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mRNA Transfection Retrofits Cell-based Assays with Xenobiotic Metabolism

Steven O. Simmons

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Office of Research and Development
National Center for Computational Toxicology

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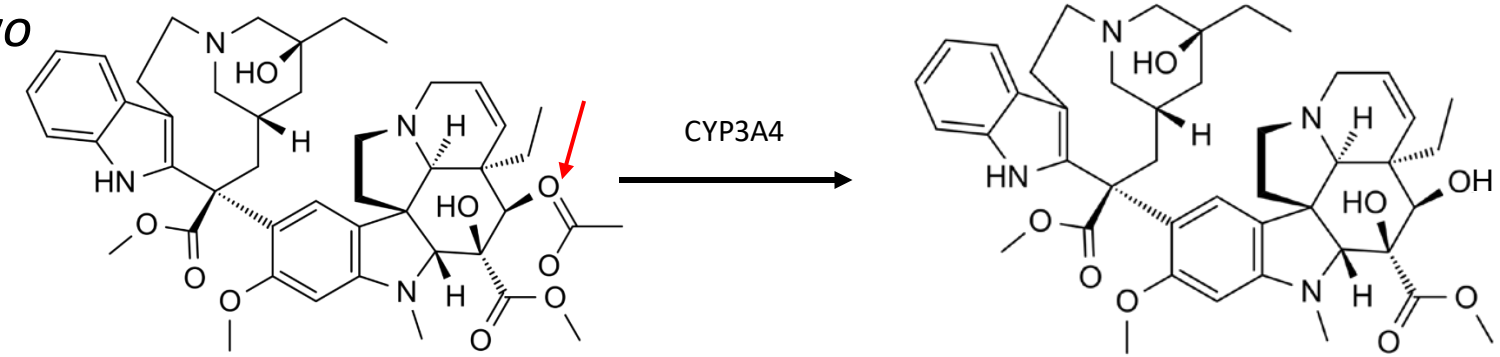
Why is Metabolic Competence Important for *in vitro* Assays?

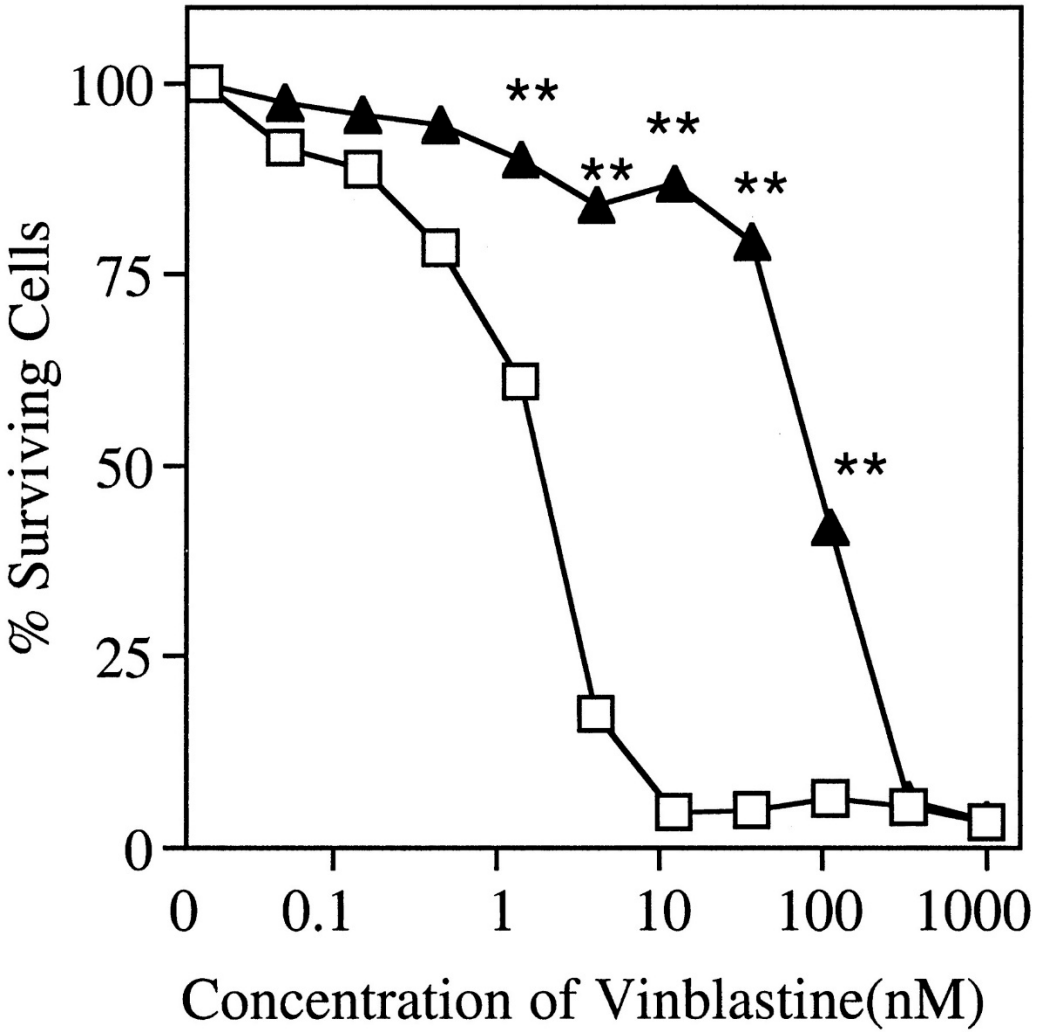
Our existing *in vitro* assays have limited or no metabolic capacity. This leads to two problems:

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

Example: Vinblastine



Vinblastine Detoxification by Human CYP3A4



- Chinese Hamster Ovary cells
- Parental cells (□) vs. cell overexpressing CYP3A4 (▲)
- 72-hour incubation

Yao et al., *Journal of Pharmacology and Experimental Therapeutics*, 2000

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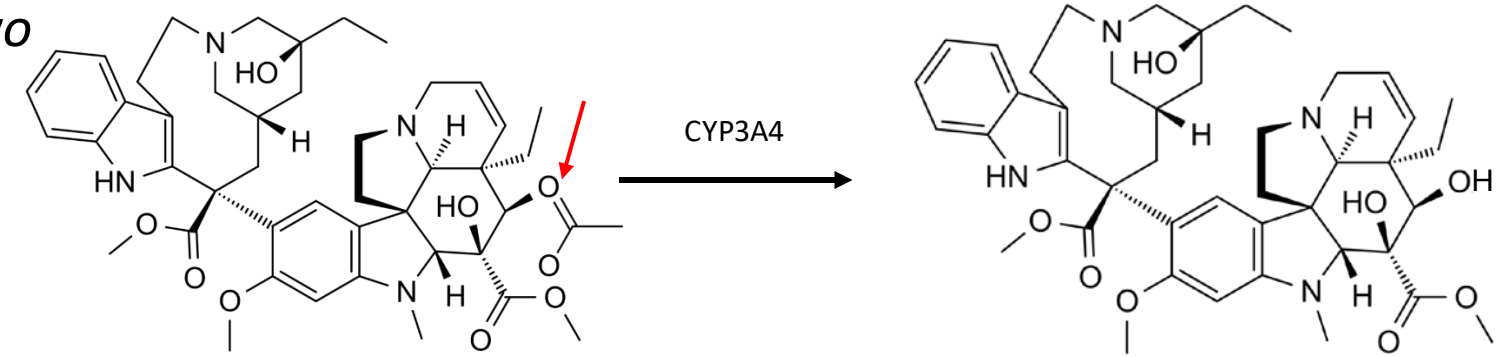
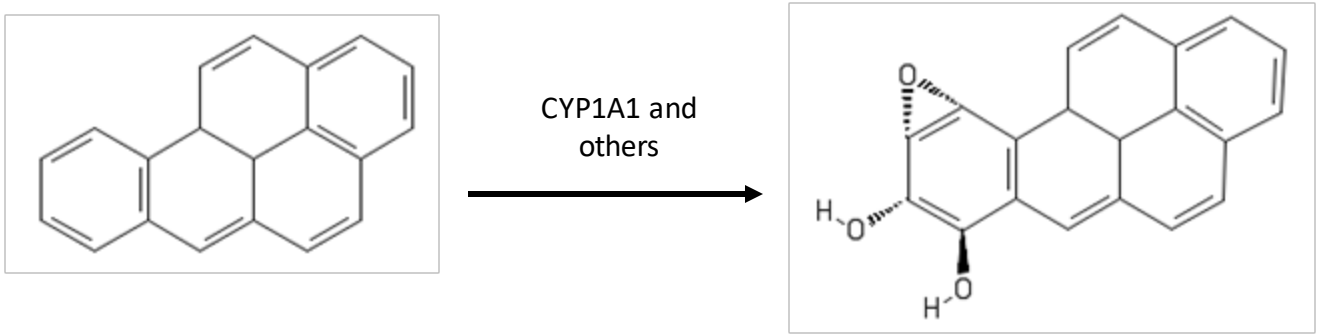
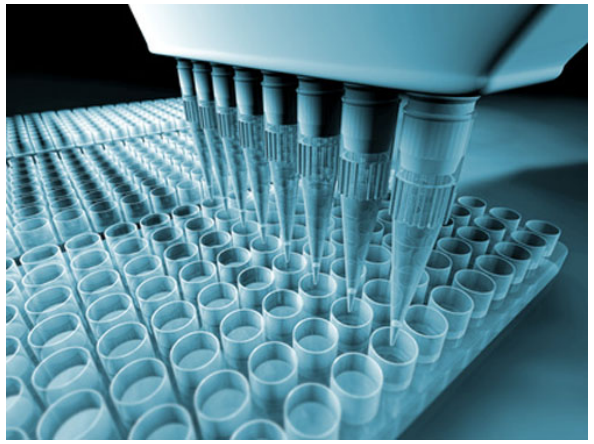


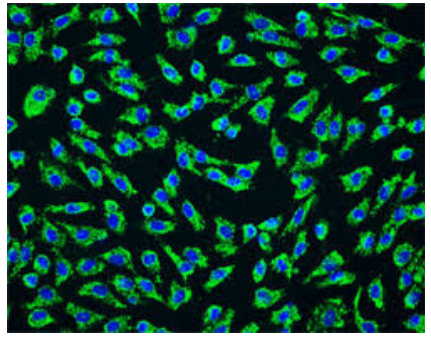
2. Underestimation of chemical hazard *in vitro* if the parent compound is activated to a more toxic metabolite *in vivo*

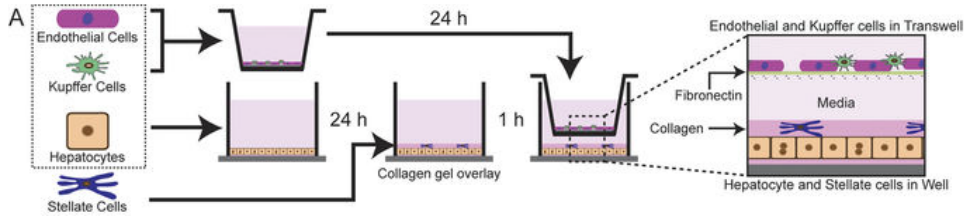
   Example: Benzo[a]pyrene

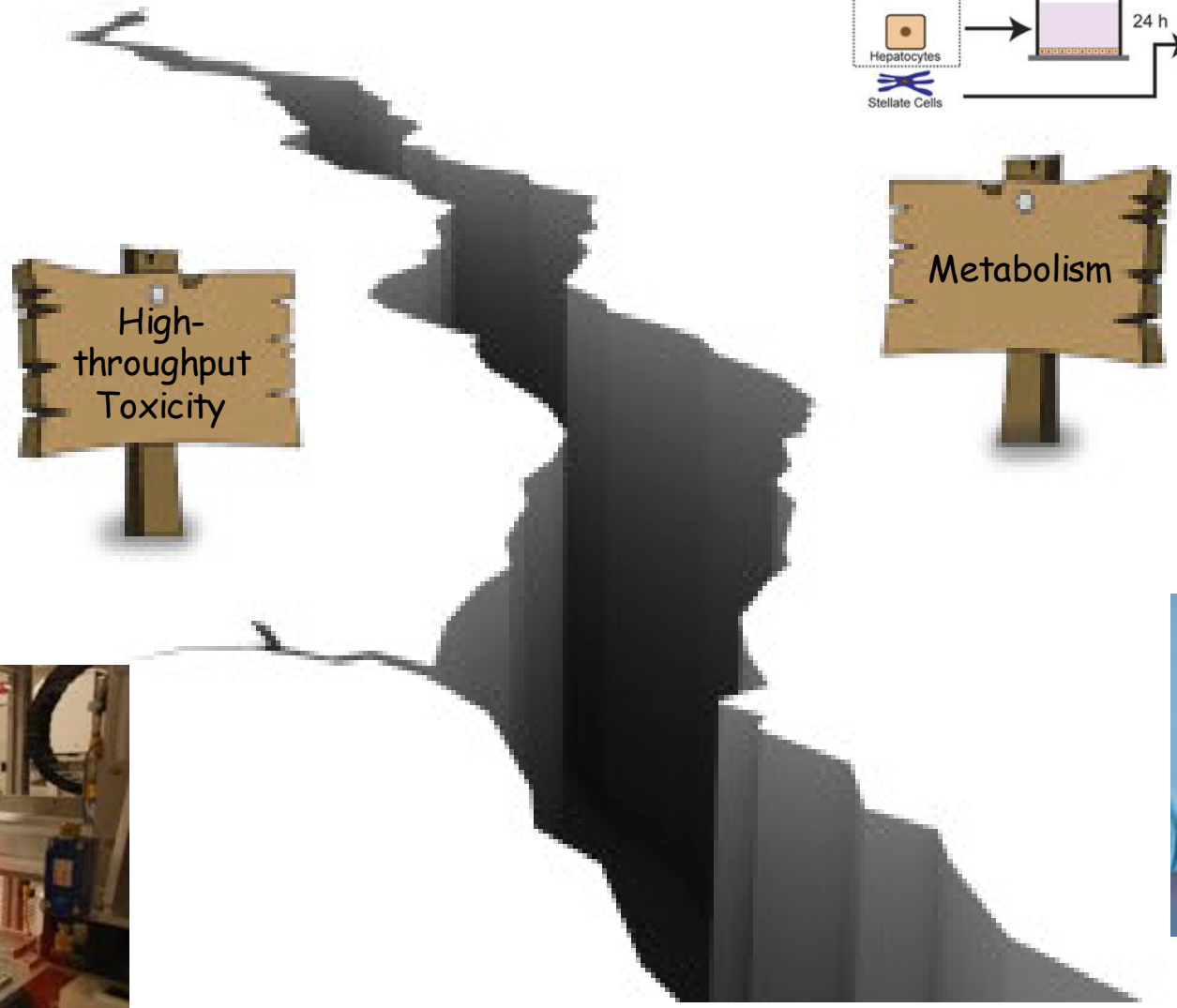


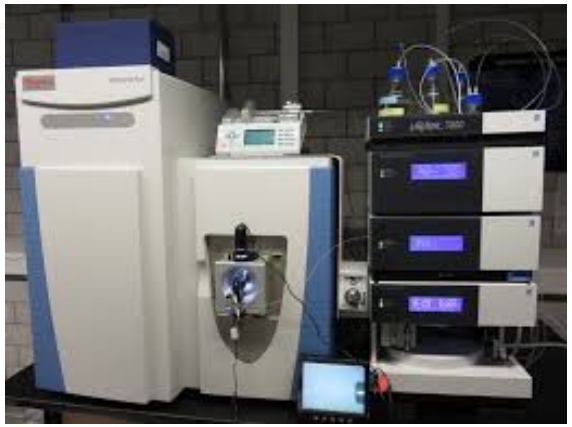
Retrofitting ToxCast/Tox21 *in vitro* Assays





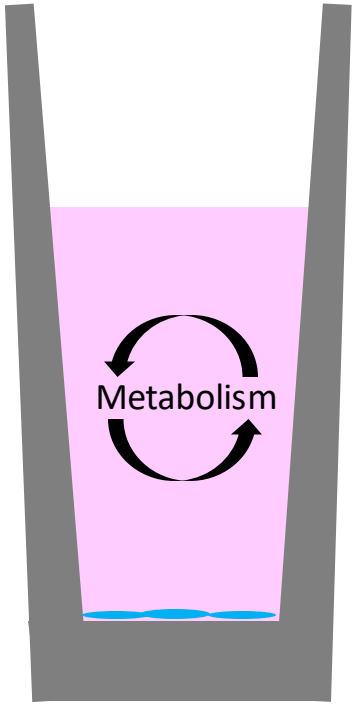






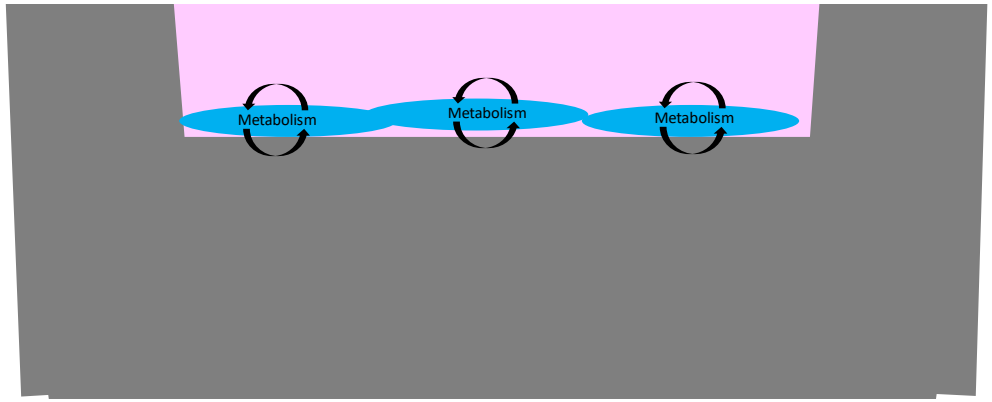
Two Scenarios- Two Strategies for Retrofitting

"Extracellular" Strategy



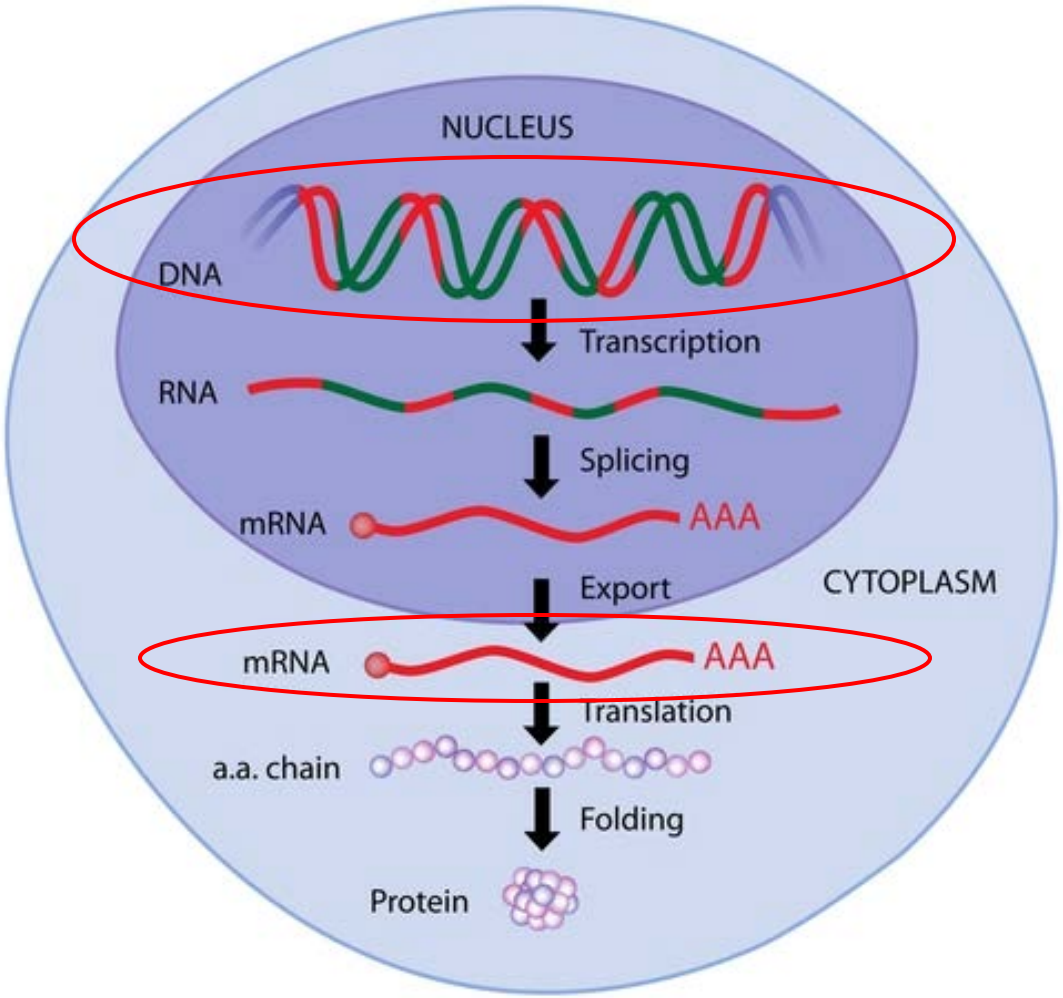
- Capable of metabolizing chemicals in the medium of both cell-based assays and cell-free assays
- More closely models hepatic metabolism and effects of circulating metabolites

"Intracellular" Strategy



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

Intracellular Metabolism

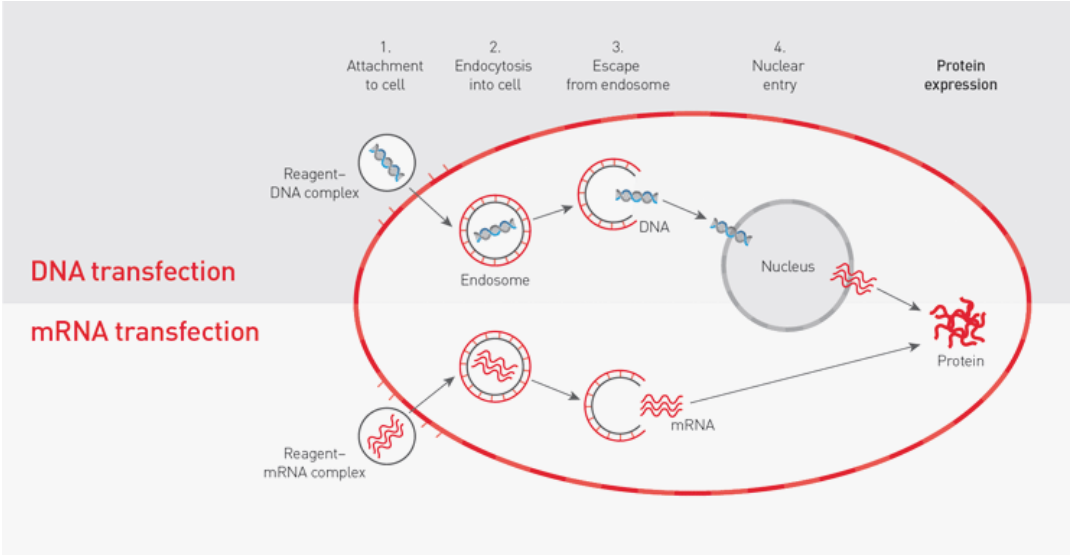


- Introducing xenobiotic-metabolizing enzyme (XME)-encoding genes back into cells with low/no expression is not a new idea
- Plasmid transfection, electroporation, and various viral vectors introduce XME-encoding genes (DNA) back into cells under control of gene promoters that drive strong expression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

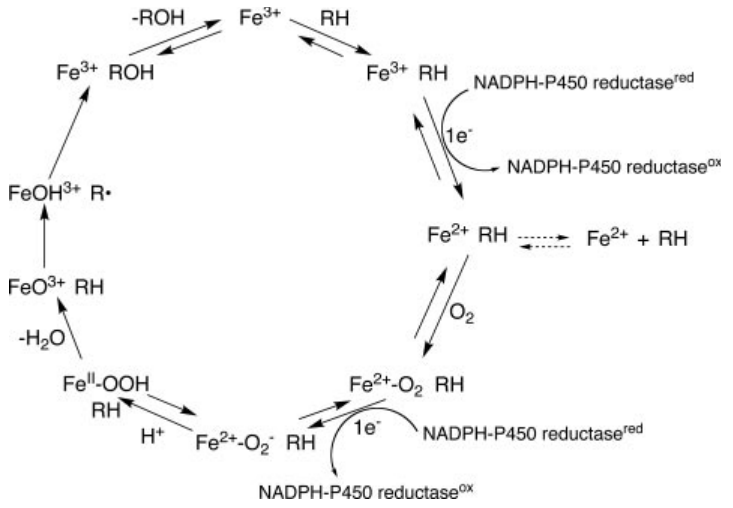
- Transfection of XME-encoding mRNAs is a novel approach that bypasses cellular transcription
- Chemically-modified nucleotides and cap eliminate the toxicity traditionally seen with RNA transfection
- Rapid XME expression and permits user to define composition and ratios of input mRNAs
- Method development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

Optimization

- Nucleic acids (DNA or RNA) are large, charged molecules that do not readily cross the cell membrane
- Cationic lipid transfection
- The most popular transfection reagents in use today are cationic lipids (Lipofectamine ™, etc.)
- Lipid:RNA was optimized empirically using CYP3A4 activity
- Payload volume was optimized empirically using CYP3A4 activity



- POR required for the electron transfer from NADPH to cytochrome P450 enzymes in ER
- Although ubiquitously expressed, if POR is rate-limiting, CYP activity will be sub-optimal
- POR co-expression was optimized empirically using CYP3A4 activity



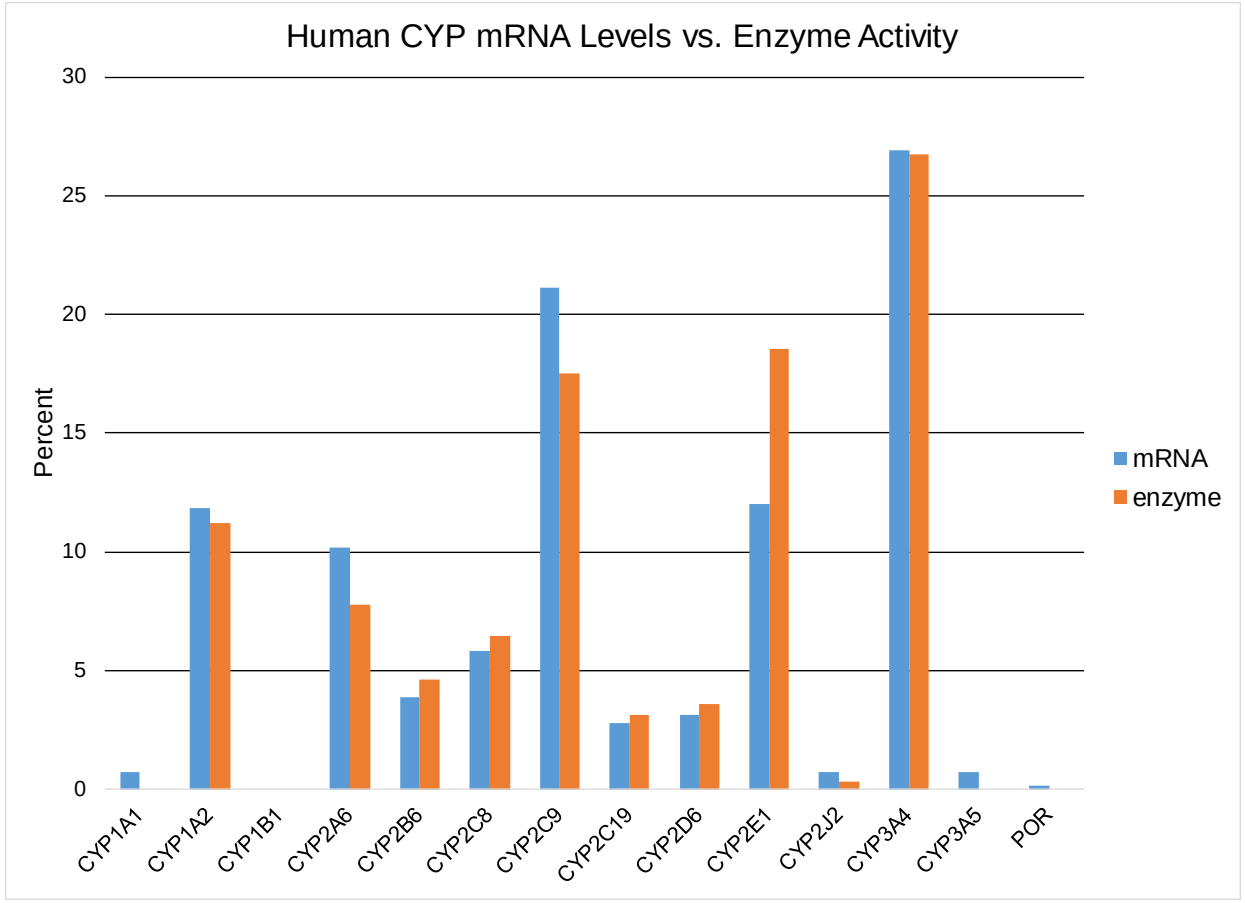
- Optimization nearly doubled CYP3A4 activity in HEK293T cells

Characterizing a Panel of CYP Enzymes

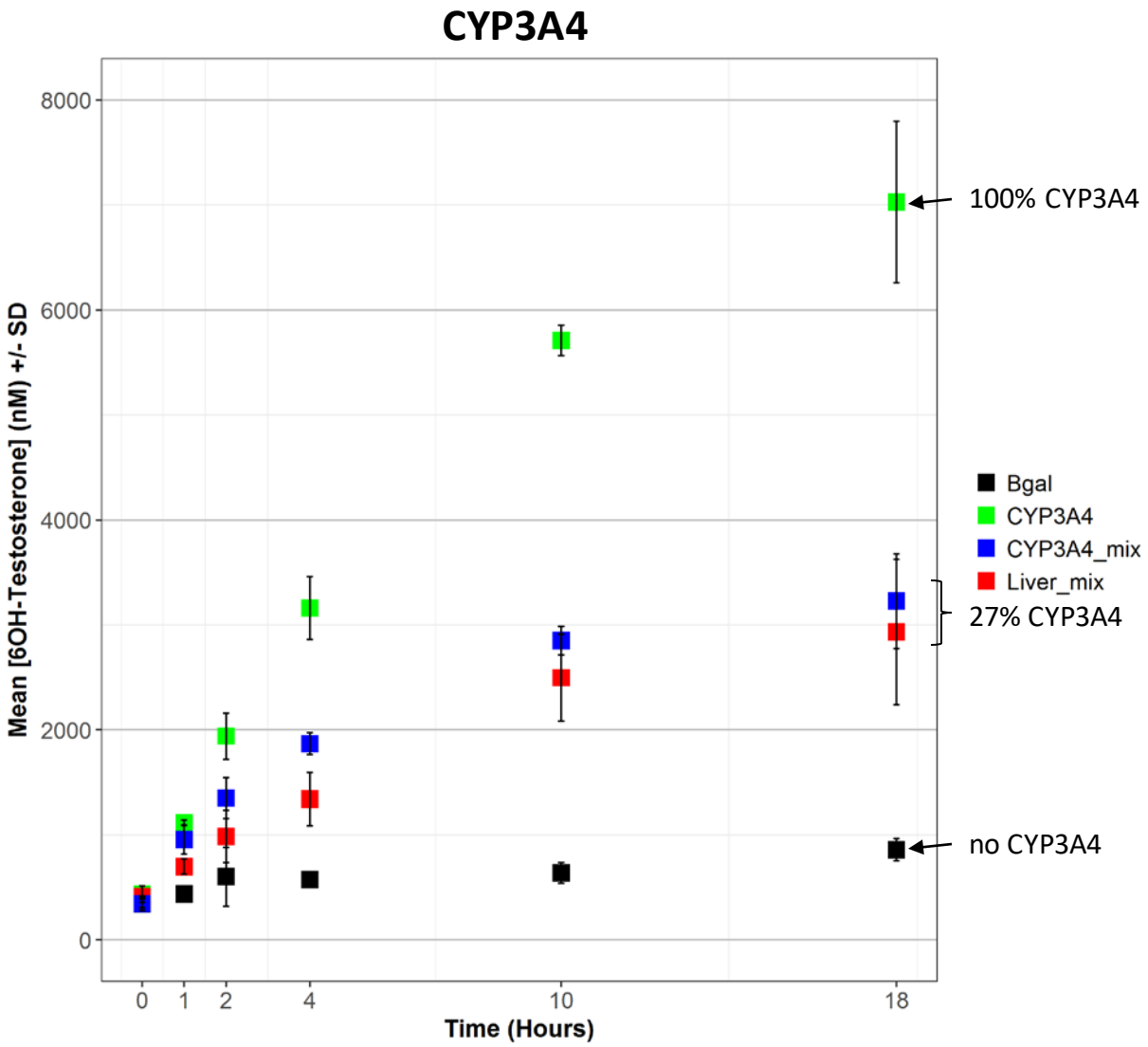
- With optimization complete, next was to characterize the activity of the 10 most prevalent CYPs in human liver identified through a meta-analysis of over 700 subjects (Zanger and Schwab, 2013):

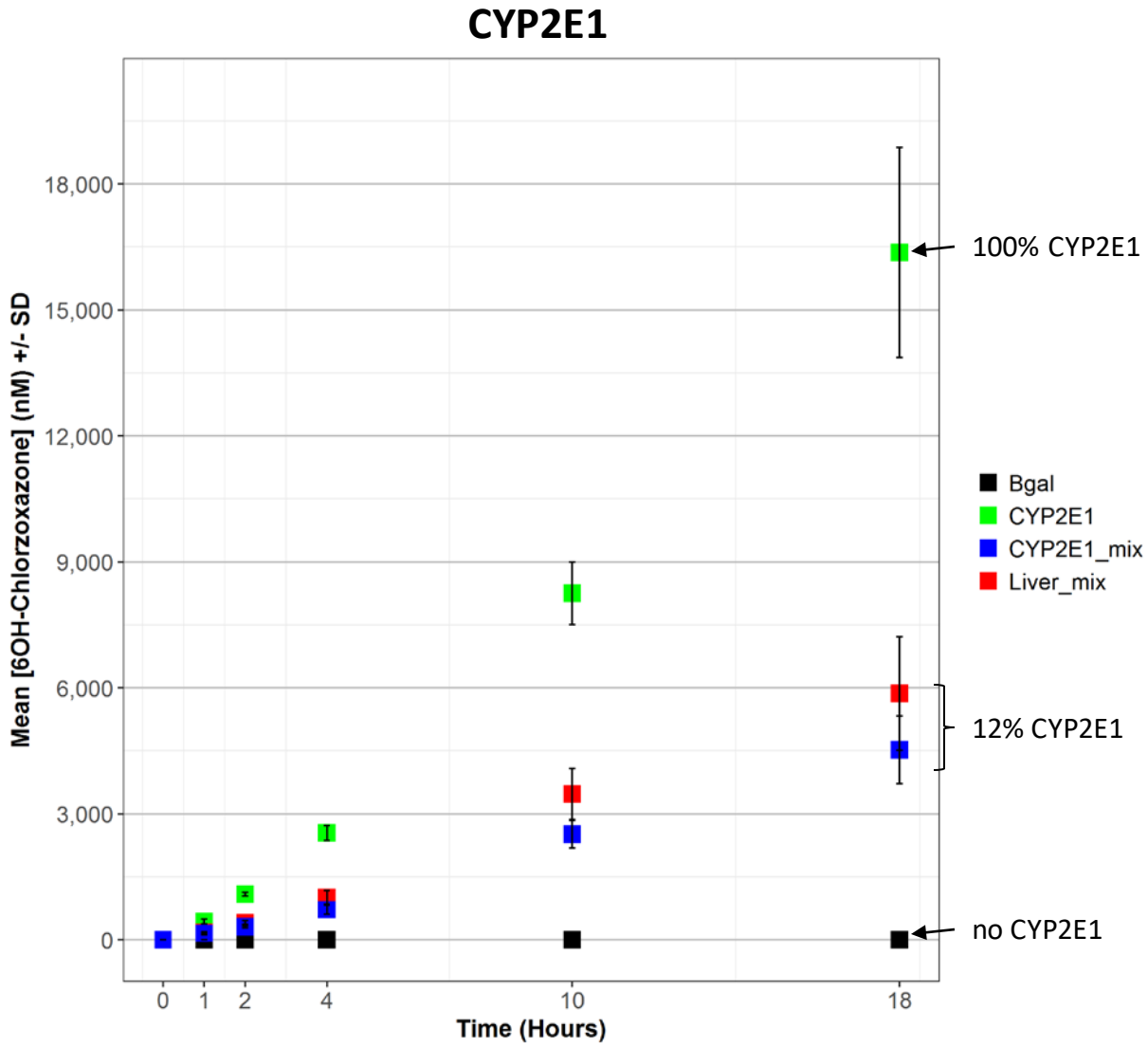
  - CYP1A2 (12%)
  - CYP2A6 (10%)
  - CYP2B6 (4%)
  - CYP2C8 (6%)
  - CYP2C9 (21%)
  - CYP2C19 (3%)
  - CYP2D6 (3%)
  - CYP2E1 (12%)
  - CYP2J2 (1%)
  - CYP3A4 (27%)

(% of pooled liver mRNA)

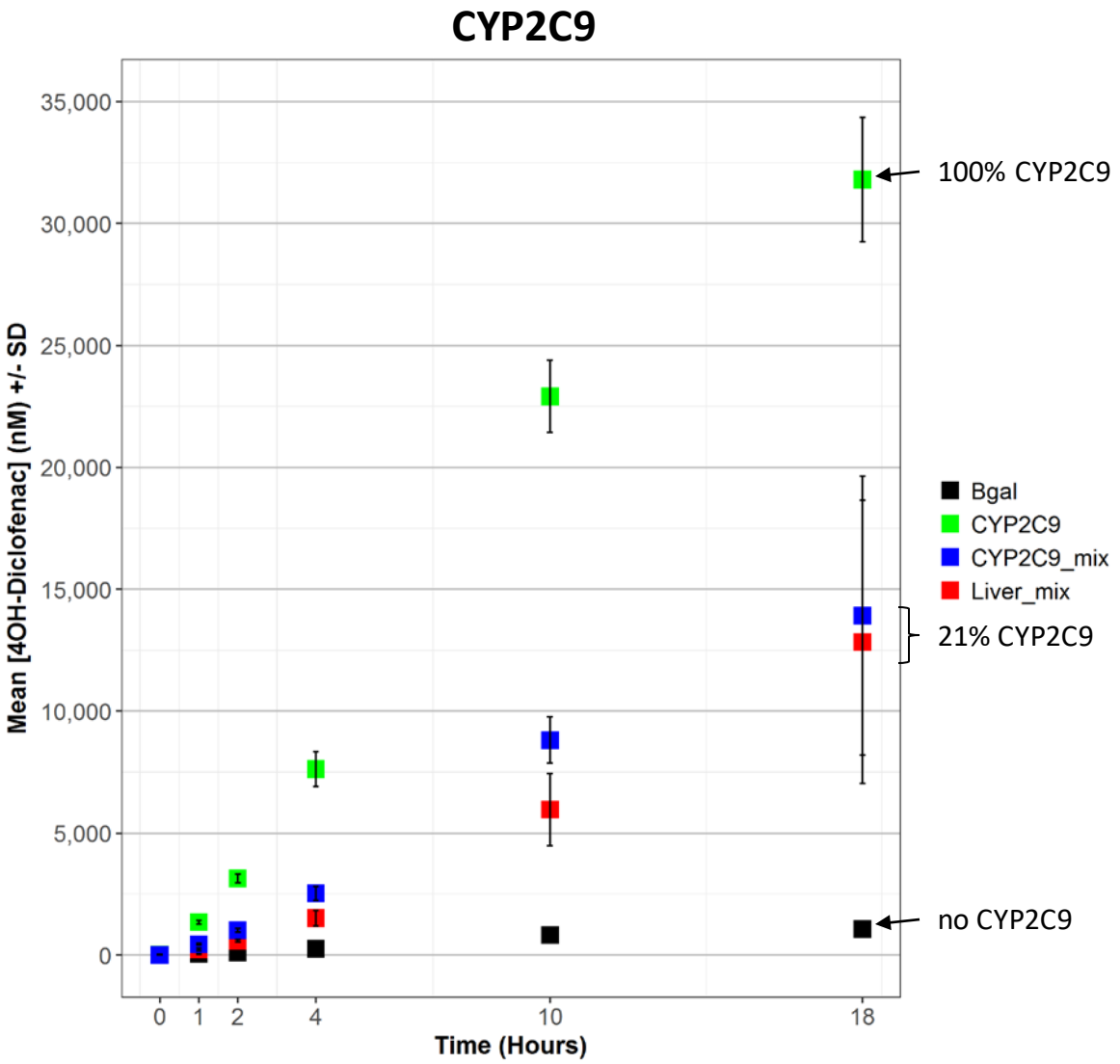


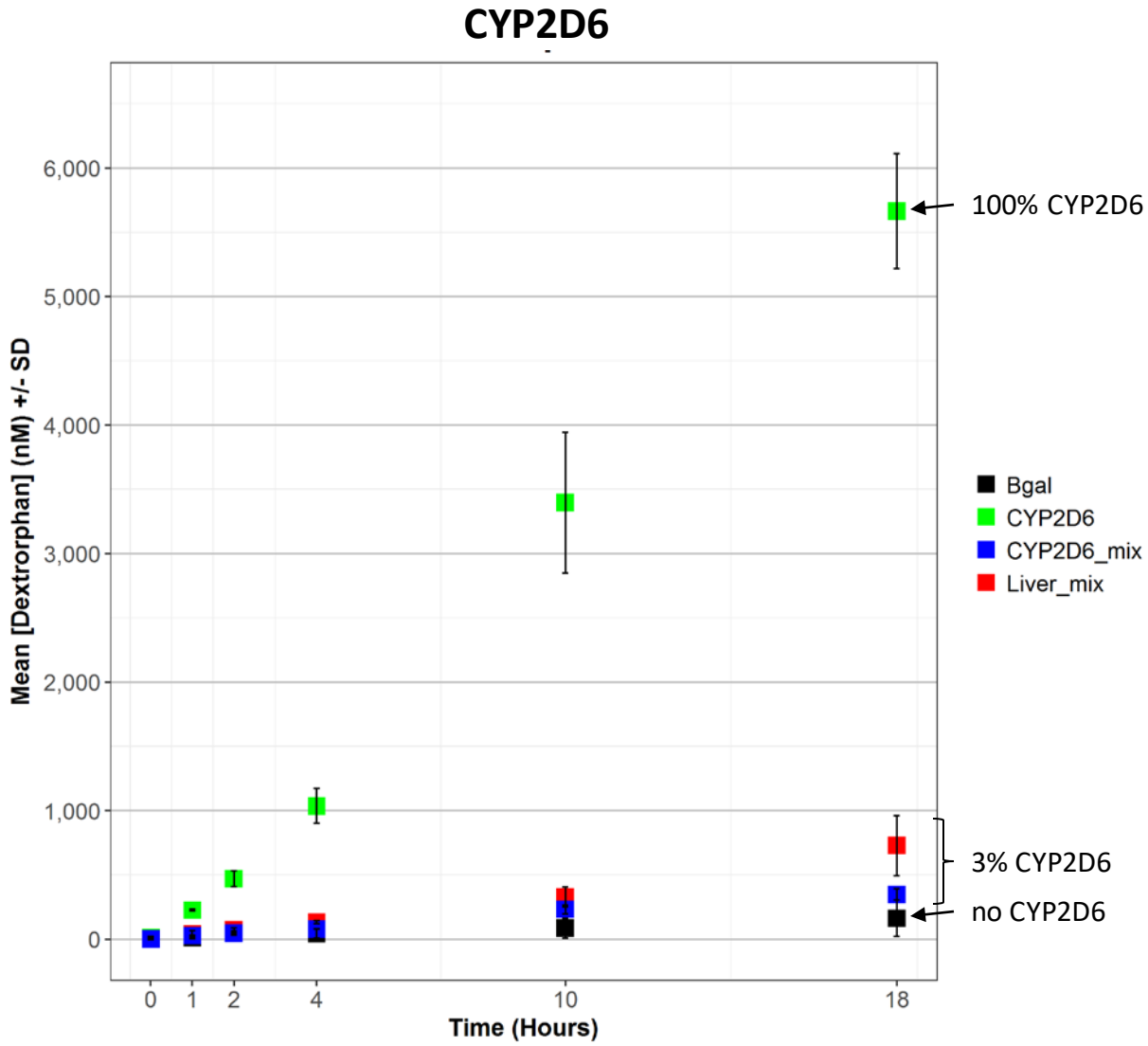
Benchmark Substrate Studies (LC-MS/MS)



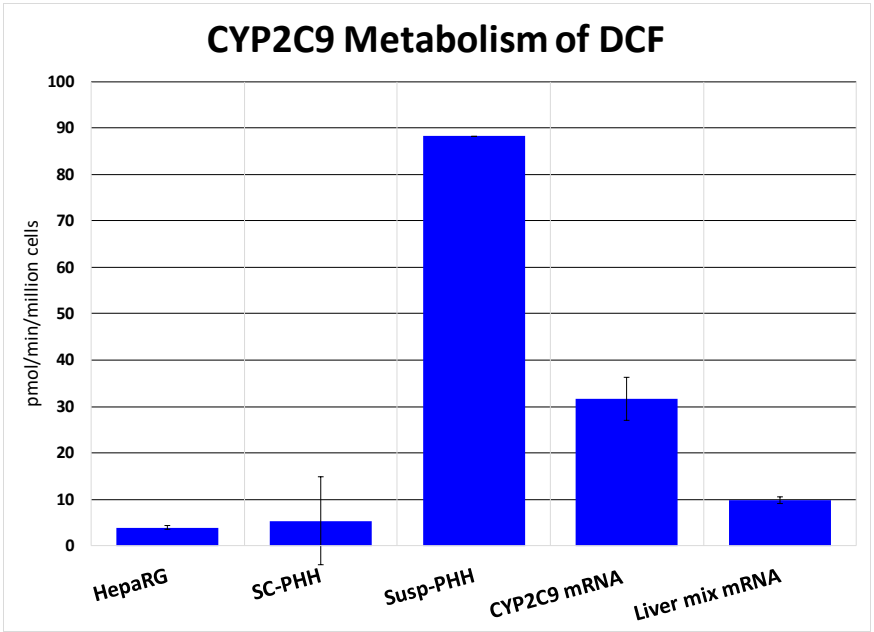


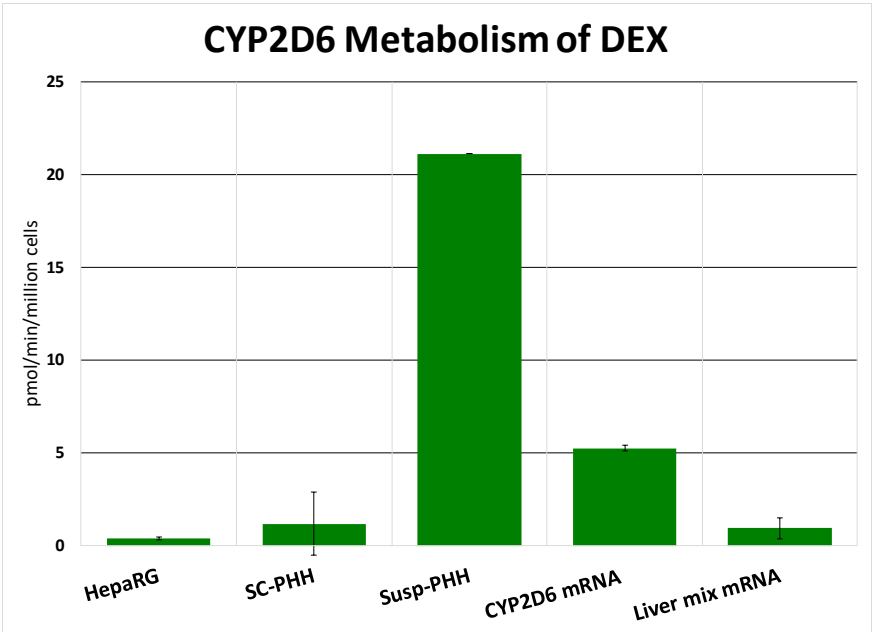
Benchmark Substrate Studies (con't)

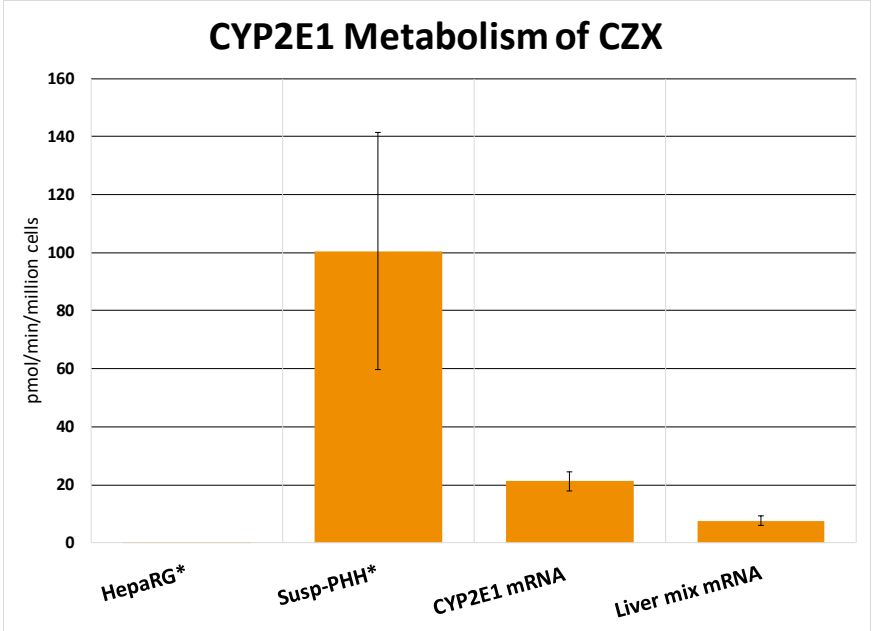


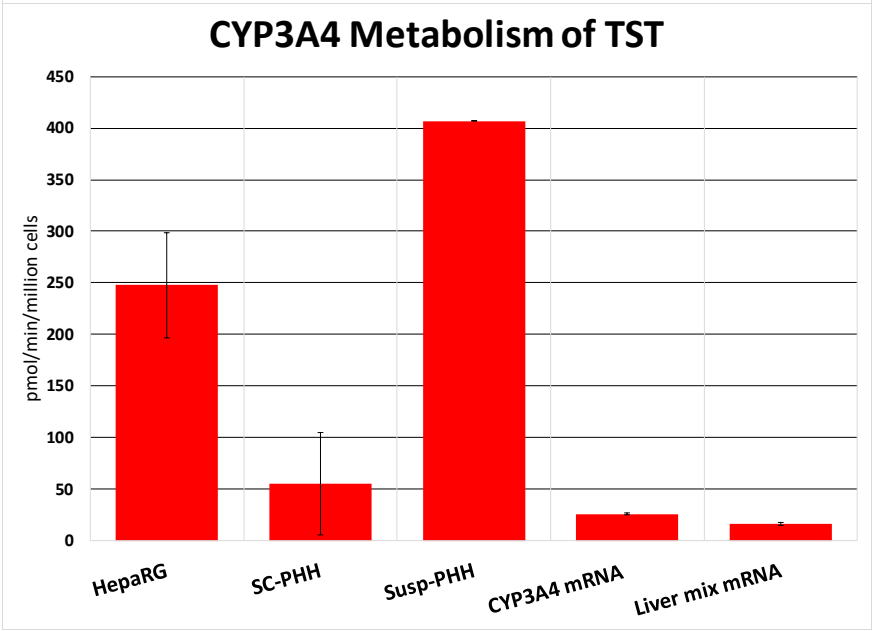


Comparison to "Gold-Standard" XM-Competent Cell Models









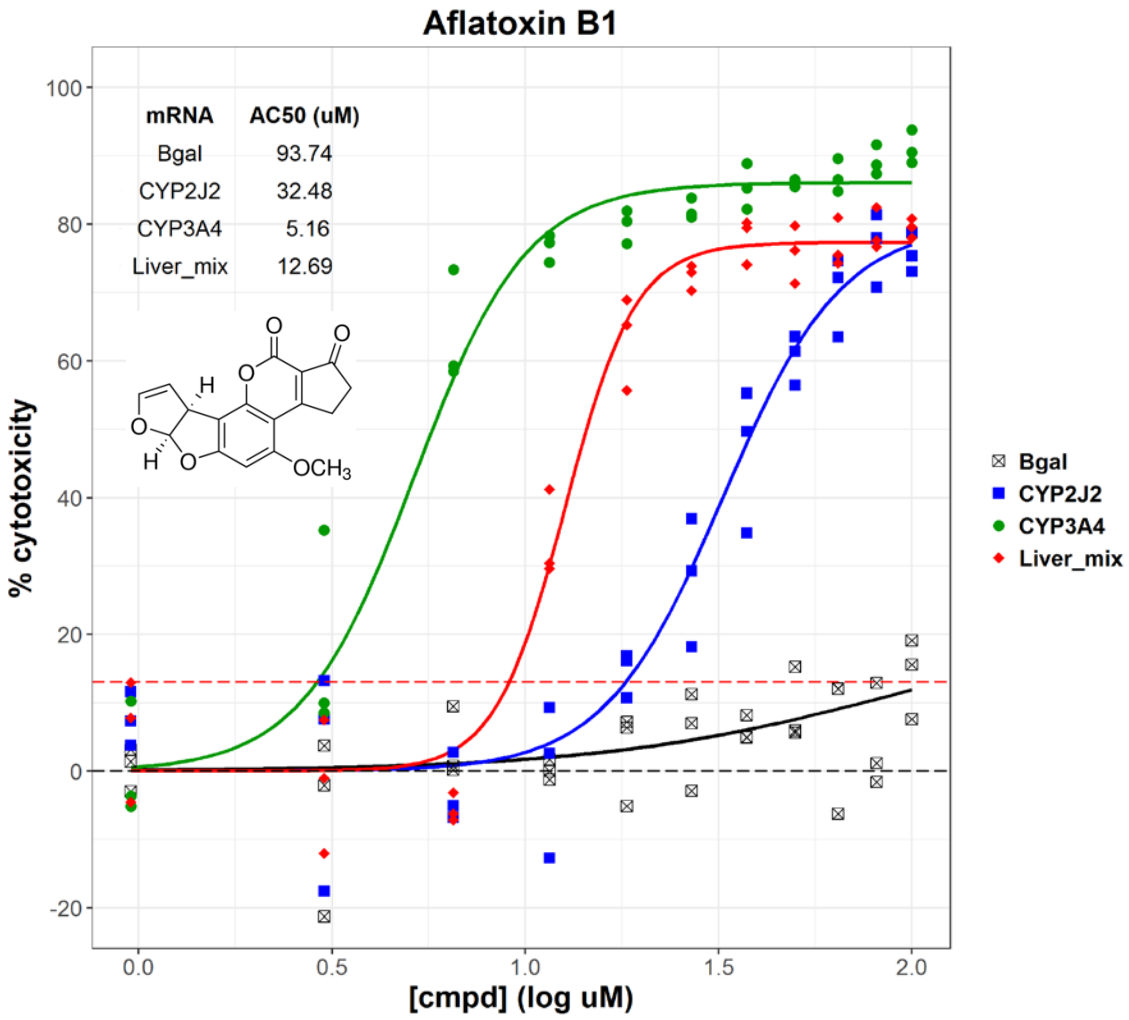
Deployment to Cell-Based Assays

- 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 enzymes produce predicted metabolites and at rates > than HepaRG and SC-PHH models, even when handicapped by HTS conditions
- Getting cells to express CYPs in 384-well plates was never going to be the hard part…

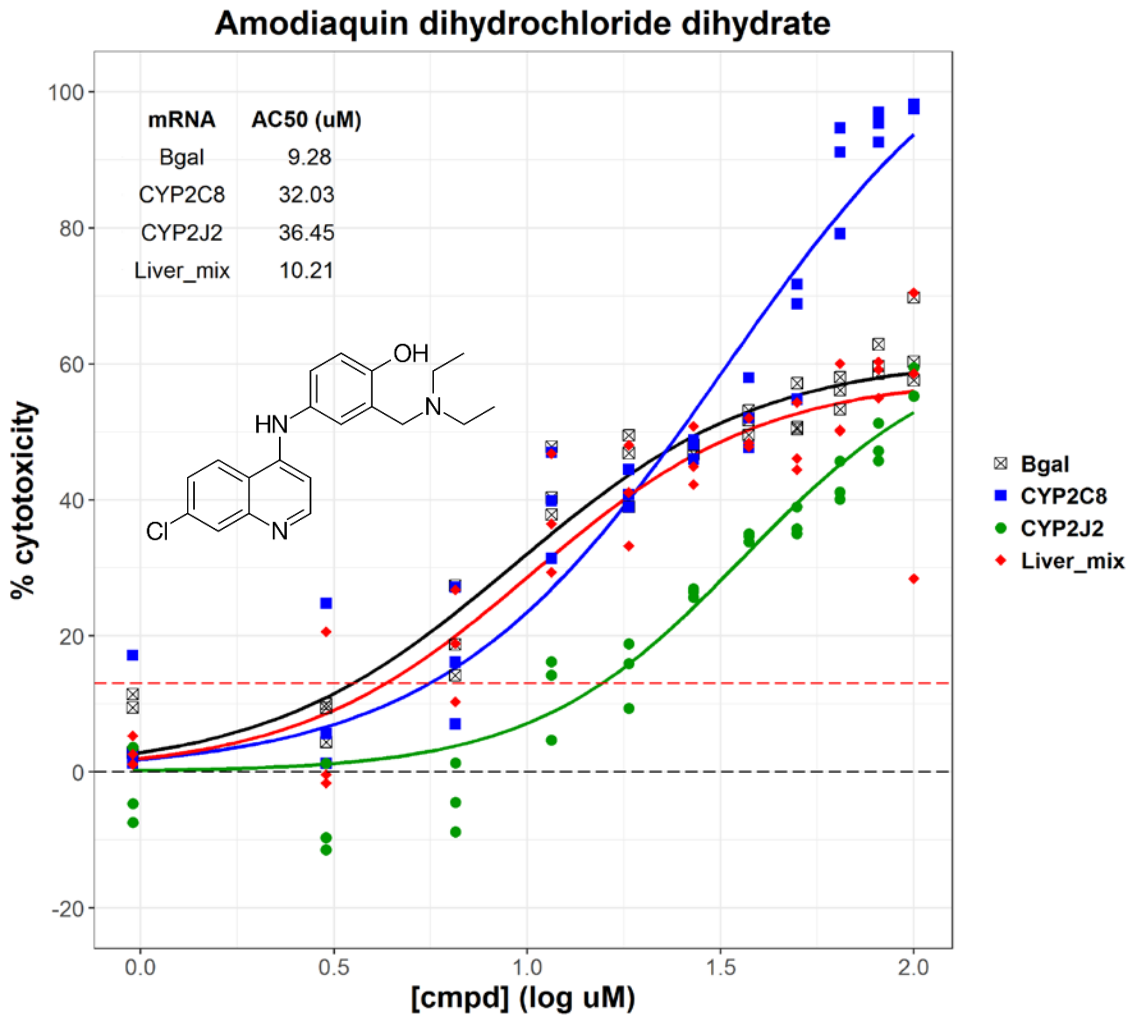
- **What happens when we couple this method with a cell-based assay?**
- **Can we observe CYP-dependent shifts in bioactivity?**

- HEK293T cells transfected with 10 x CYP singlets, Liver mix, and β-gal control (12 biogroups)
- 56 test compounds
- 11 concentrations
- 36 hour exposure
- N = 3
- Cytotoxicity measured using Cell Titer Glo™ Assay

Cytotoxicity Screening Results

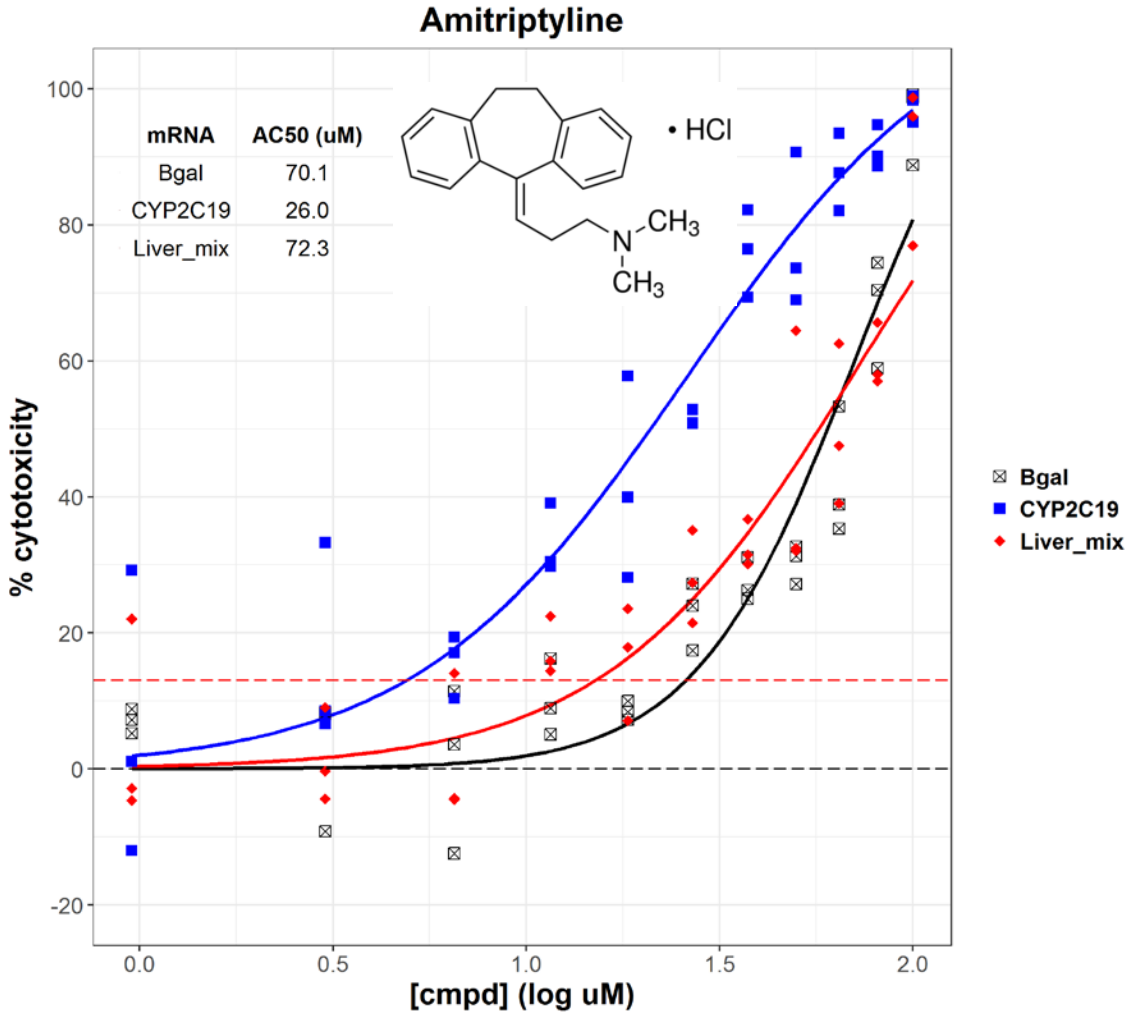


Not in ToxCast or Tox21 inventory

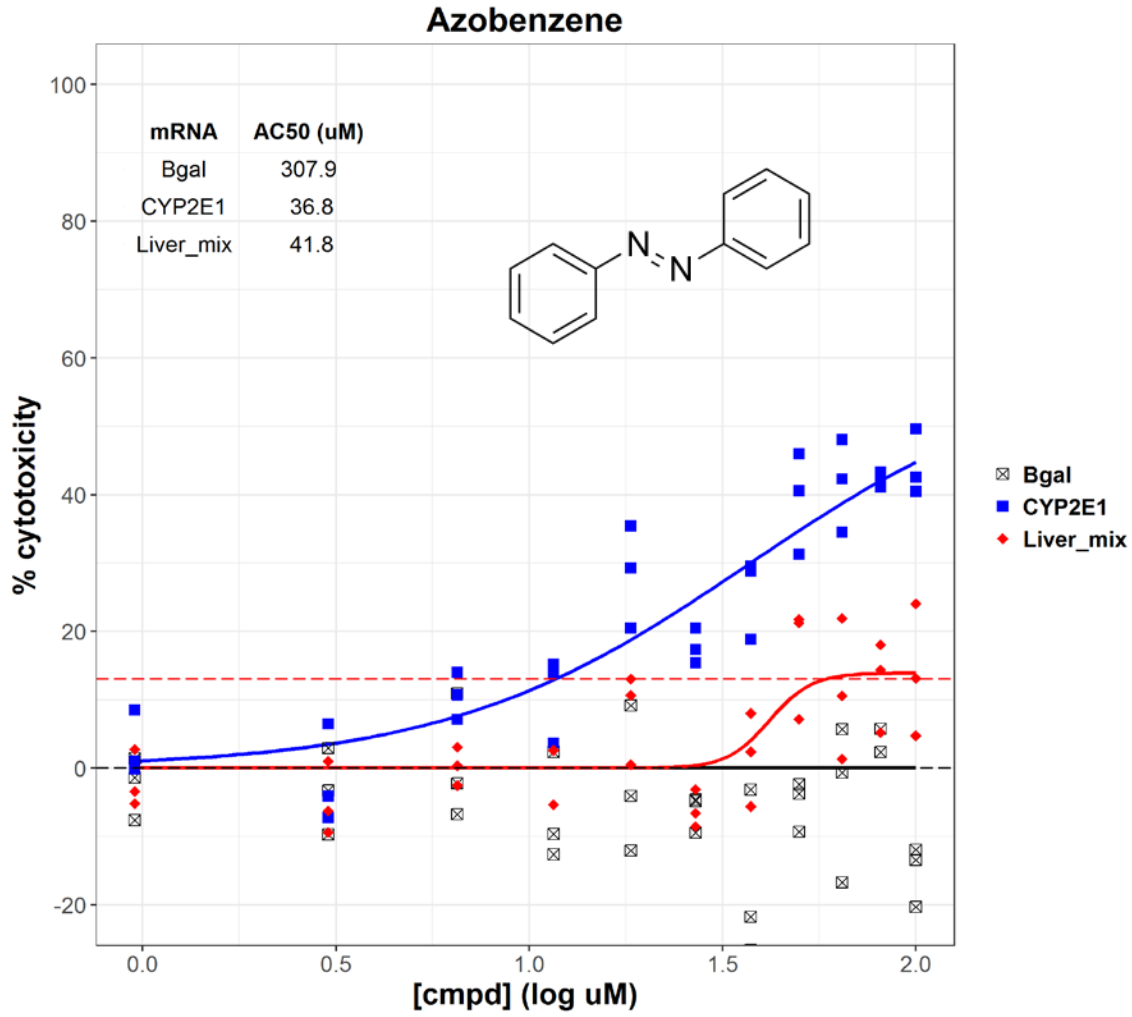


Active in 2 of 64 assays (3.1%)

Cytotoxicity Screening Results (con't)



Active in 35 of 279 assays (12.5%)

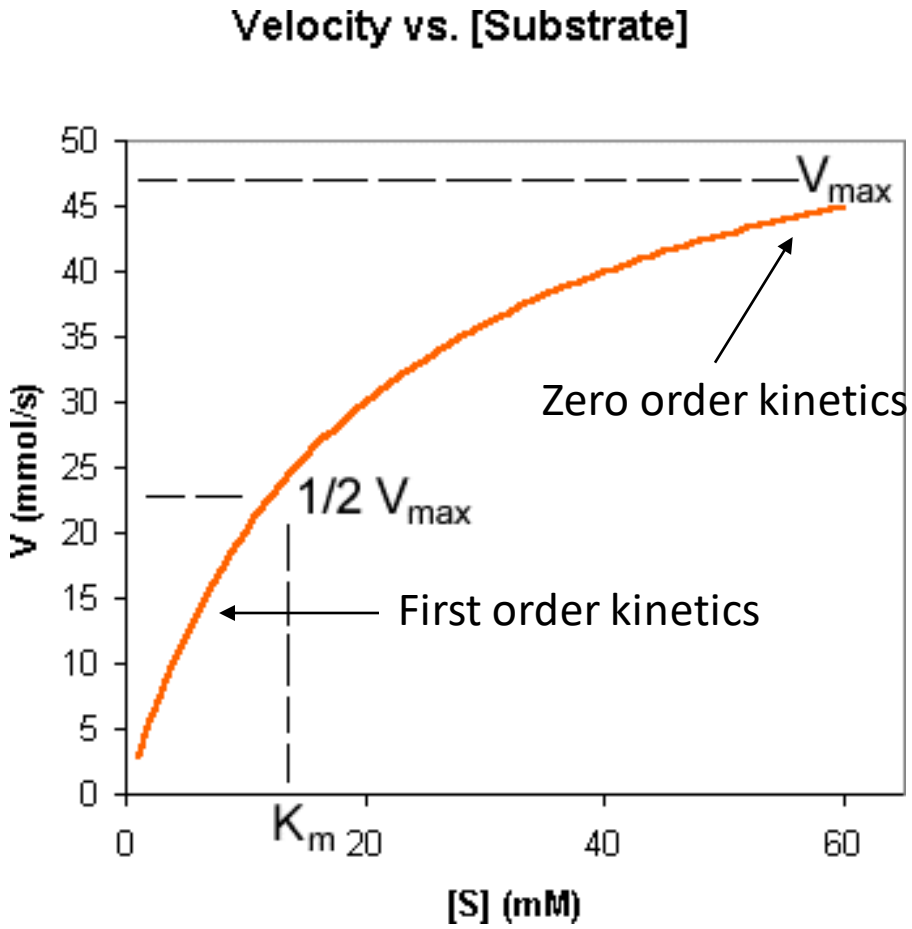


Active in 14 of 882 assays (1.6%)

What Did We Learn???

- We did not observe much detoxification with CYP expression, which is odd considering the role metabolism plays in toxicokinetics
- Why???
  1. Wrong test chemicals
  2. Enhanced Phase I metabolism (oxidations) overwhelmed Phase II metabolism (conjugations)
  3. Kinetics

Michaelis-Menten Kinetics

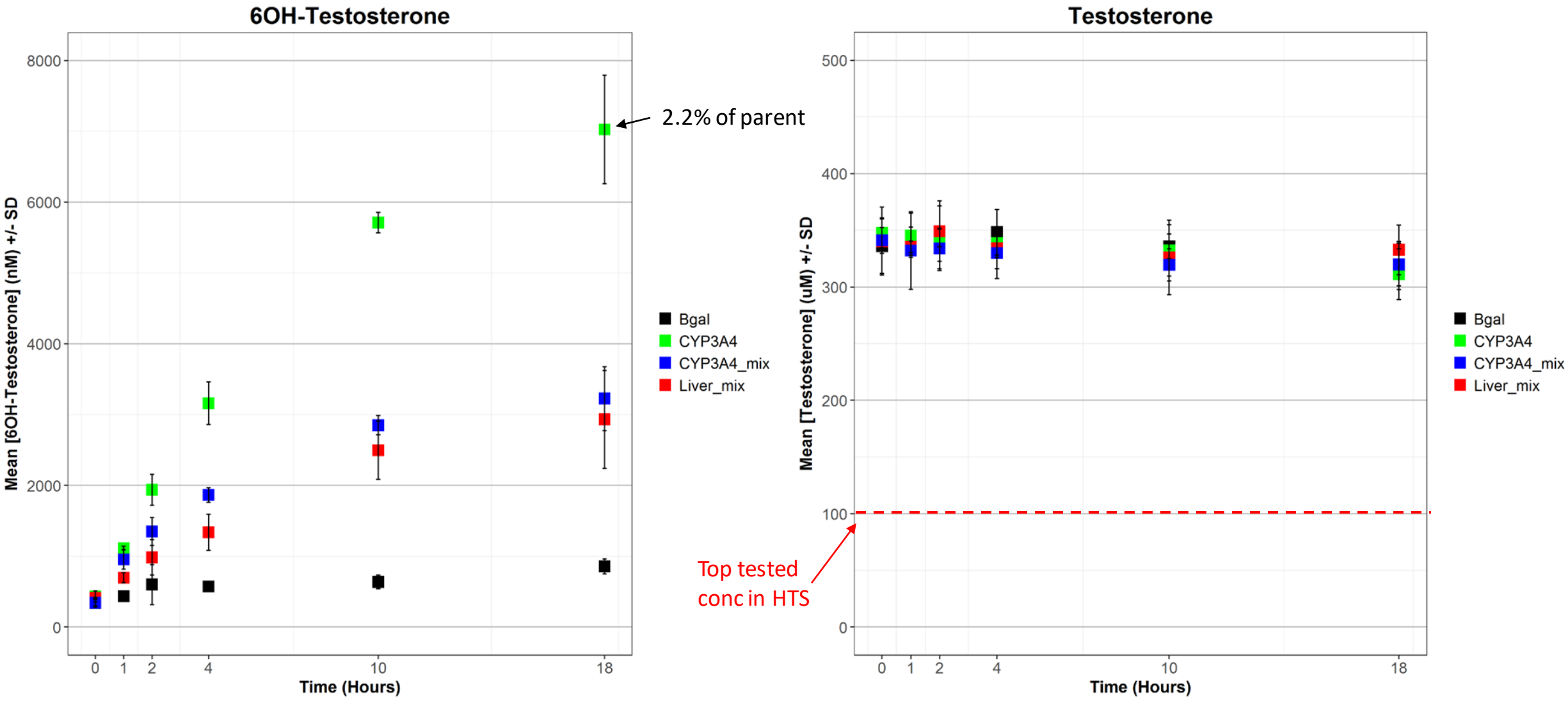


www.chem.wisc.edu

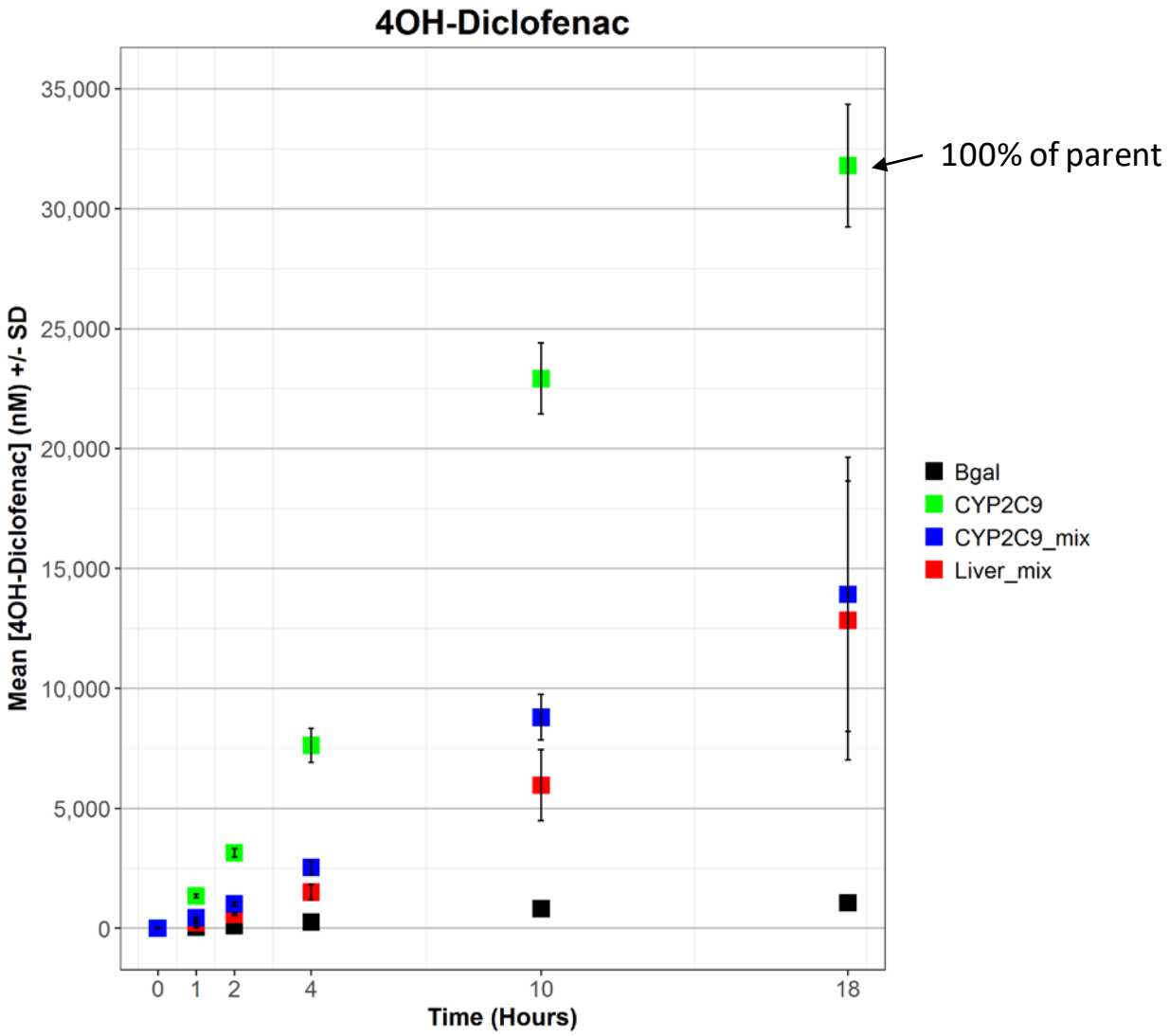
- Rate of metabolite formation (V) increases as a function of substrate concentration [S] (parent chemical)
- At Vmax is maximal reaction rate where increasing [S] has no added effect
- At the [S] at ½ Vmax is called the Km
- If [S] is well below the Km, V is very slow (Km ÷ 10 → 9% Vmax)
- If [S] is well above the Km, V is very fast (Km x 10 → 91% Vmax)

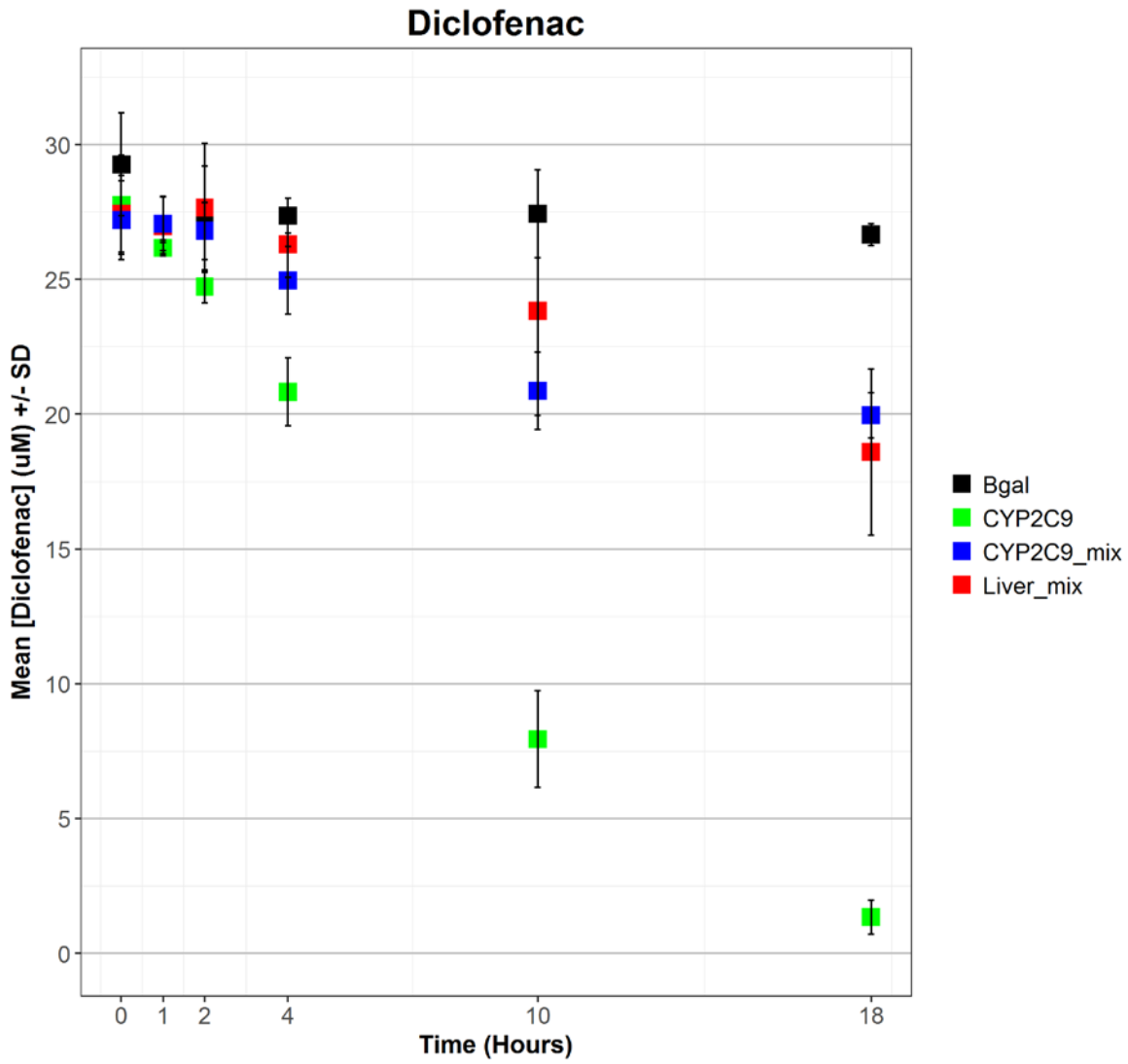
- This has real-world consequences as we deploy metabolic retrofits to HTT screening

Metabolite Formation vs. Parent Depletion: CYP3A4



Metabolite Formation vs. Parent Depletion: CYP2C9





What Did We Learn???

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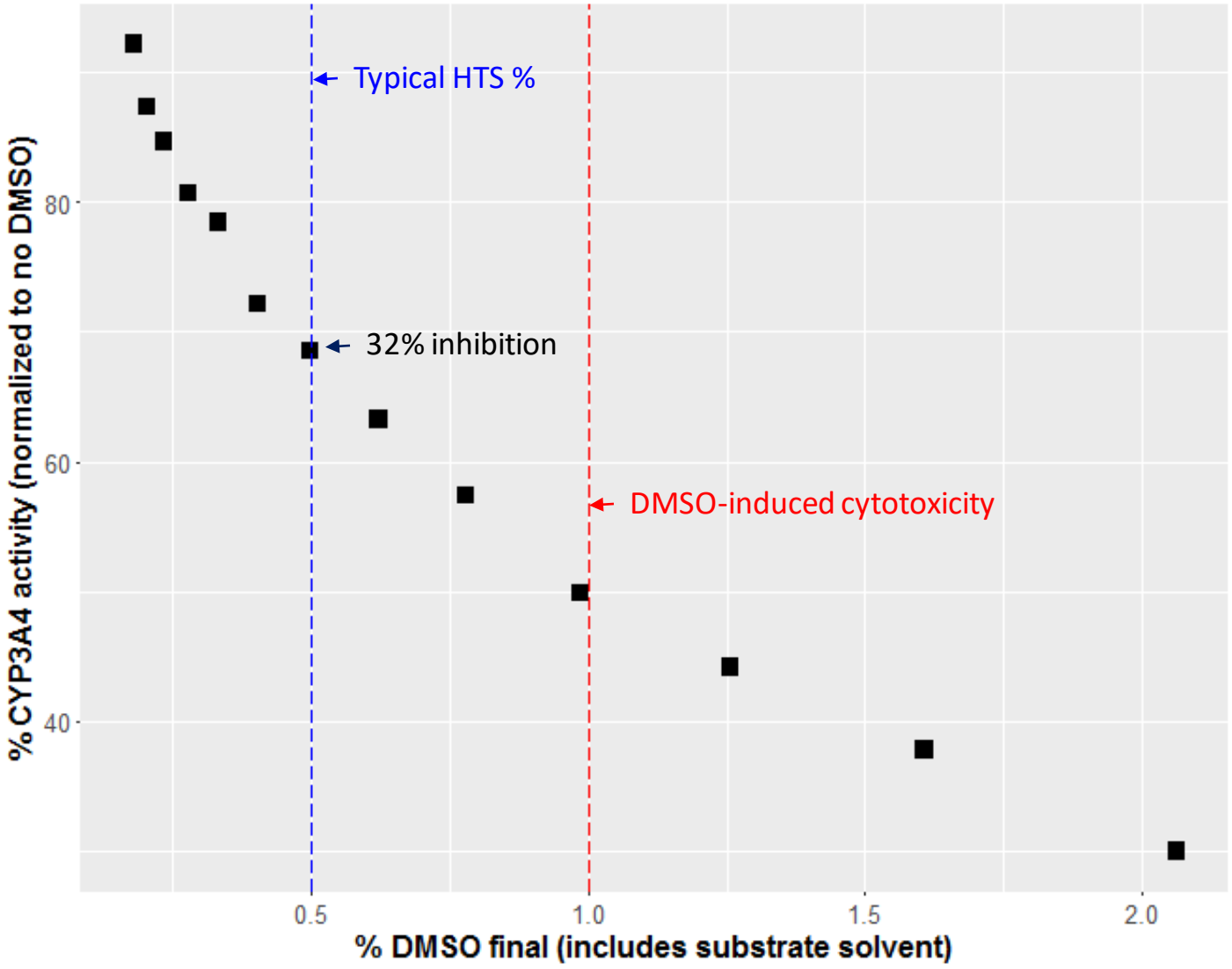
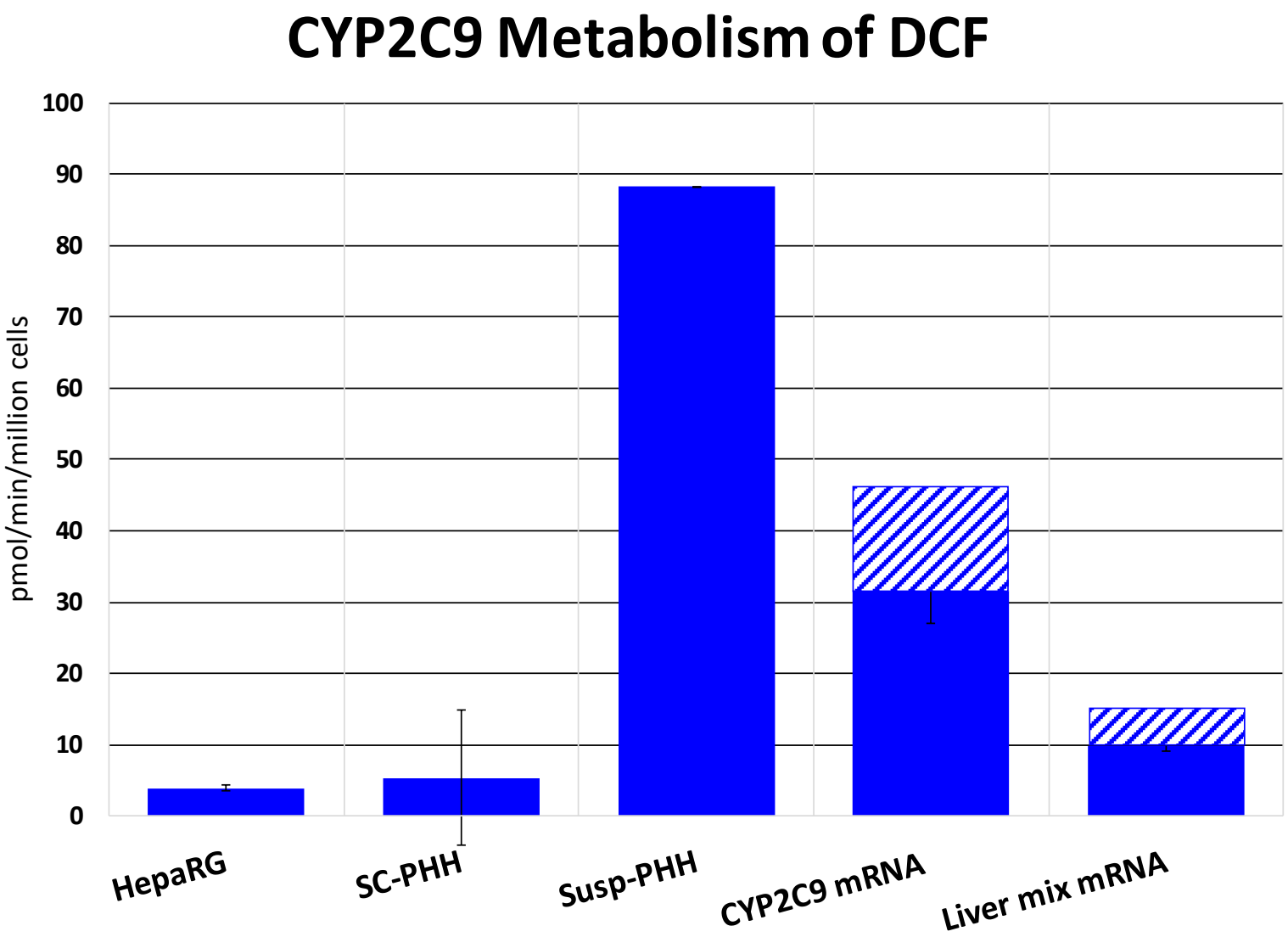
- Why???
  1. Wrong test chemicals
  2. Enhanced Phase I metabolism (oxidations) overwhelmed Phase II metabolism (conjugations)
  3. Kinetics- since we do not know every enzyme x chemical interaction, we cannot know how our test chemical concentrations relate to Km or what the Vmax is…
     - Testosterone x CYP3A4 → 53-128µM with low Vmax (~40 pmol/min/pmol enzyme)
     - Diclofenac x CYP2C9 → 9-11µM with high Vmax (+400 pmol/min/pmol enzyme)
     - We cannot change Vmax or Km
     - We could theoretically increase V by increasing our test chemical concentrations, but DMSO (library solvent) inhibits CYP activity, even at typical HTS screening levels

DMSO Inhibits Cell-based CYP3A4 Activity



Impact of DMSO Inhibition



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What Did We Learn???

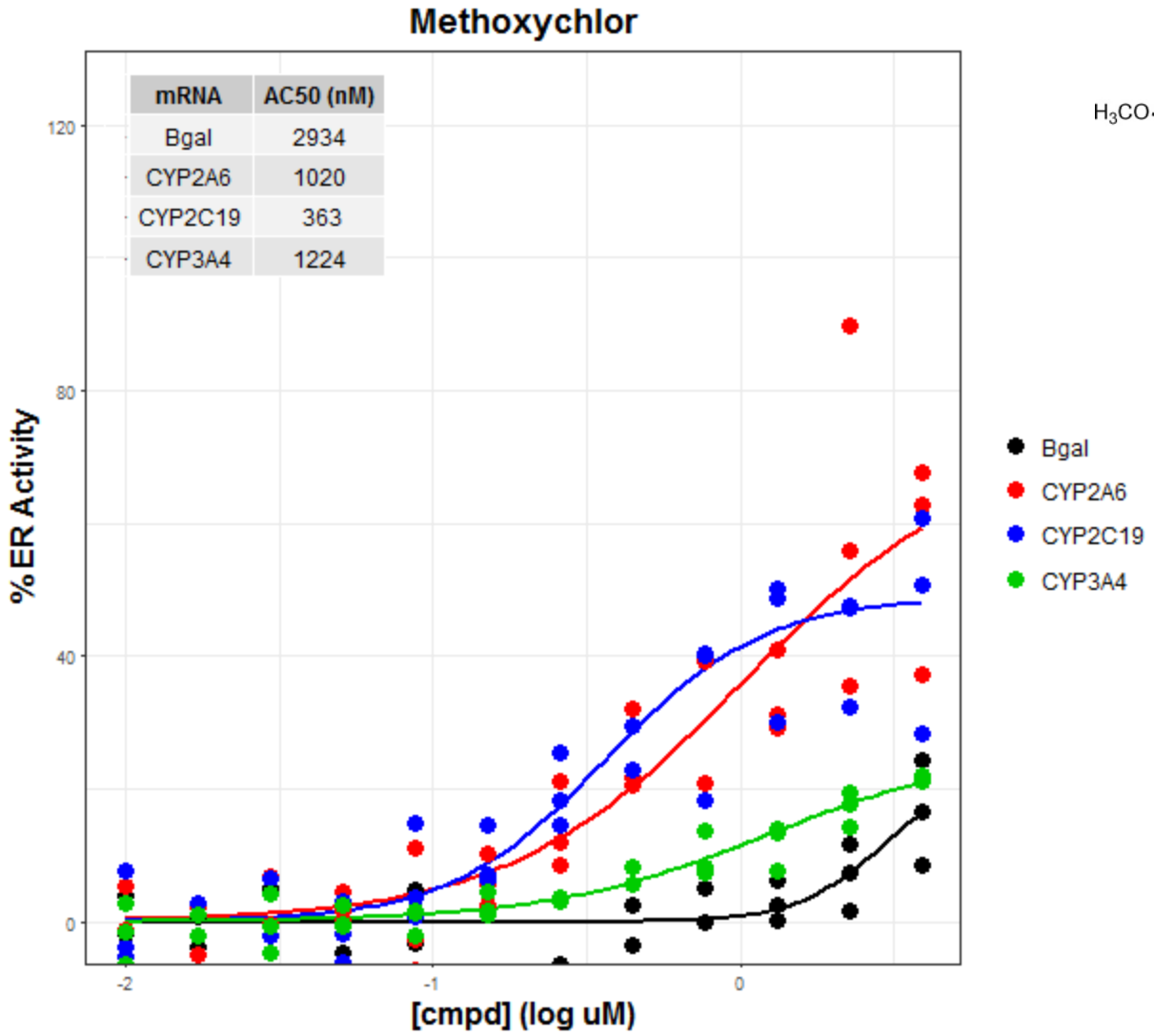
- We did not observe much detoxification with CYP expression, which is odd considering the role metabolism plays in toxicokinetics
- If we do not appreciably deplete the a toxic parent compound, we should not see detoxifications
- If the metabolites of toxic parents are also toxic, we will not see detoxifications
- While not unimportant, detoxifications are not a chief concern for screening
- Bioactivated metabolites were detected, even using when cytotoxicity as the endpoint
- Pathway-based assays (endocrine, stress responses) are likely to prove more sensitive endpoints
- Pooling mRNAs to mimic tissue metabolism dilutes the effectiveness of any single enzyme compared to using single mRNA transfections, especially for poorly expressed gene (CYP2D6, CYP2C19, CYP2J2)

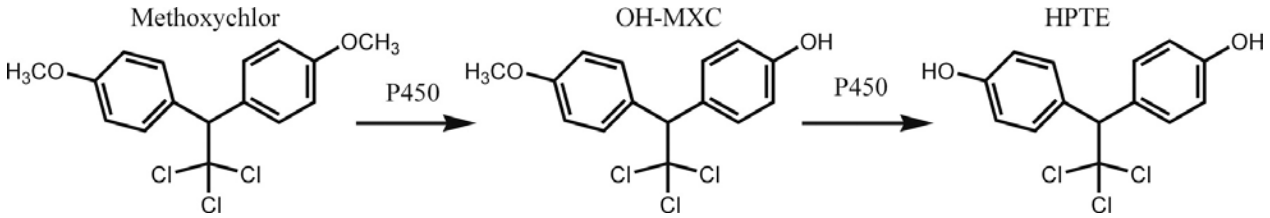
Conclusions and Future Directions

- mRNA transfection provides a method to imbue deficient cell models with robust XM activity
- mRNA mix can be tightly controlled by user in ways alternative gene delivery methods cannot
- Bypassing transcription and RNA processing gives rapid expression ideal for HTS applications in plates with low working volumes (10-80 µl) where time is critical (evaporation/edge effects)
- Very cost-effective → Less than $20 total per 384-plate ($0.05 per well) at pilot scale synthesis
- No imposition on current cell-based assay protocols: forward and reverse transfections work effectively, so transfection mix can be added to cells at seeding

- A proposed cross-partner partnership with NTP and NCATS is under consideration
- Compare mRNA transfection to direct microsome addition
- Several assays proposed to get a more complete picture of what works well
    - Cell stress/DNA damage assays- p53, ATAD5, Nrf2
    - Endocrine assays- ER, AR

Deployment to ER Transactivation Assay





- Methoxychlor (MXC) has minimal ER agonist activity
- MXC is demethylated by certain human CYP450 enzymes to HPTE: 1A2, 2A6, 2C18, 2C19 > 2B6, 2C9
- HPTE is a more potent and efficacious agonist of ER

- VM7 cells (formerly BG1) transfected with CYP-encoding mRNA or B-gal control for 6 hours (384w)
- Exposed to MXC (10nM – 5µM) for 24 hours
- Activity normalized to maximal E2-induced activity (parallel wells on same plate)
- A minimal ER response was seen in cells transfected with B-gal or CYP3A4 mRNA
- Increases in both efficacy and potency of MXC was observed in cells transfected with CYP2A6 or CYP2C19

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NCCT

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