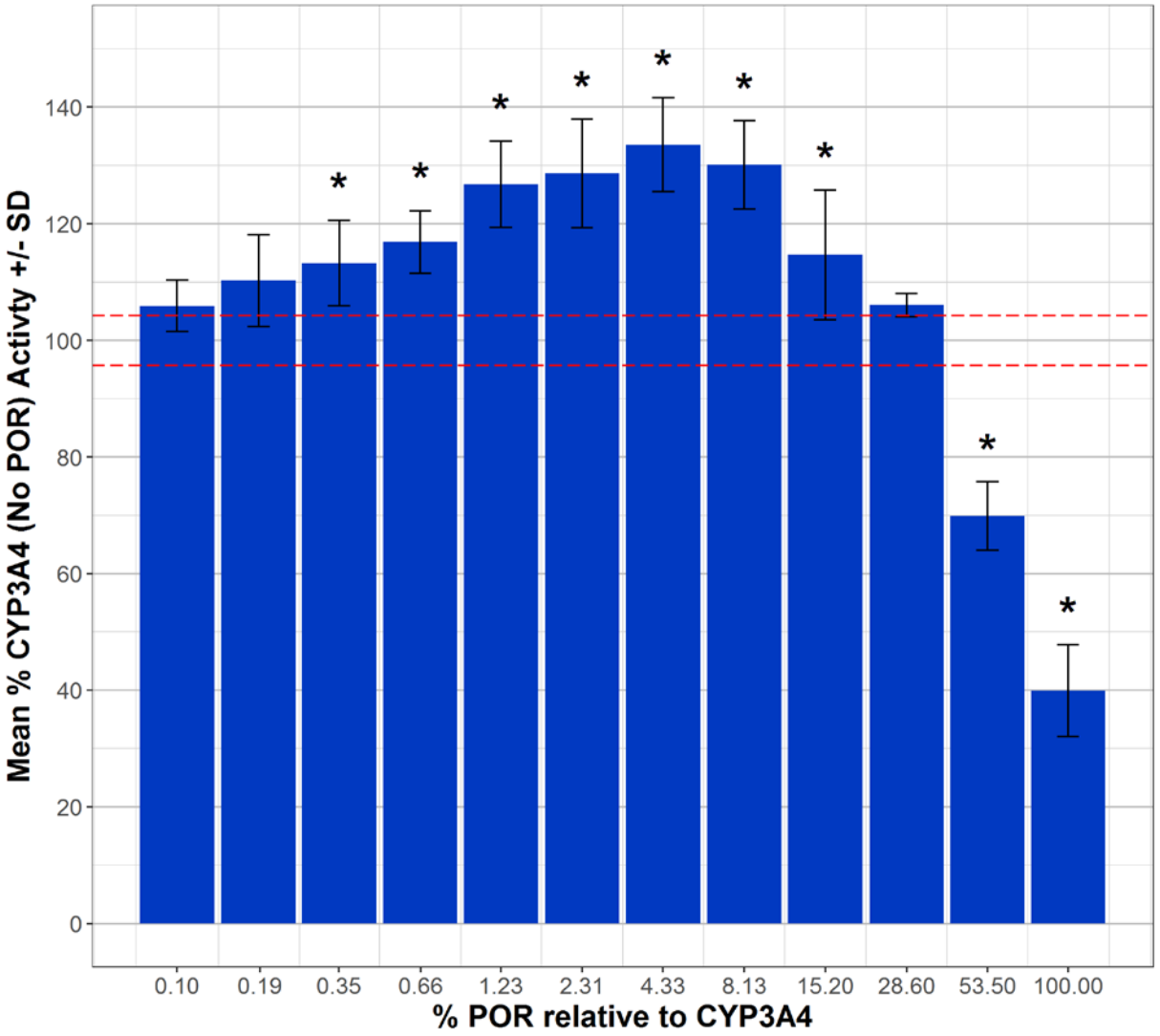
CYP Genes Are Not Expressed in Immortalized Cells



- Immortalized Human Kidney Epithelial Cells (HEK293T)
- 25,000 cells/well in 384-well plates
- Luminescent CYP3A4 substrate (Luciferin-IPA)
- Standard RNA transfection protocol (no prior optimization)
- 25ng mRNA per well
- Luciferin-IPA added 6 hours post-transfection
- 12 hour metabolism

- HEK293T cells have no intrinsic CYP3A4 activity (black)
- Simply introducing CYP3A4-encoding mRNA into these cells generates robust CYP3A4 activity

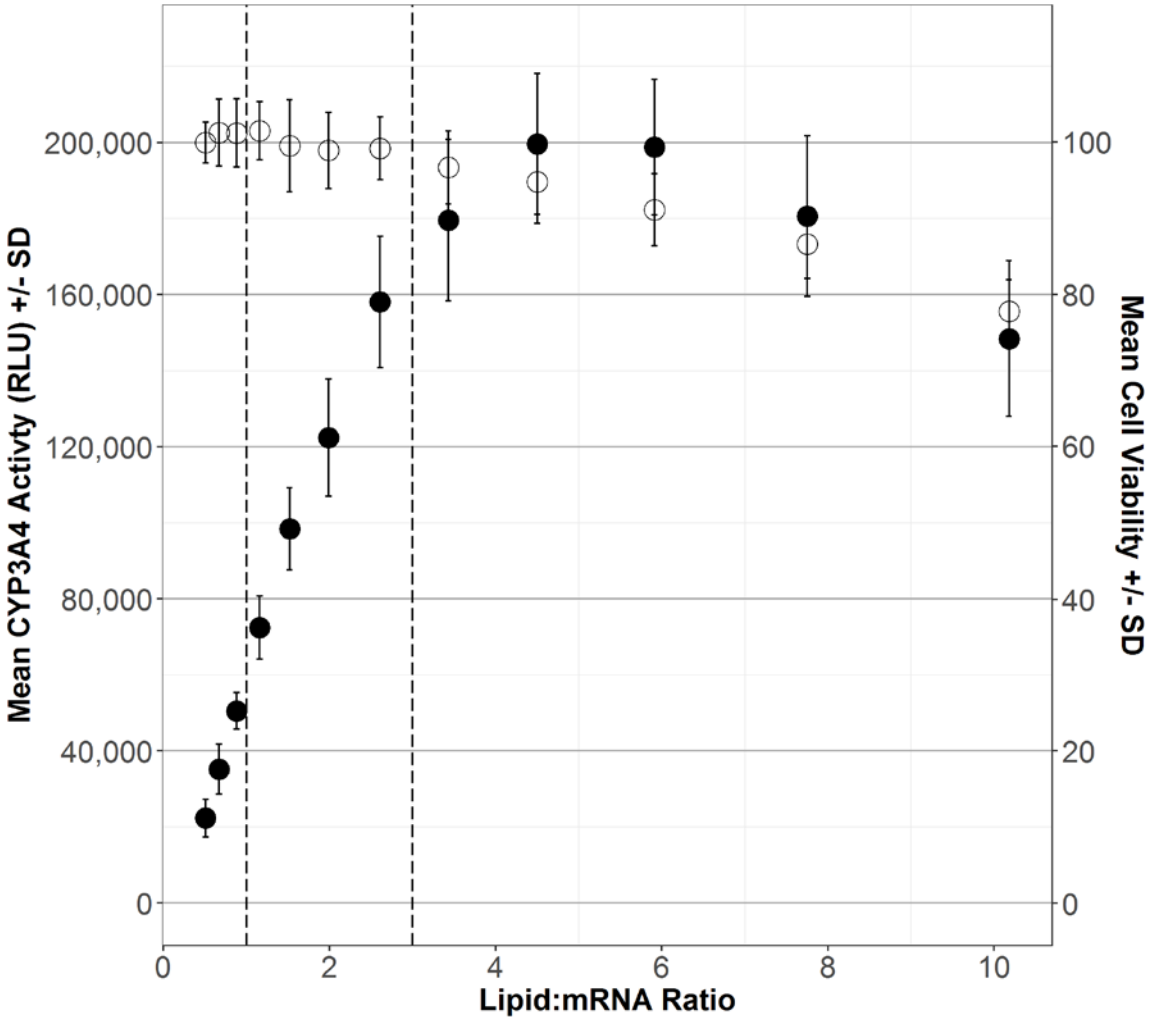
P450 Oxidoreductase Co-expression

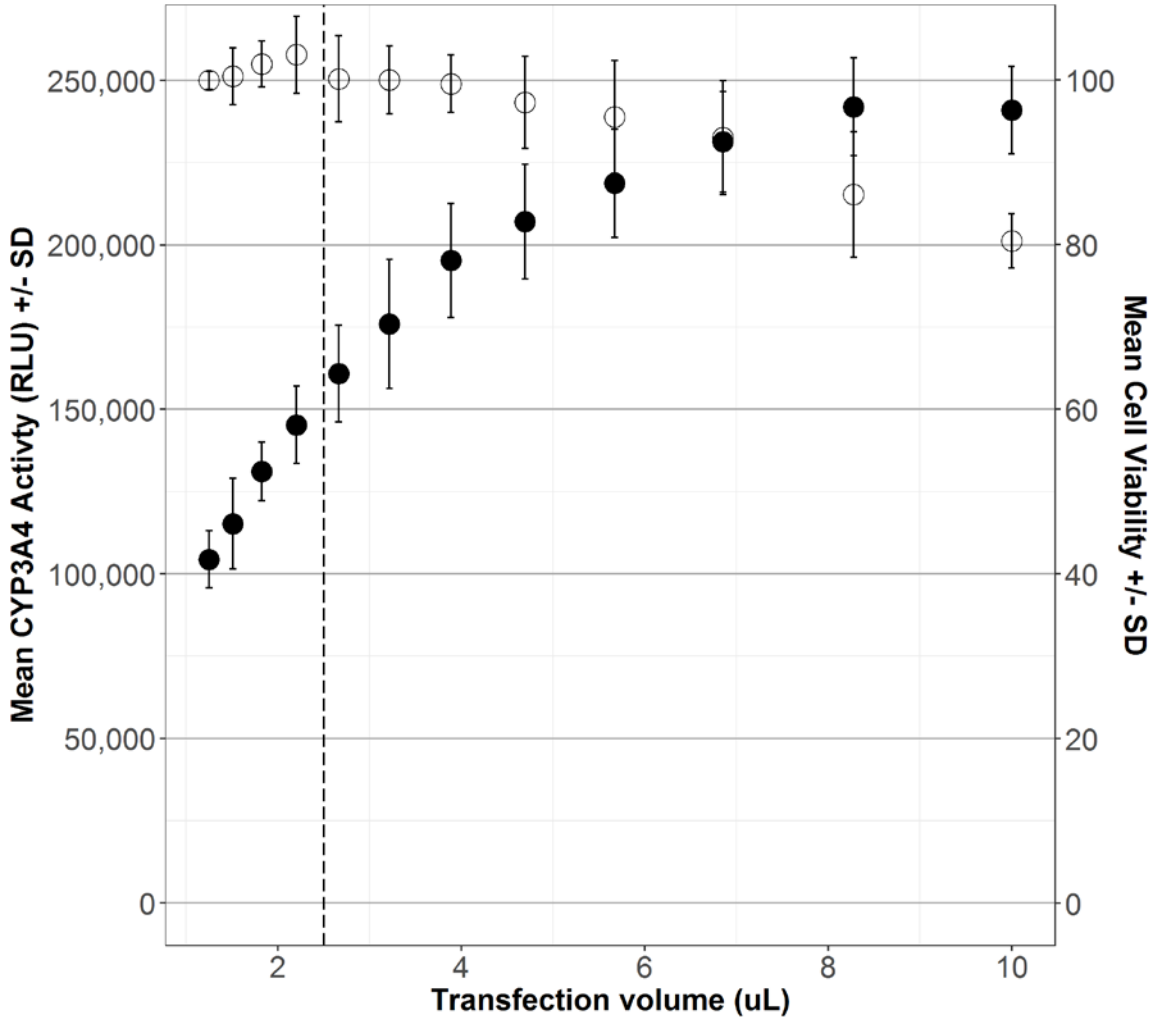


- POR required for the electron transfer from NADPH to cytochrome P450 enzymes in ER
- 12.5ng CYP3A4 mRNA per well
- 0.0125 – 12.5ng POR mRNA per well co-transfected
- β-galactosidase mRNA (negative control) added to ensure total 25ng mRNA input to all wells

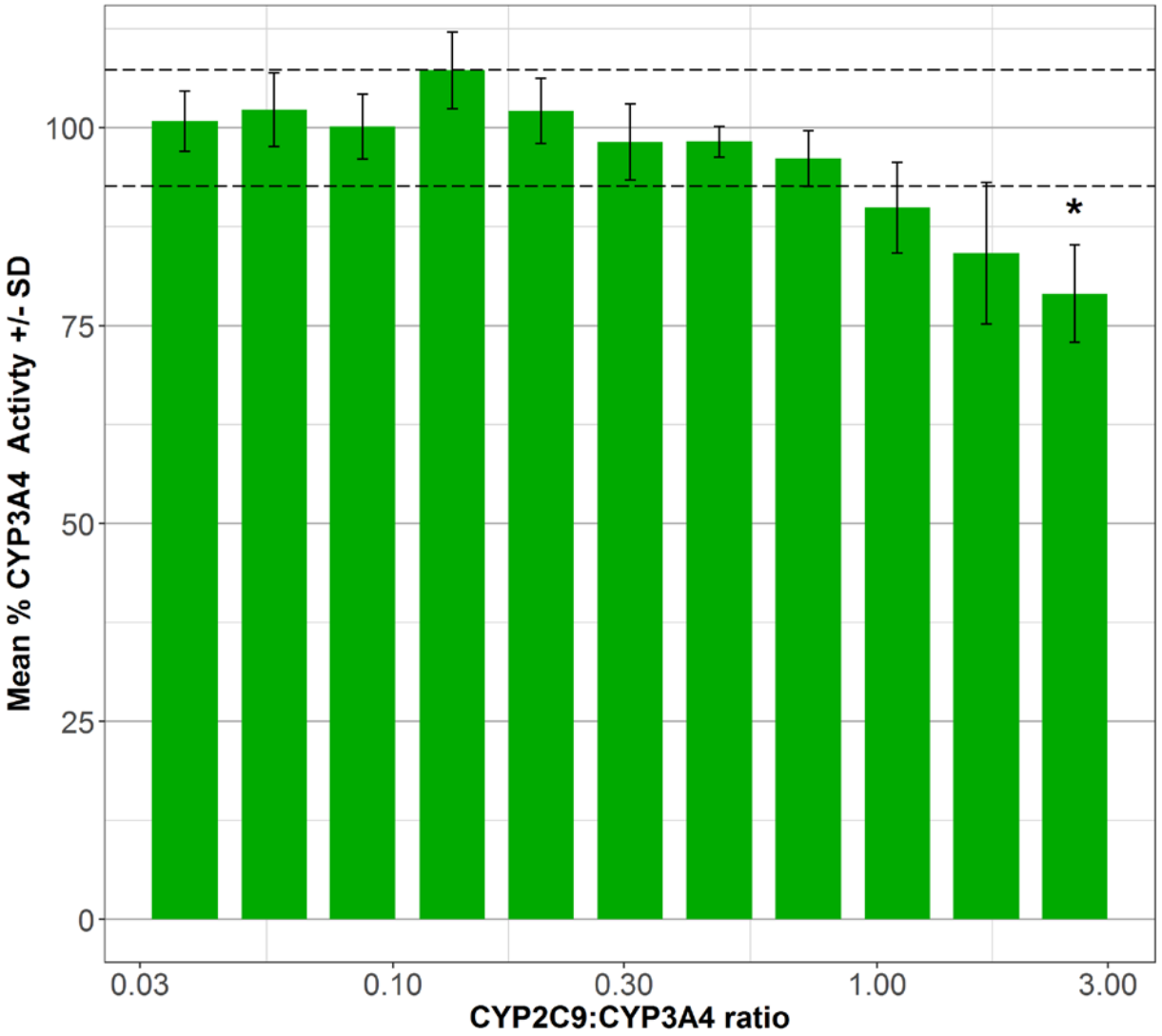
- Endogenous POR levels in HEK293T are rate-limiting
- Co-transfecting POR-encoding mRNA at 4% relative to CYP3A4 mRNA increased CYP3A4 activity by 34%
- The returns on POR mRNA compositions > 4% relative to CYP3A4 quickly diminish
- This 24:1 CYP:POR ratio would be very difficult to achieve with traditional gene overexpression methods

mRNA Transfection Optimization





Co-Expression of CYP mRNAs

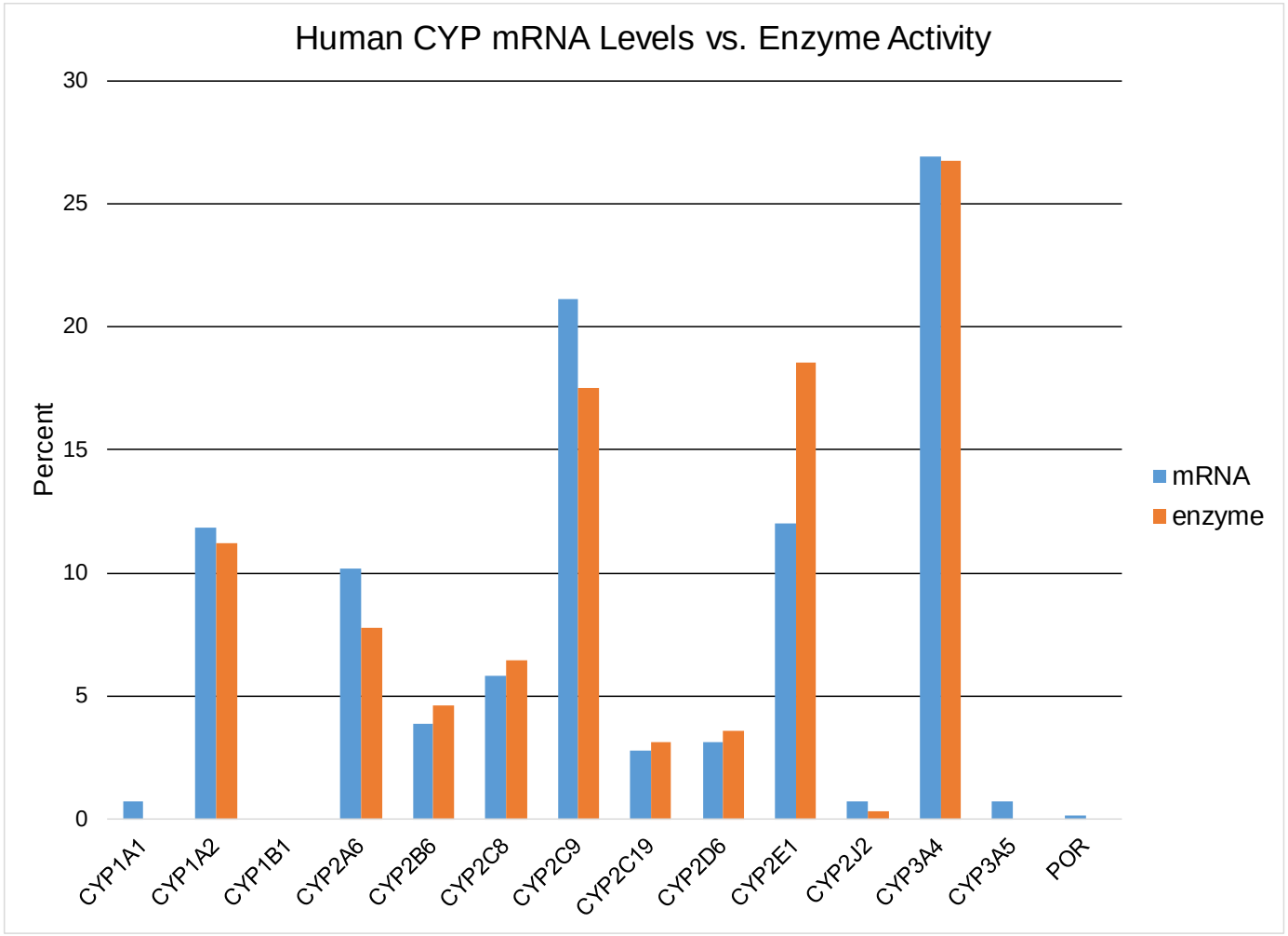


- Ectopic CYP expression can exhaust cellular resources
- What happens to activity of CYP A when co-expressed with increasing amounts of CYP B?
- 13ng CYP3A4 mRNA + 2ng POR per well
- 27% of total CYP payload ~ human liver
- 0.132 – 35ng CYP2C9 mRNA per well co-transfected
- β-galactosidase mRNA (negative control) added to ensure total 50ng mRNA input to all wells

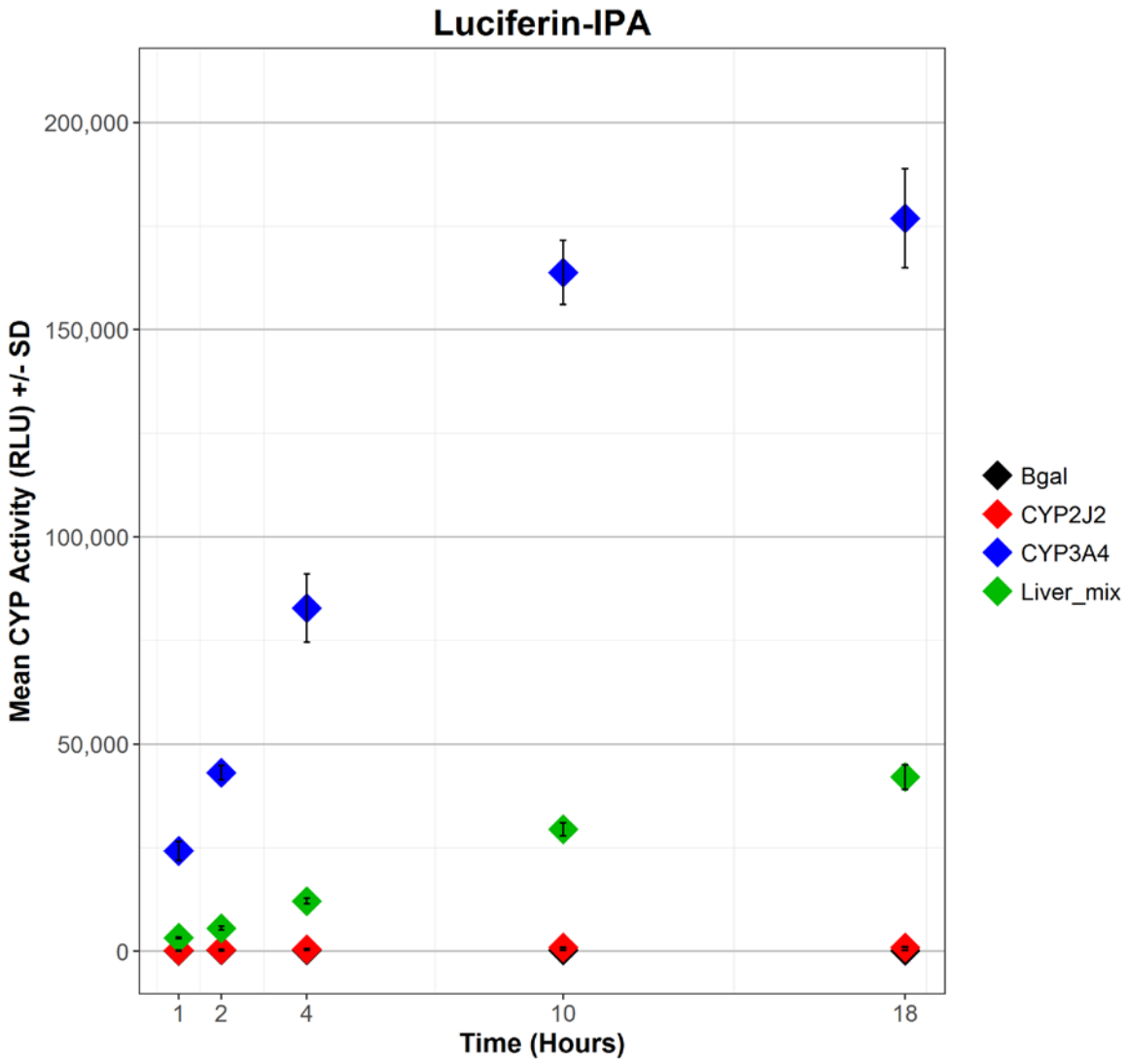
- Co-transfecting CYP2C9-encoding mRNA adversely impacts CYP3A4 activity by as CYP2C9 mRNA levels reach parity
- There is a 21% activity penalty for CYP3A4 when co-expressed with other CYP mRNAs at human liver ratios

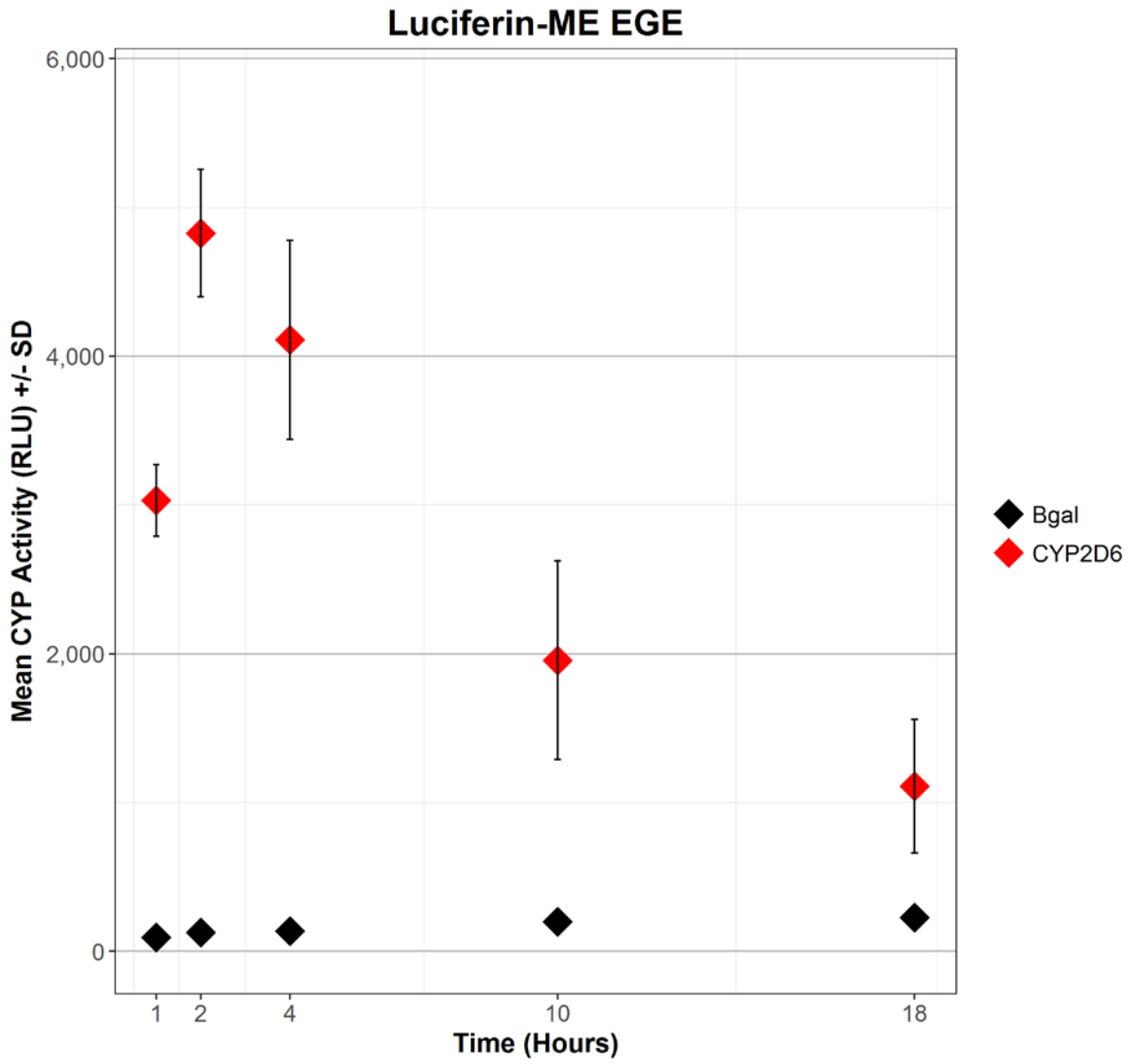
Characterizing a Panel of CYP Enzymes

- With optimization complete, next was to characterize the activity of the 10 most prevalent CYPs in human liver identified through a meta-analysis of over 700 subjects:
  - CYP1A2
  - CYP2A6
  - CYP2B6
  - CYP2C8
  - CYP2C9
  - CYP2C19
  - CYP2D6
  - CYP2E1
  - CYP2J2
  - CYP3A4
- Each CYP was characterized as:
  - Singlet (96% CYP + 4% POR)
  - CYP_mix (liver % CYP + β-gal)
  - Liver_mix (all 10 CYPs @ liver %)

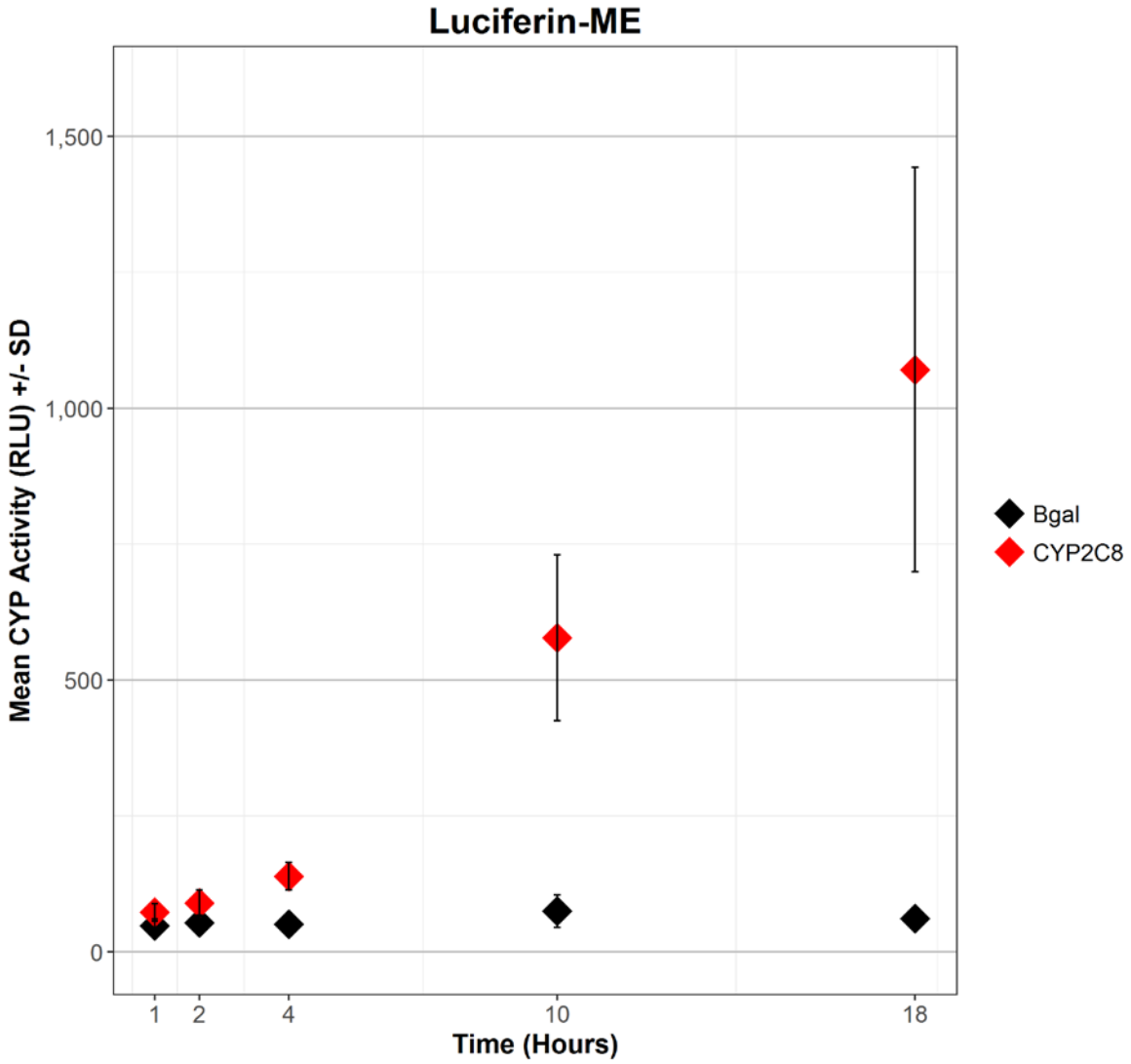


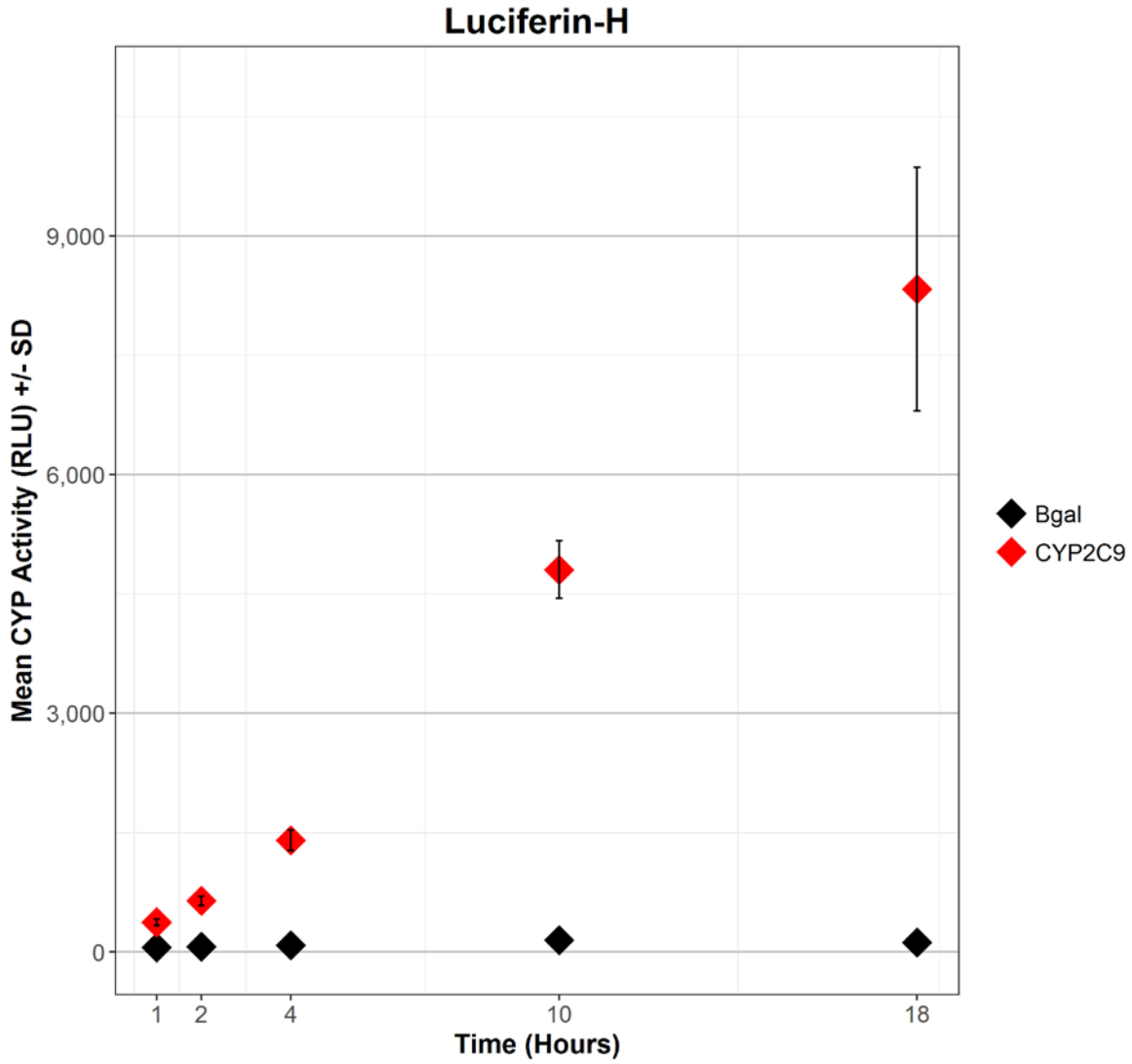
Time Course of CYP Activity



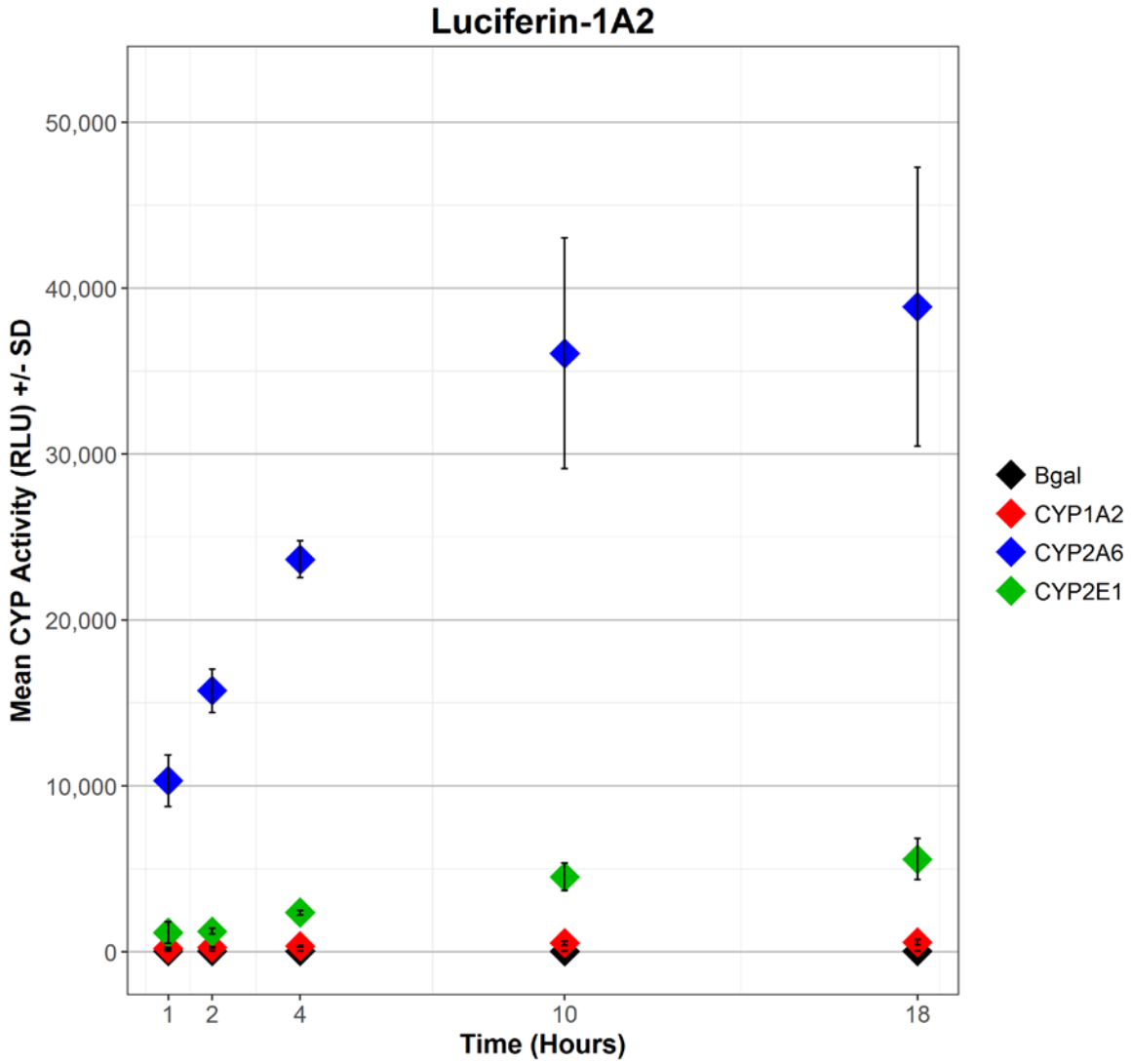


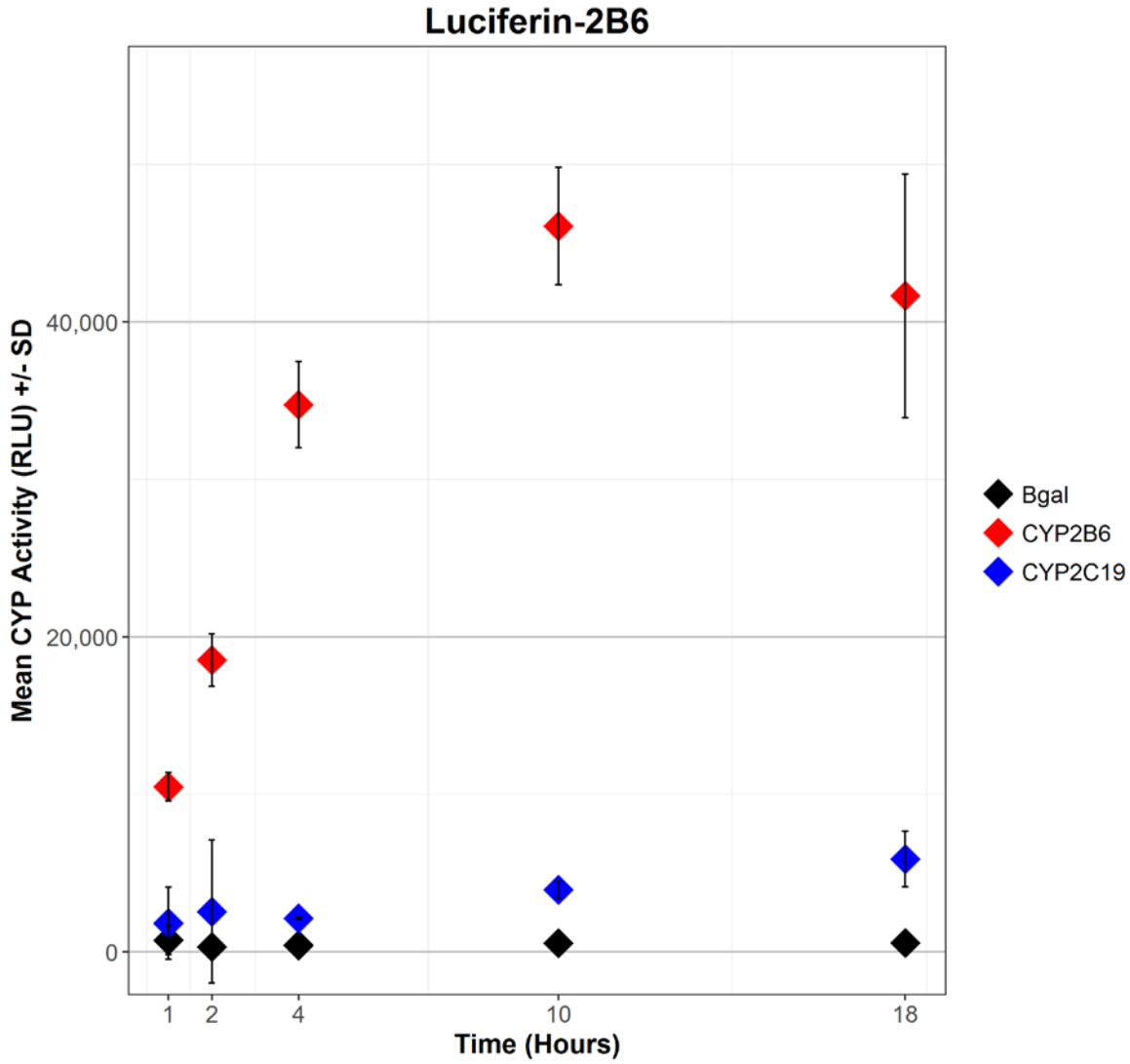
Time Course of CYP Activity (con't)



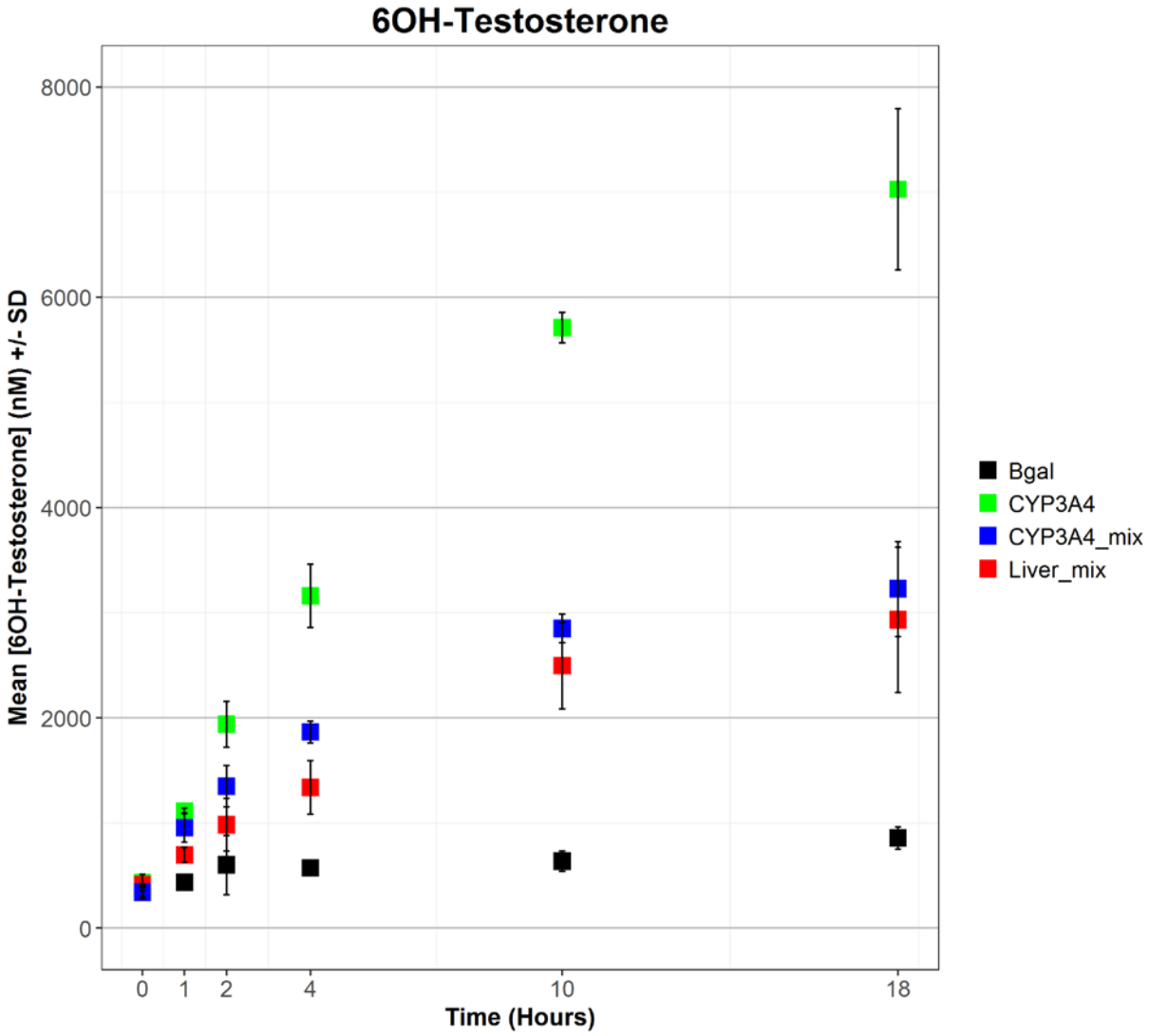


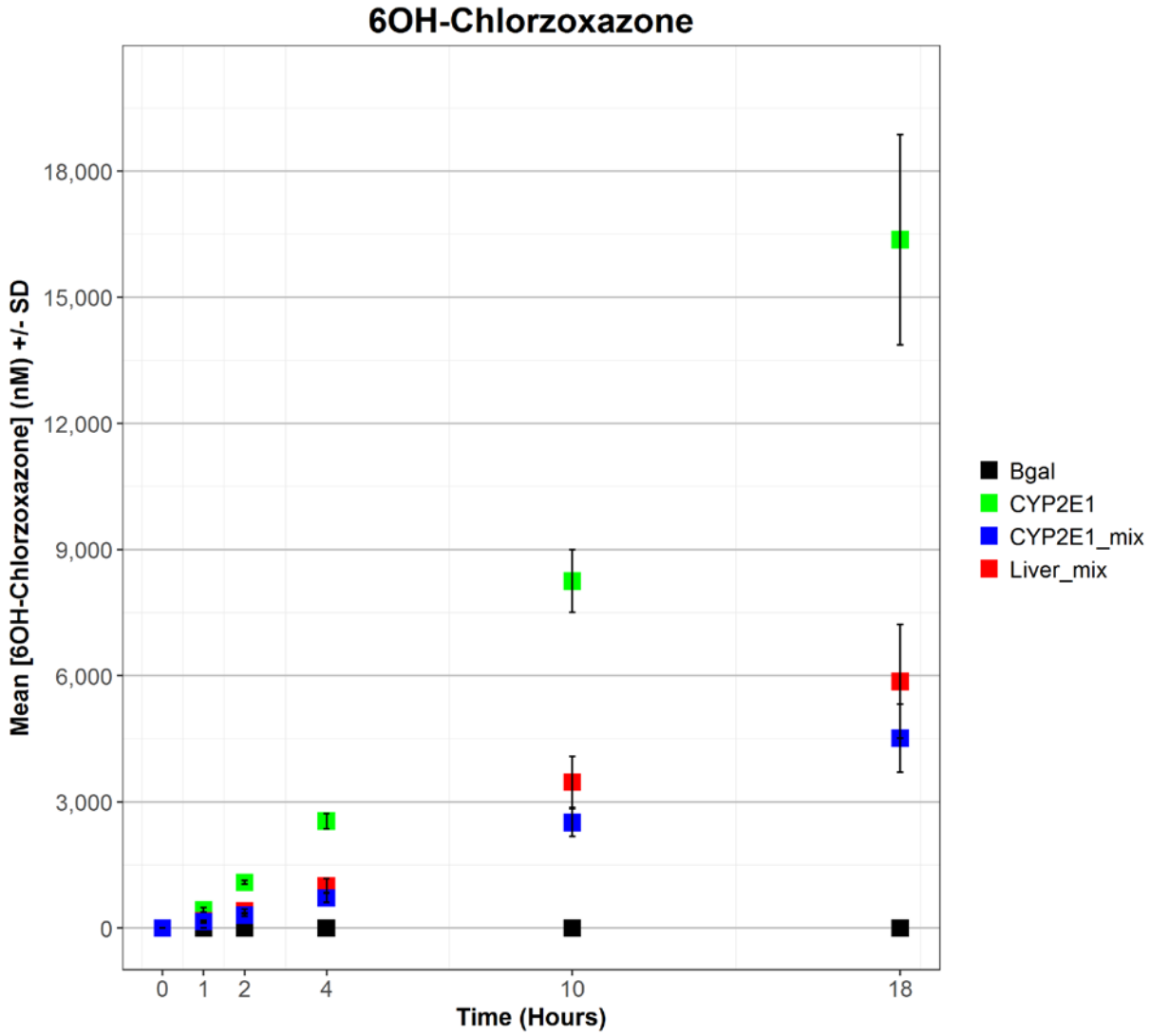
Time Course of CYP Activity (con't)



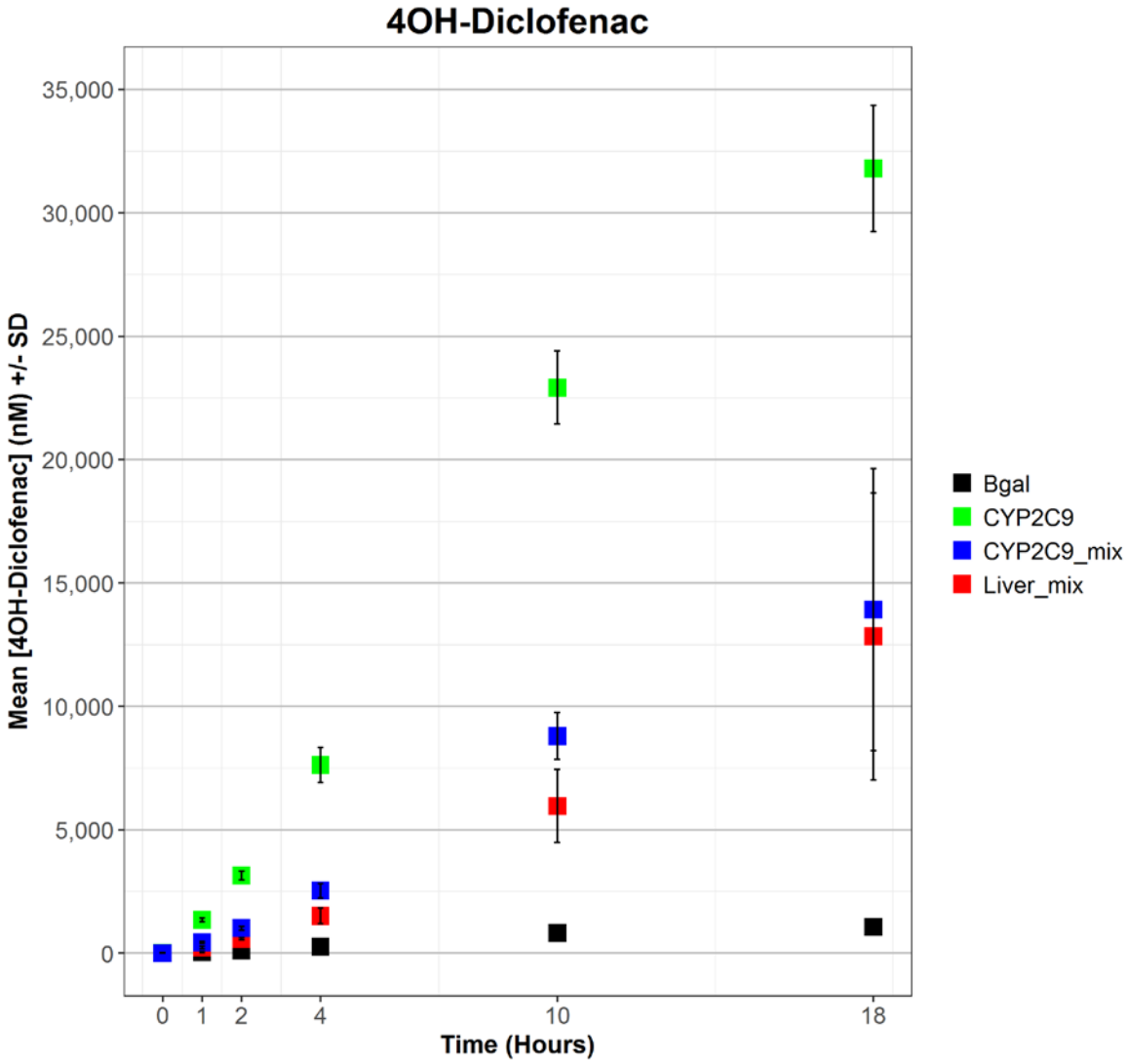


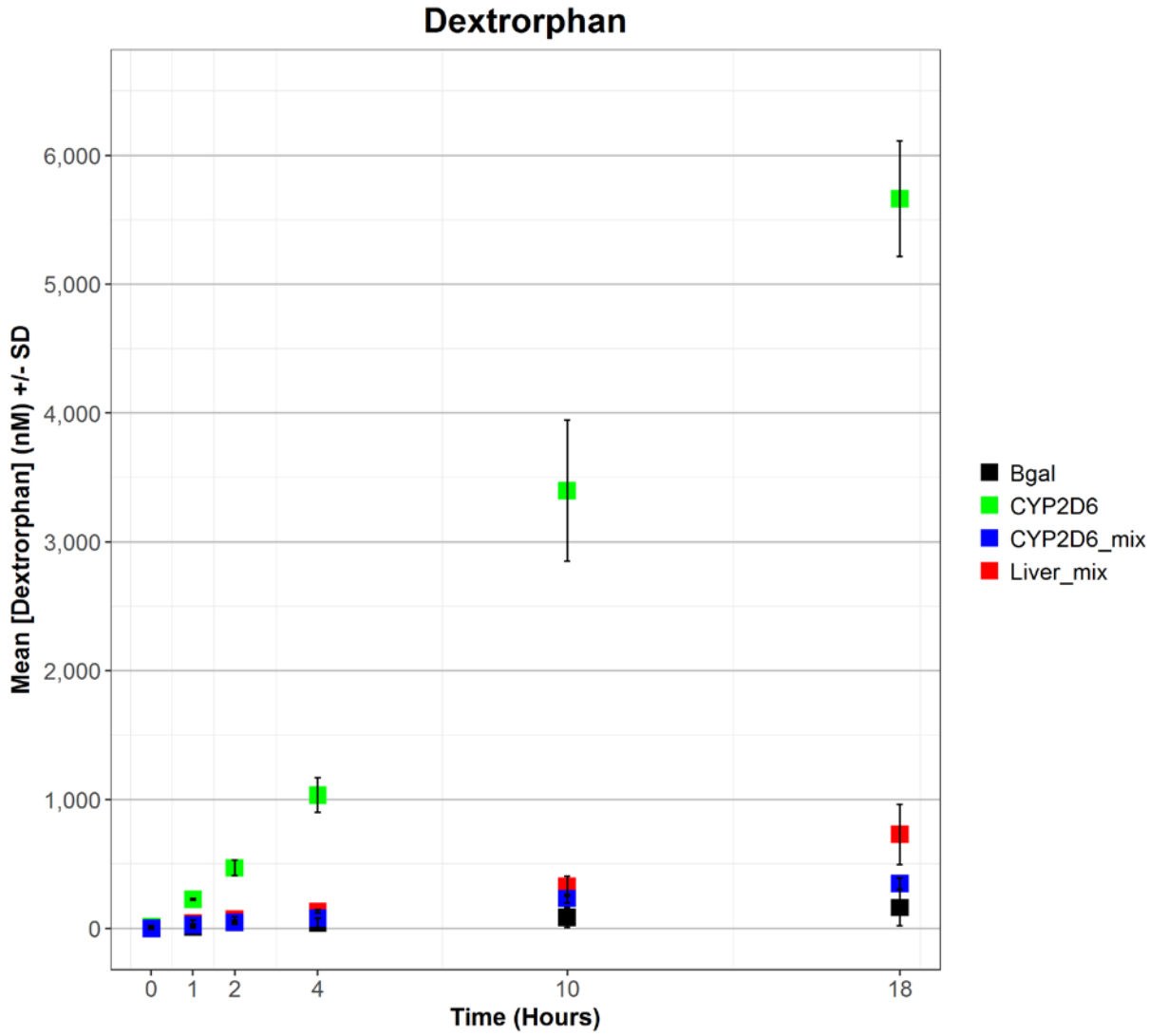
Benchmark Substrate Studies



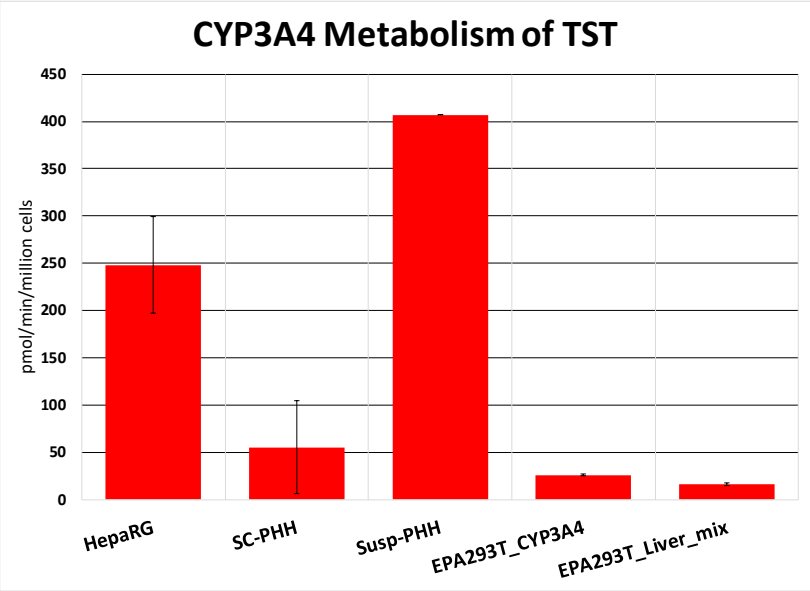


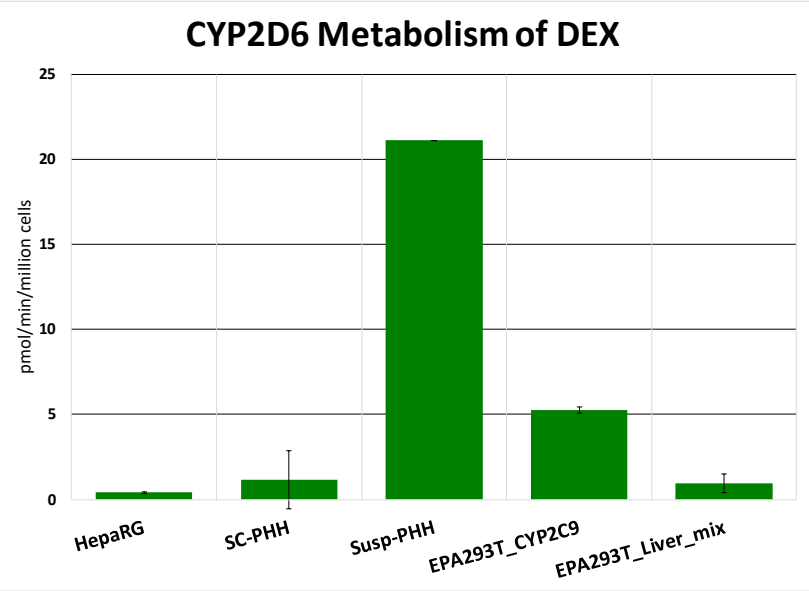
Benchmark Substrate Studies (con't)

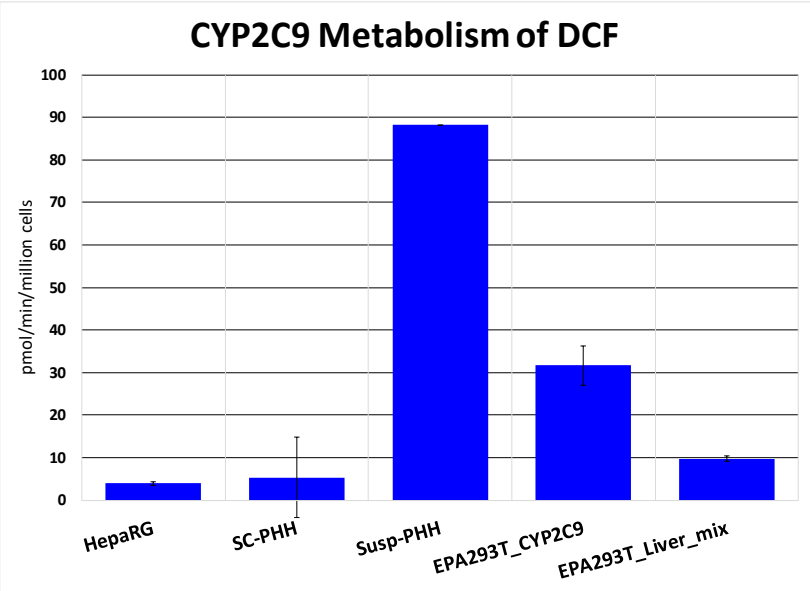


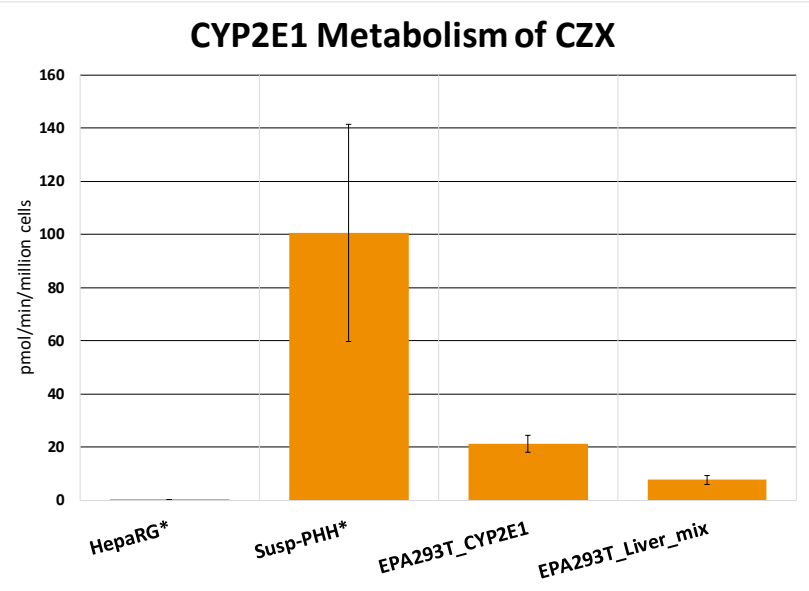


Comparison to “Gold-Standard” Cell Models



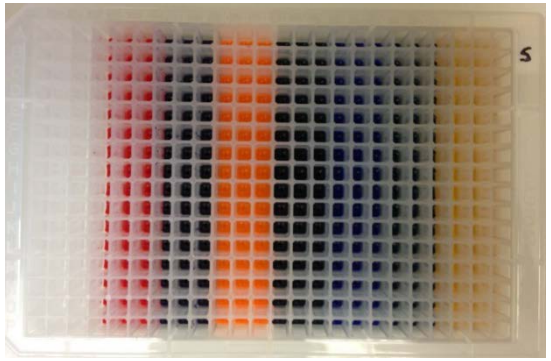




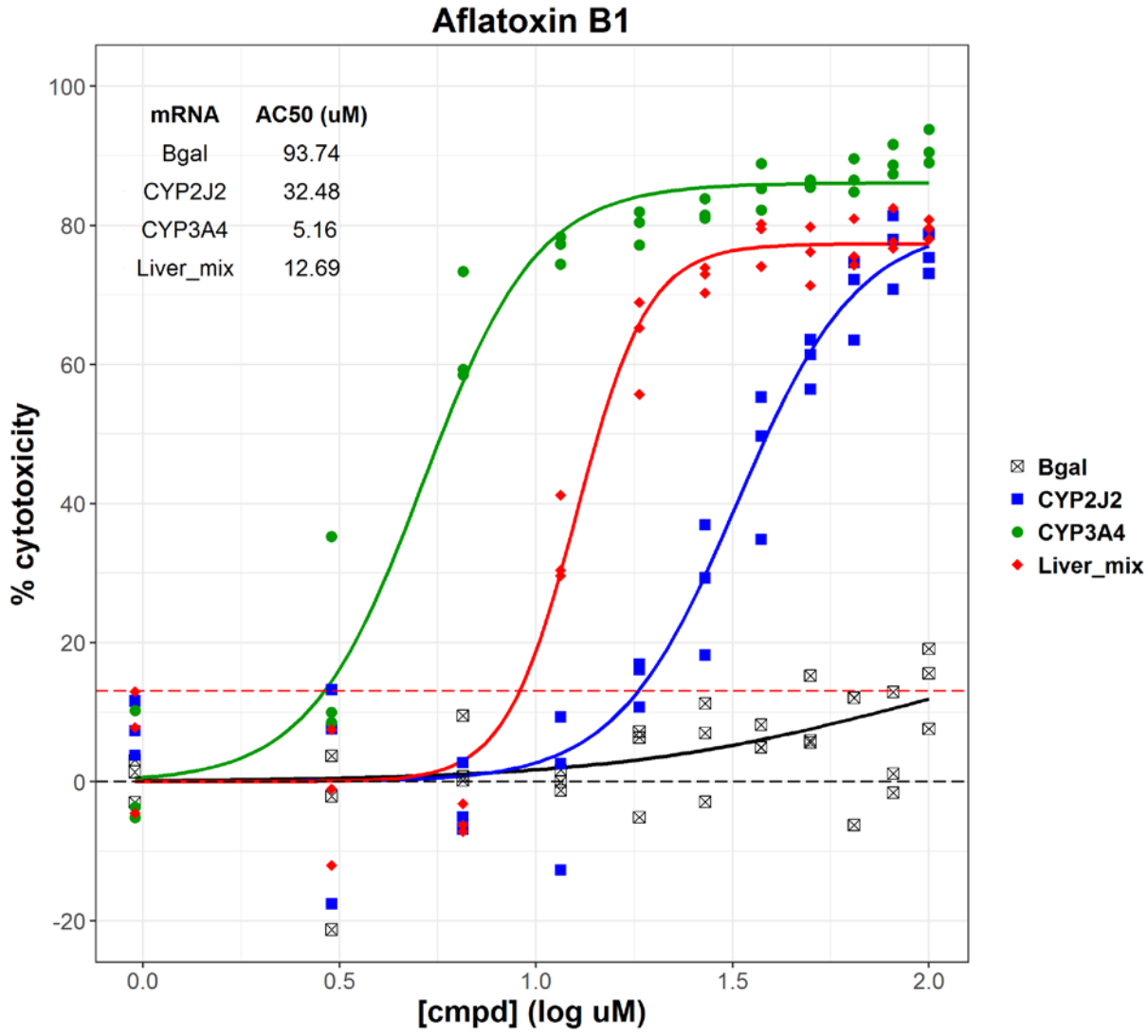


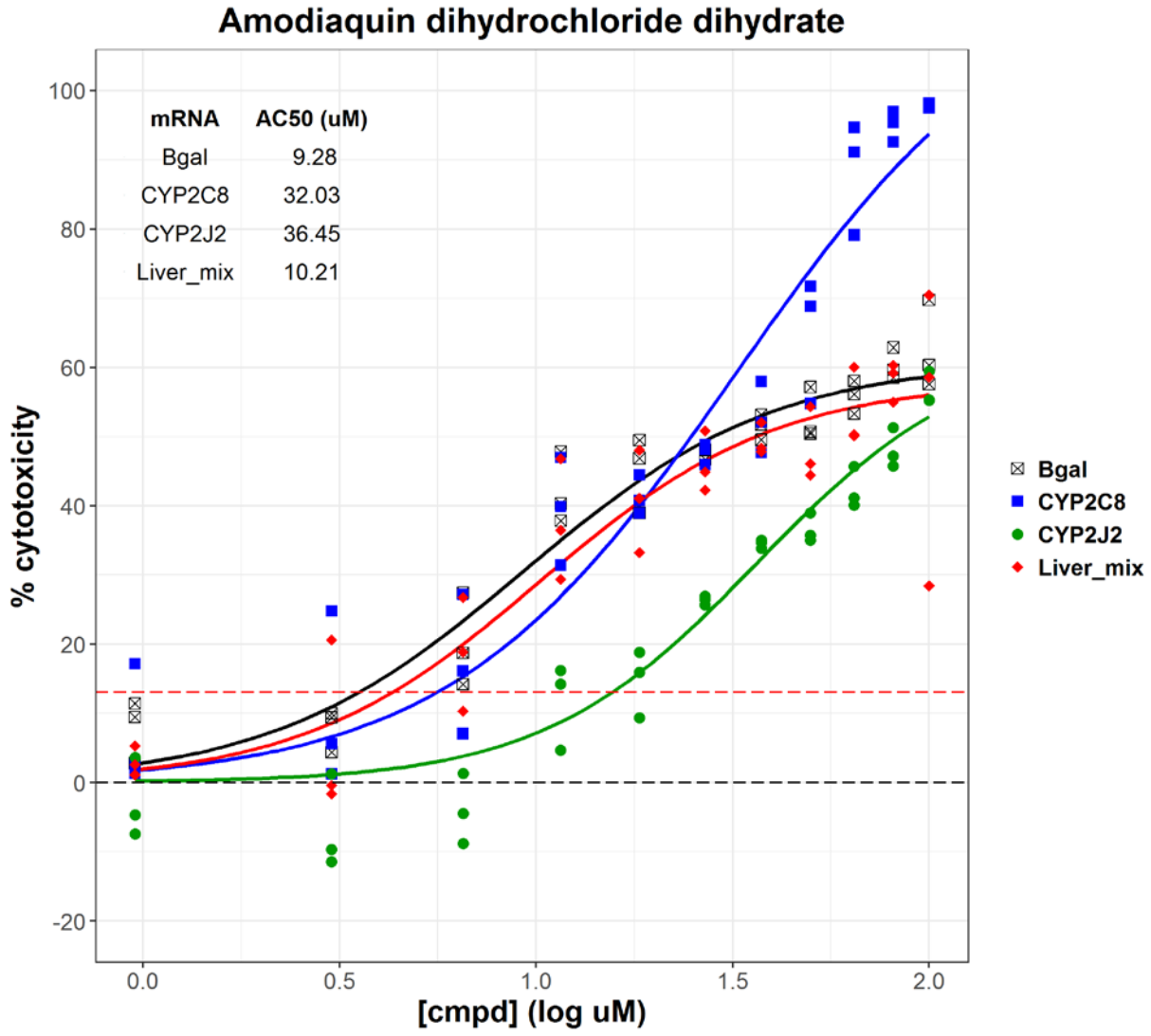
Deployment to Cell-Based Assays

- mRNA transfection retrofits CYP-deficient cell model with robust CYP activity
- Onset of CYP activity is rapid (~6 hours post-transfection) and is sustained for at least 18 hours
- CYP activities produce predicted metabolites and at rates > than HepaRG and SC-PHH models, even when handicapped by HTS sonditions
- What happens when we couple this method with a cell-based assay? Are there any CYP-dependent shift in bioactivity?
- HEK293T cells transfected with 10 x CYP singlets, Liver mix, and β-gal control
- Cells treated with 56 test compounds at 11 concentrations with randomized dispense pattern using acoustic liquid handler → randomization minimizes impact of “edge effect” observed with long assay durations
- 32 hour exposure
- Cytotoxicity measured using Cell Titer Glo™ Assay

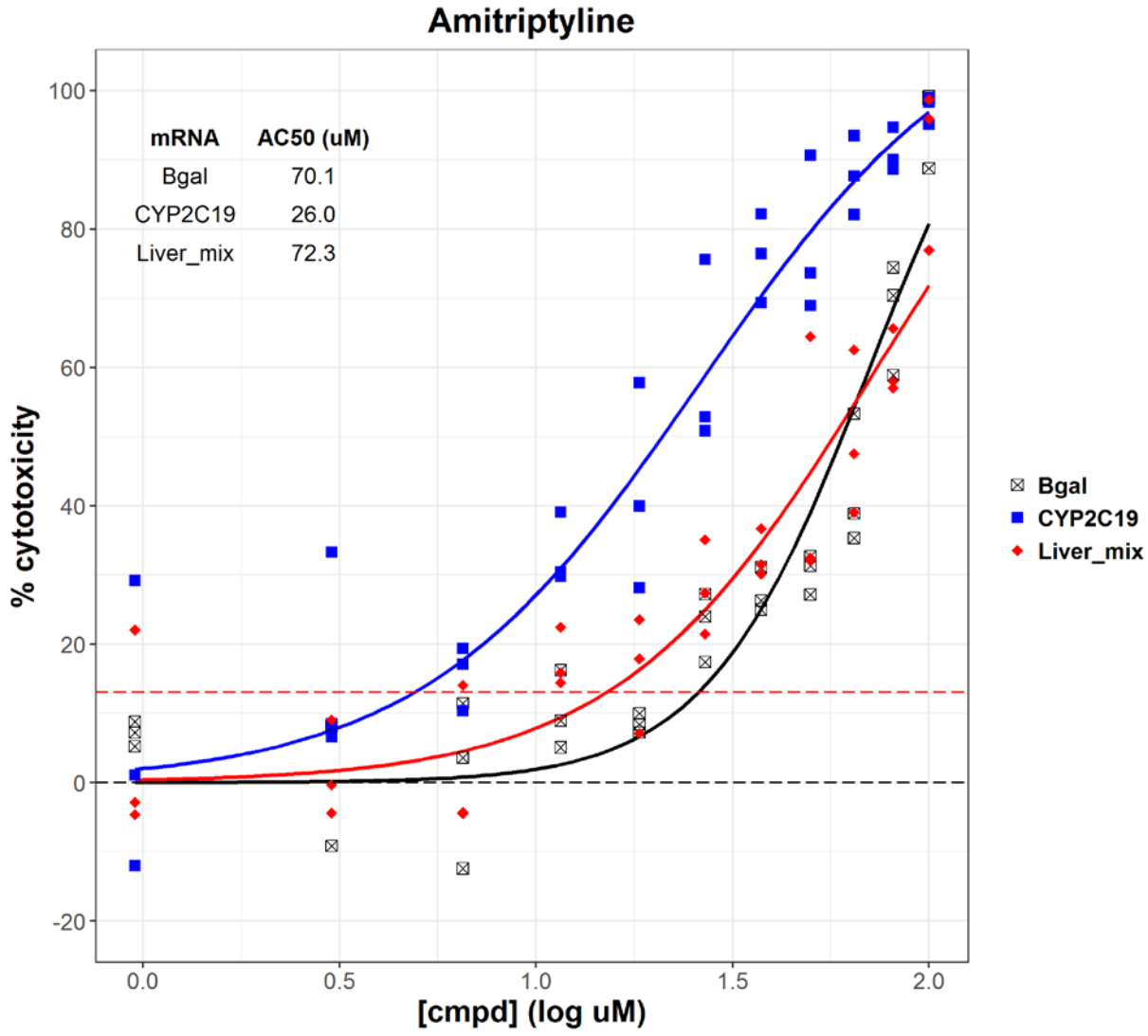


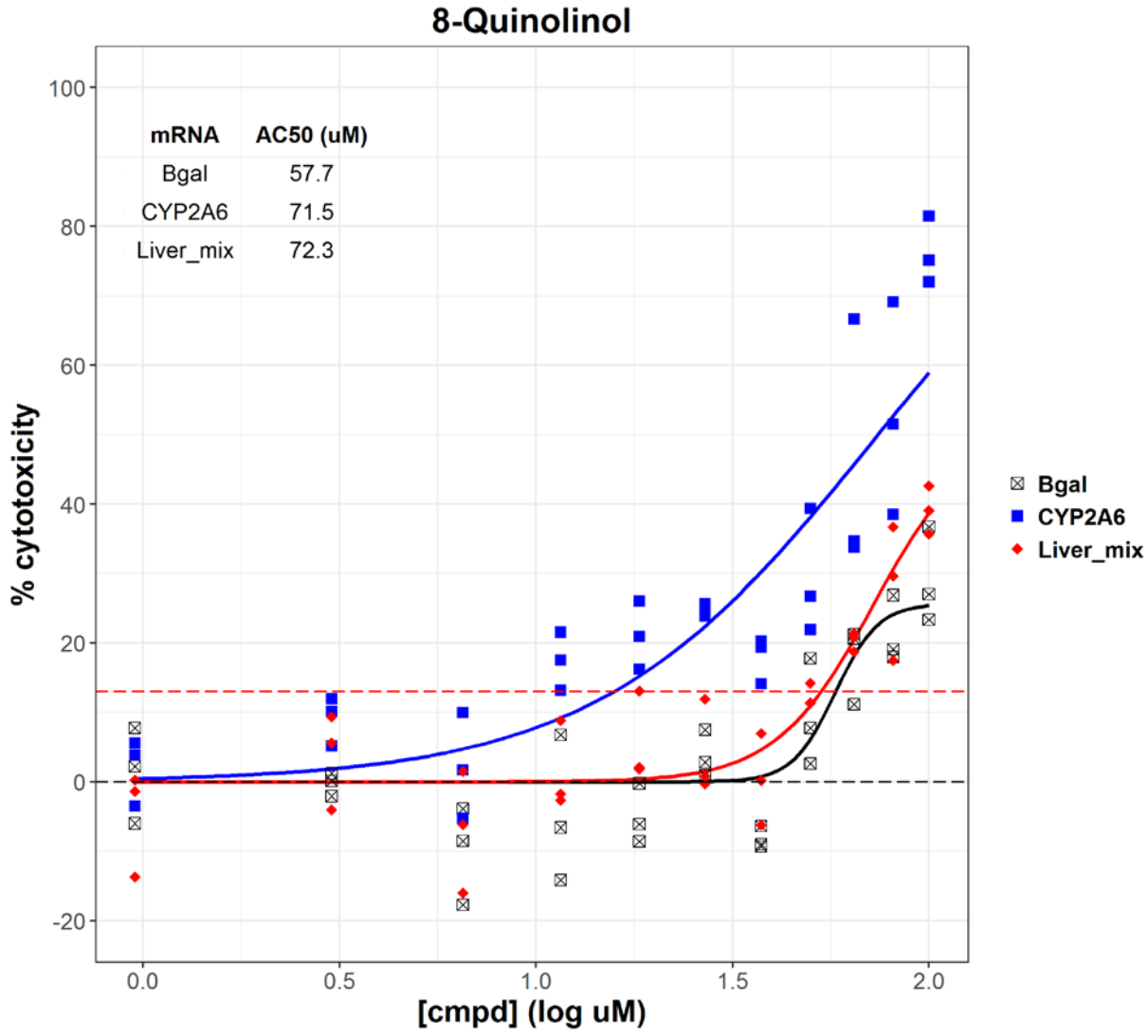
Cytotoxicity Screening Results





Cytotoxicity Screening Results (con't)





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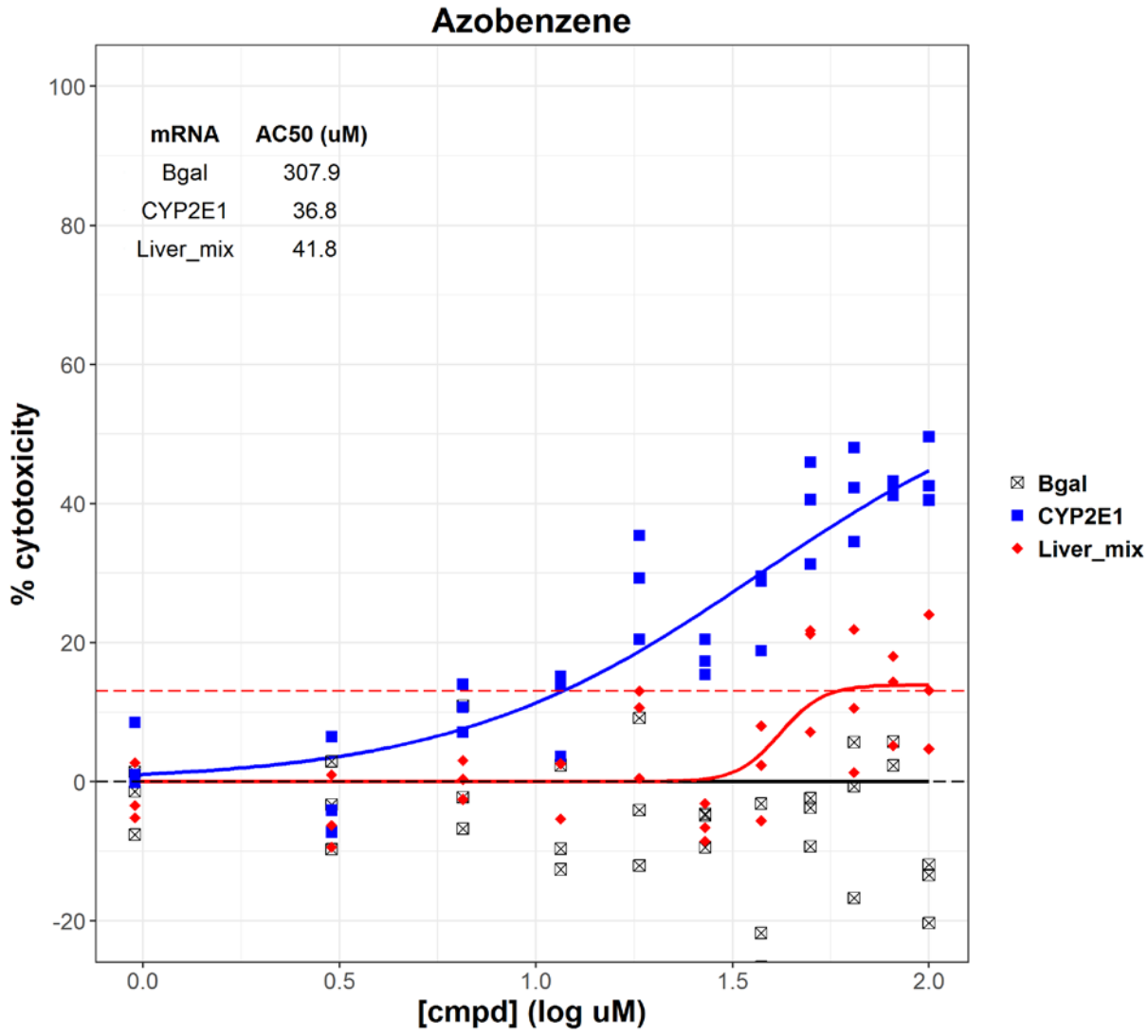

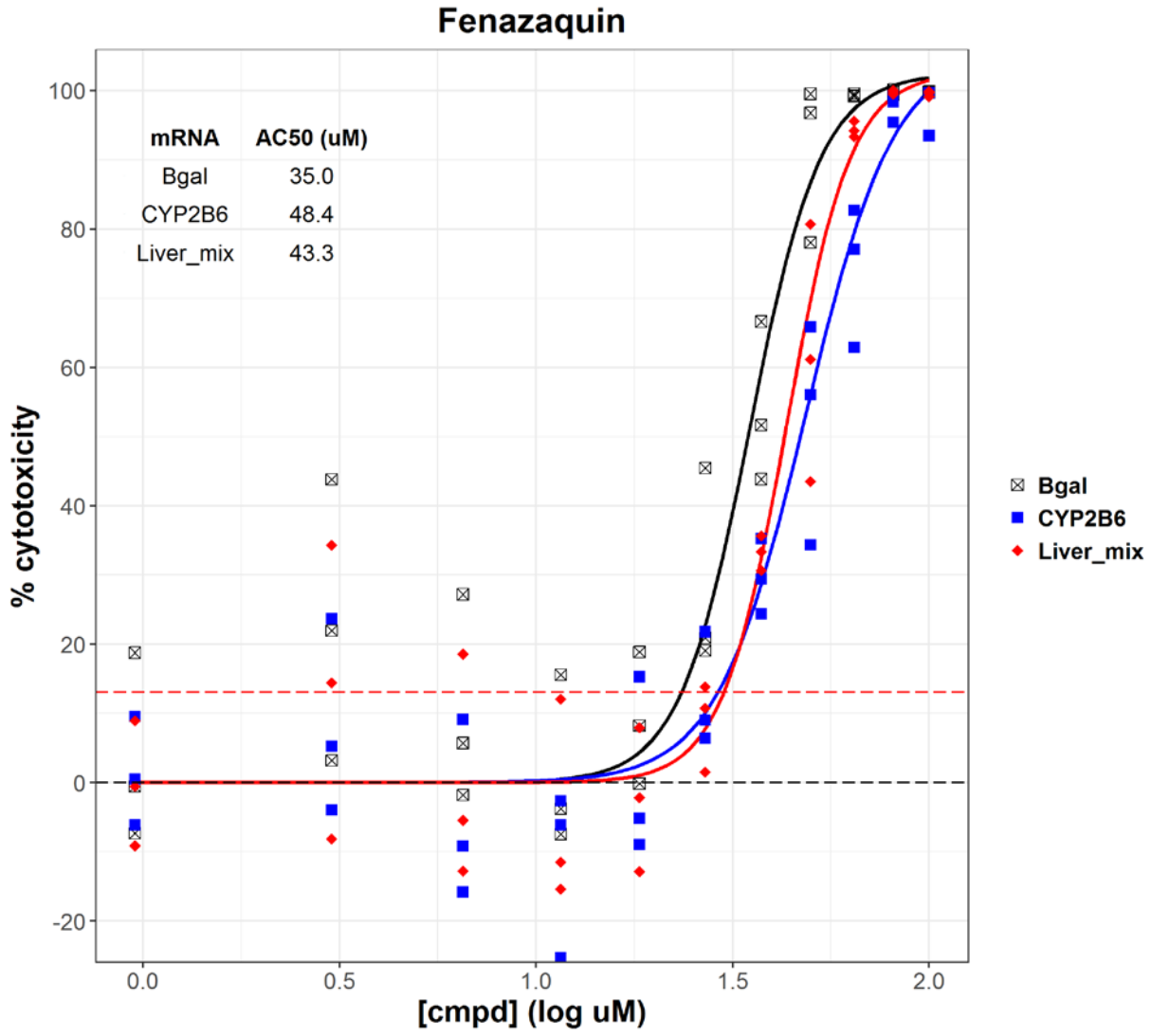

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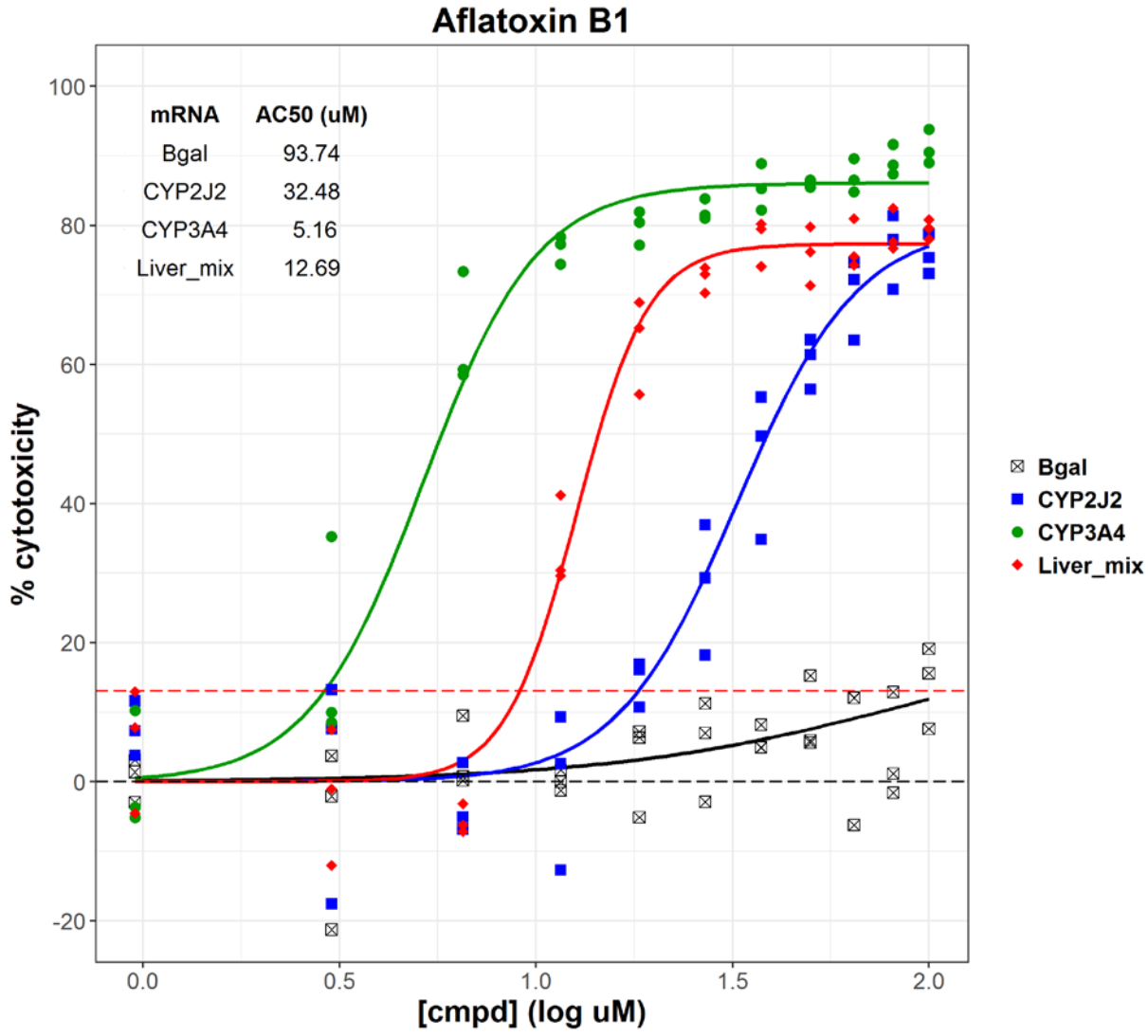
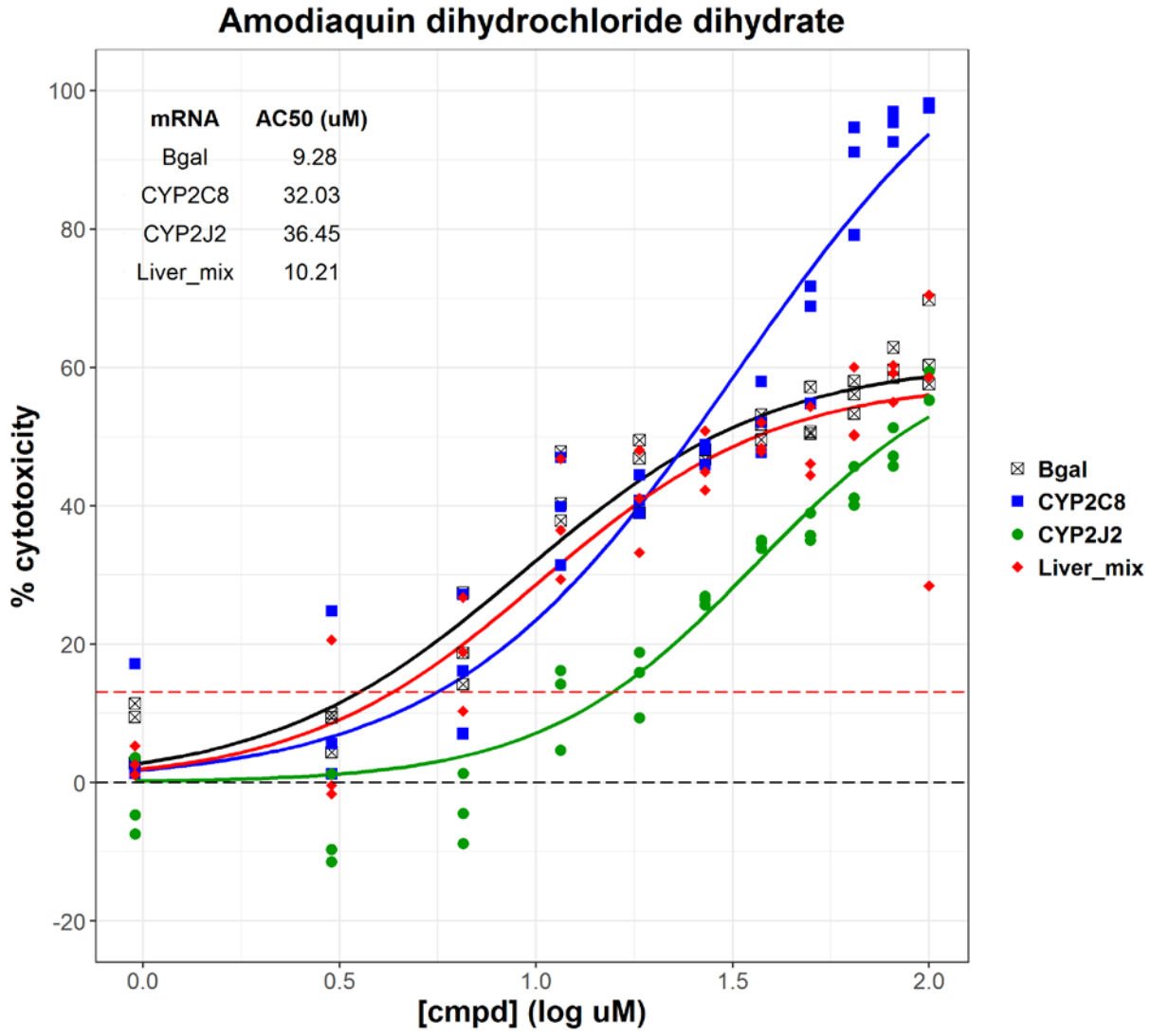


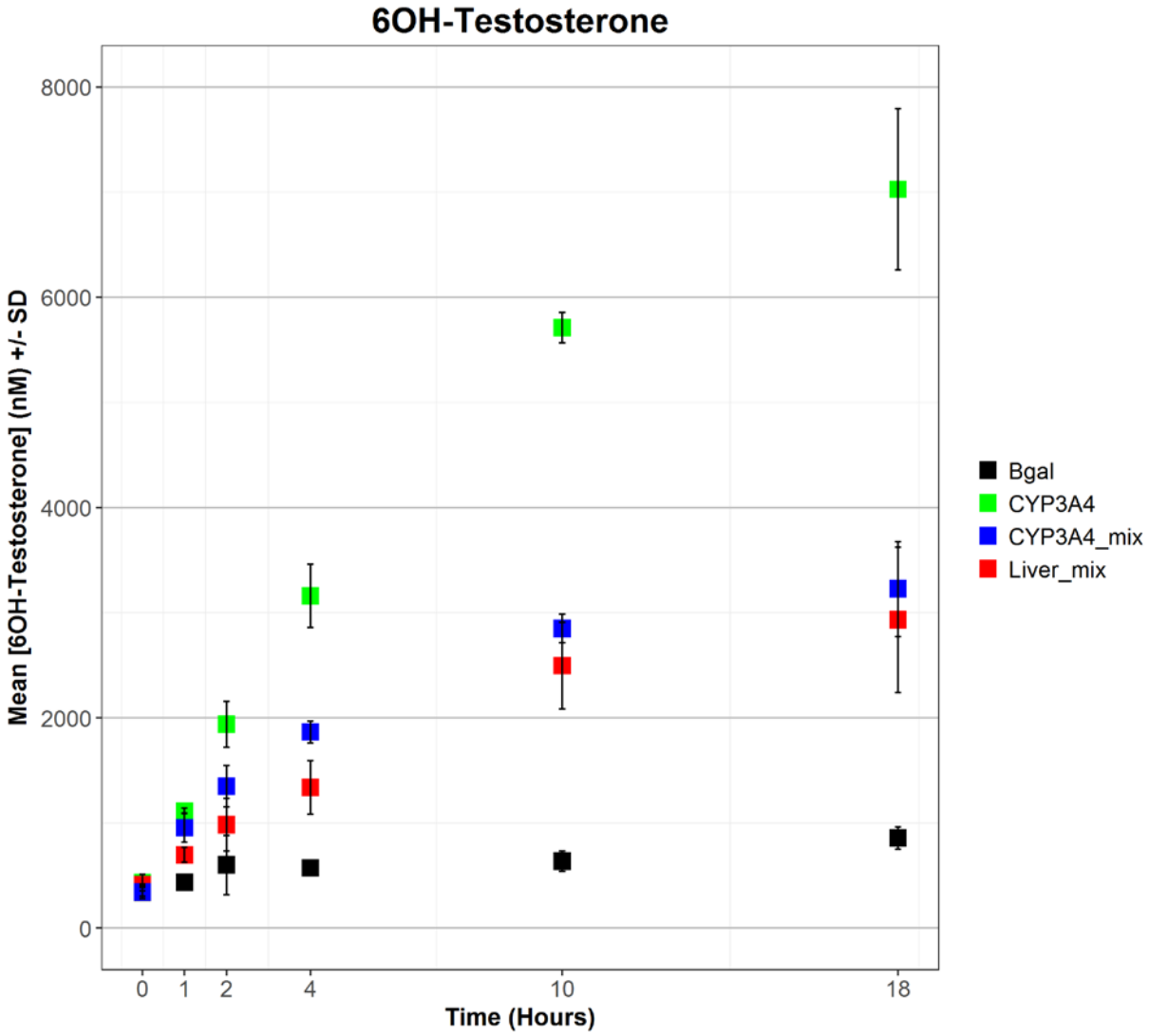


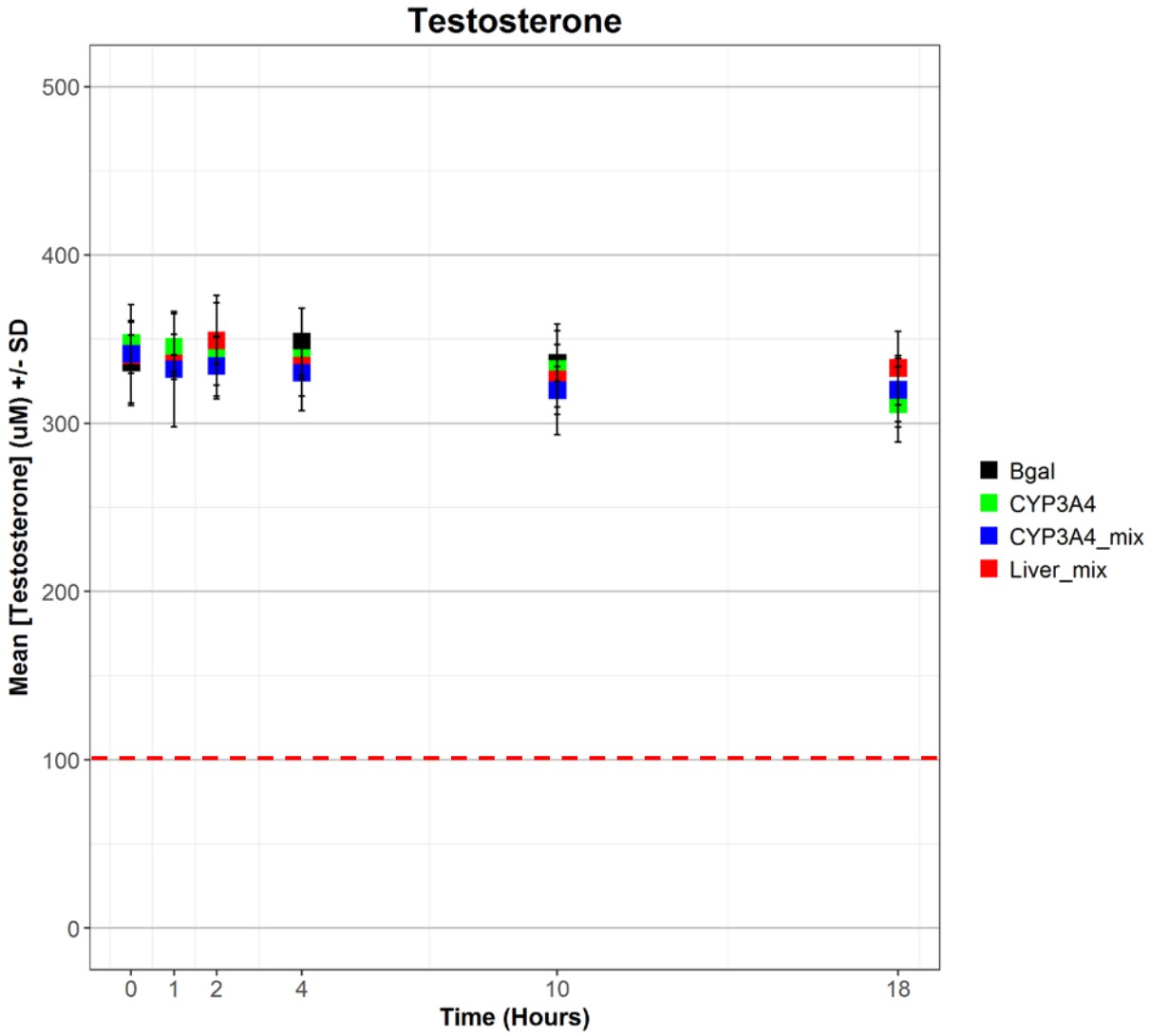
United States Environmental Protection Agency

What Have We Learned???

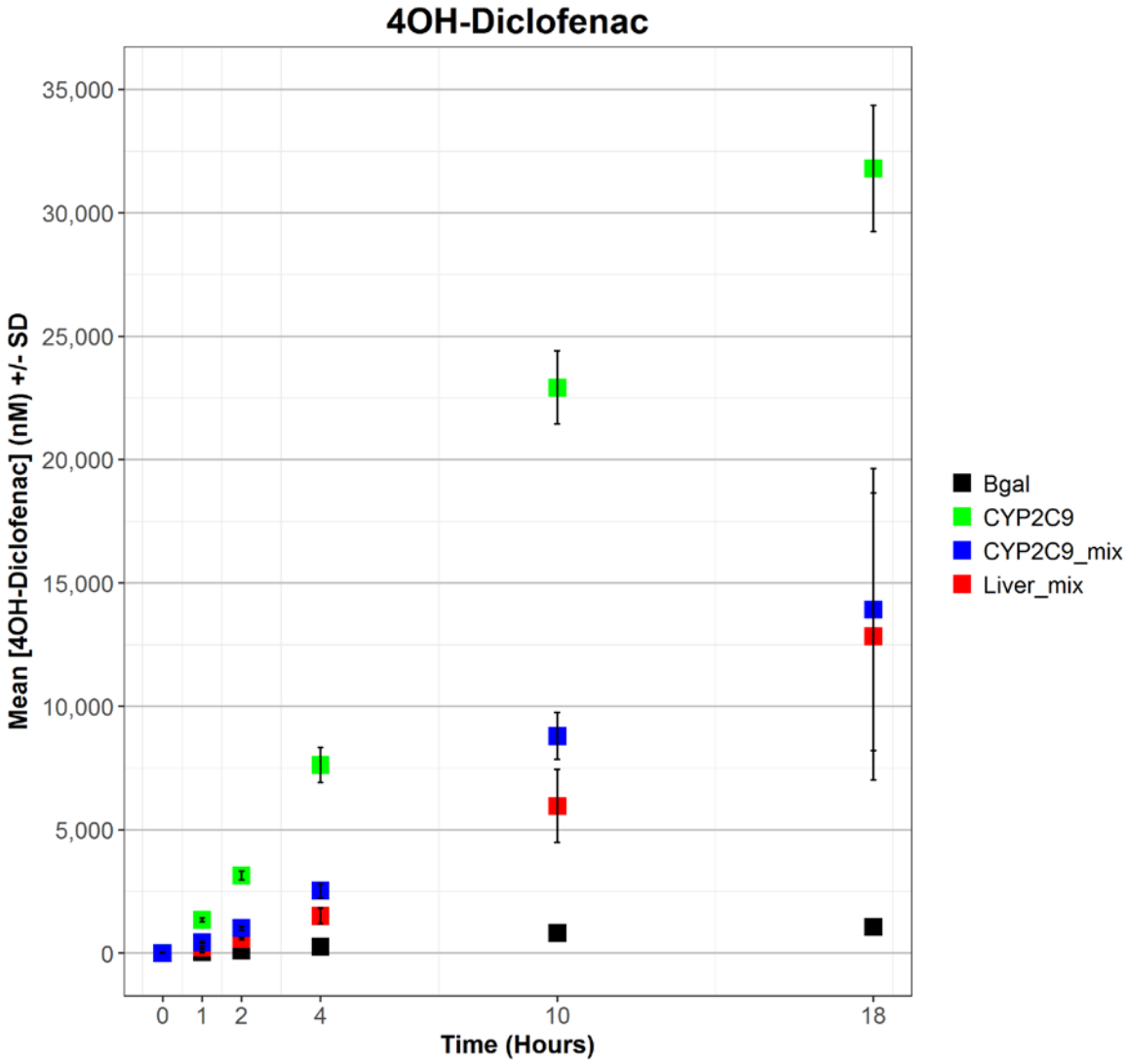
- We did not observe much detoxification with CYP expression, which is odd considering the role metabolism plays in toxicokinetics
- Why???

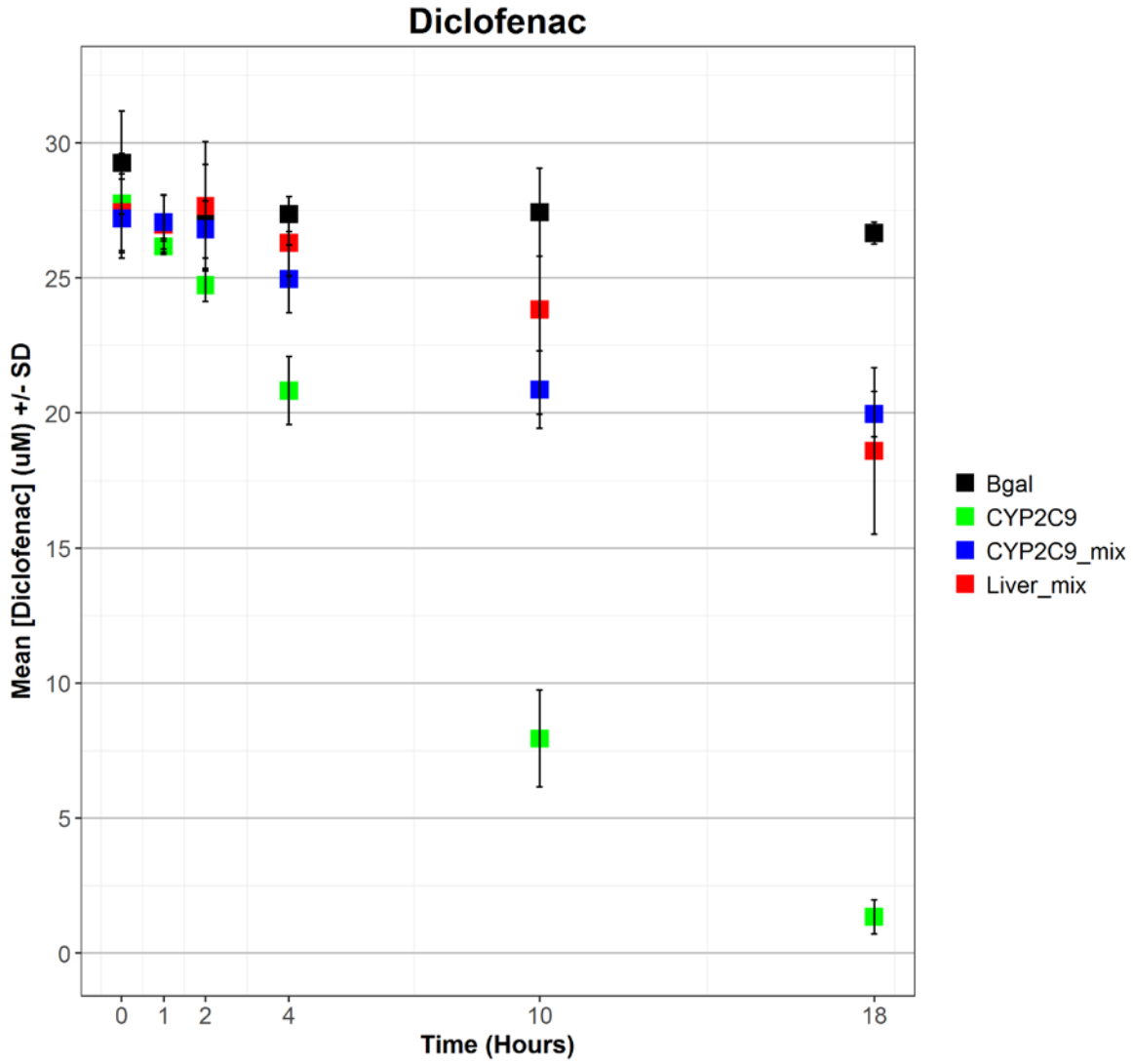
Metabolite Formation vs. Parent Depletion





Metabolite Formation vs. Parent Depletion (con't)

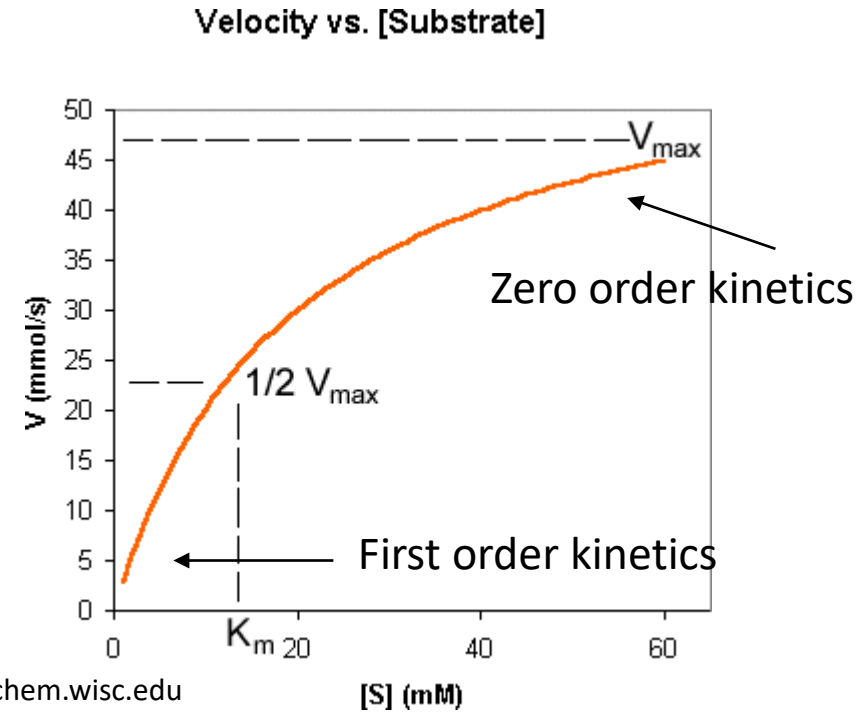




What Have We Learned???

- We did not observe much detoxification with CYP expression, which is odd considering the role metabolism plays in toxicokinetics
- Why???
- It seems to be easier to detect potent metabolites than to appreciably clear toxic parents
- This also assumes that the metabolites of toxic parents are themselves not toxic
- Faster clearance will at some point be hampered by DMSO concentrations

Km, Vmax, and Cytochrome P450s vs. DMSO levels



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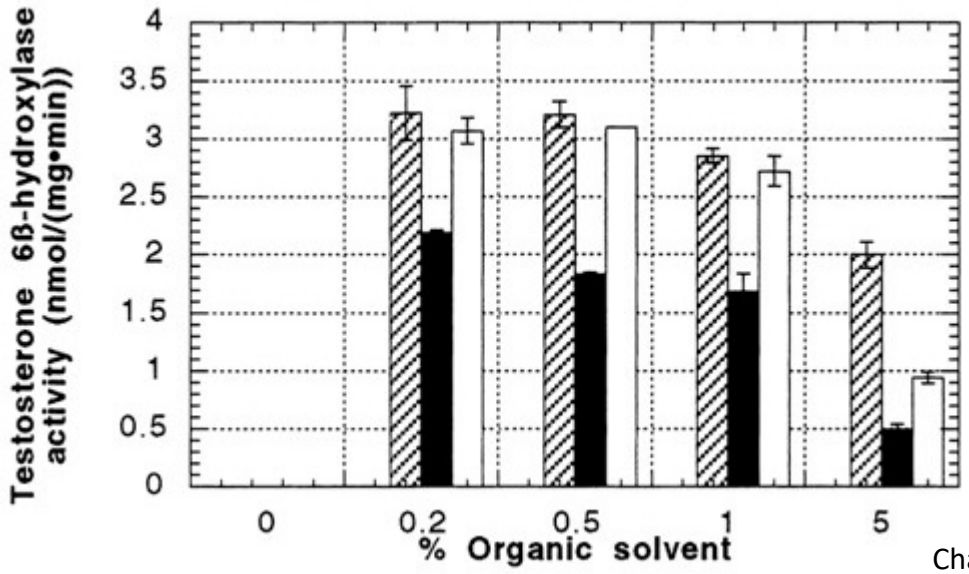
Increasing [DMSO] Inhibits CYP3A4 Activity



Chauret et al., 1998

- Metabolic reaction velocity is a function of substrate concentration.
- Testing at higher concentrations quickly becomes an issue because DMSO (library solvent) inhibits CYP activity.

↑[chemical]
↑ reaction velocity

↑[DMSO]
↓ CYP activity

EPA United States Environmental Protection Agency

What Have We Learned???

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- Why???
- It seems to be easier to detect potent metabolites than to appreciably clear toxic parents
- This also assumes that the metabolites of toxic parents are themselves not toxic
- Faster clearance will at some point be hampered by DMSO concentrations
- Cytotoxicity is the “wrong” assay to look for bioactivated metabolites
- There is no single “typical” human liver with respect to CYP expression, there are at least five (Slatter et al., 2006)

- mRNA transfections do provide a method to imbue deficient cell models with robust XME activity
- mRNA mix can be tightly controlled in ways alternative gene delivery methods cannot
- Rapid expression ideal for HTS applications in high-density, multi-well plates with low working volumes (10-80 µl) where time (e.g.evaporation) is critical
- Very cost-effective → Less than $20 total per 384-plate ($0.05 per well) at pilot scale synthesis

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