National Institute of Environmental Health Sciences
Division of Translational Toxicology

Insights from Profiling Transcription Factor Transactivation with CYP450 Metabolism Integration

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Developing Assays for Specific Context of Use

- A "context of use" clearly describes the manner and purpose of use for a particular method or approach.
  - A specific context of use may require that a testing model replicates physiology as closely as possible to encompass all biological effects. Alternatively, the objective may be better addressed by engineering a model to control biological variables to enable better mechanistic understanding.
- In vitro assay systems provide a biological system in which mechanistic endpoints can be evaluated to better characterize how chemicals elicit toxicity.
- Immortalized cell lines are not physiologically "normal" and generally lack metabolic activity, but can be easily engineered via transfection to enable multiplexed endpoint detection and introduce metabolic capacity in a controlled manner.
- Transcription factors regulate gene expression. Chemicals can interact with transcription factors and/or upstream signaling pathways in ways that initiate or promote toxicity.
- The Attagene cis-FACTORIAL assay profiles chemical effects on 46 transcription factors to provide insight into their role in toxicity mechanisms. By assessing the activity of multiple transcription factors the assay also allows us to monitor the state of signal transduction networks and therefore to identify the effect of toxicants on general cell health (Medvedev et al. 2018, https://doi.org/10.1126/sciadv.aar4666). The new Attagene CYP-Factorial assay enables the evaluation of chemical effects on transcription factor activity with CYP-mediated Phase 1 metabolism integrated.
- In this study, we explored how introduction of select metabolic enzymes in known quantities into Attagene assays can help characterize Phase I metabolism impacts on chemical-mediated transcription factor activation. **By engineering a model for a specific context of use we demonstrate that human-relevant mechanistic insight can be gained**.

Main goals/questions evaluated in this study:

1. **CYP450 effect on transcription factor activation**
   Are there differences in response with/without CYP450 enzymes present?
2. **Insight on potential mechanistic targets**
   Which transcription factors are potential targets for chemicals or their metabolites?
3. **Defining transcription factor profiles to infer "toxicity"**
   Can comparing profiles across the panel of transcription factors yield signatures that help infer putative biological outcomes?

Cytochrome P450 Phase I metabolic enzymes in the Attagene CYP-Factorial assay

CYP1A1	CYP2A6	CYP2D6
CYP1A2	CYP2B6	CYP2E1
CYP1B1	CYP2C9	CYP3A4

Summary

Attagene cis-FACTORIAL and CYP-Factorial Assays

- These assays represent complementary systems that can be leveraged to gain mechanistic insight into characterizing chemical-mediated effects on transcription factor transactivation and the impact of Phase I metabolism.
  - The cis-FACTORIAL assay characterizes chemical effects on a panel of 46 human transcription factors.
  - The CYP-factorial assay integrates specific human CYP450 enzymes into the platform.
- Comparing results between assays can identify chemicals that have active parent and/or metabolites and differences in transcription factor transactivation.
- Both assays provide mechanistic human-relevant insight into chemical or metabolite-elicited effects on transcription factor activation.

Results from this Study

- Human Phase 1 metabolism can be introduced into these assays via CYP450 integration by transfection, as confirmed with the positive control, aflatoxin B1.
- We identified chemicals such as DBP for which CYP450 metabolism alters the profile of transcription factor transactivation, confirming different activities between parent vs. metabolite compounds.
- Profiling across all 46 transcription factors can produce toxicity signatures for chemicals.
- "Biological read-across" can identify chemicals with similar effects.
- Profiles for "toxic" vs. "non-toxic" chemicals yield insight into the biological mechanisms underlying adversity.

Study Design

- **Cell line:** HepG2 (human hepatocellular carcinoma cell line)
- **Concentration-response screening:** 24 chemicals at 4 concentrations in triplicate
- **Attagene transcription factor profiling in cis-FACTORIAL and CYP-Factorial assays**

Day 1
- Cis-factorial transfection
- **CYP450 addition (CYP-Factorial assay only)**

Day 2
- Remove growth media of Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS)
- Add assay media (DMEM with 1% CS-FBS)
- Compound addition (24-hour exposure)

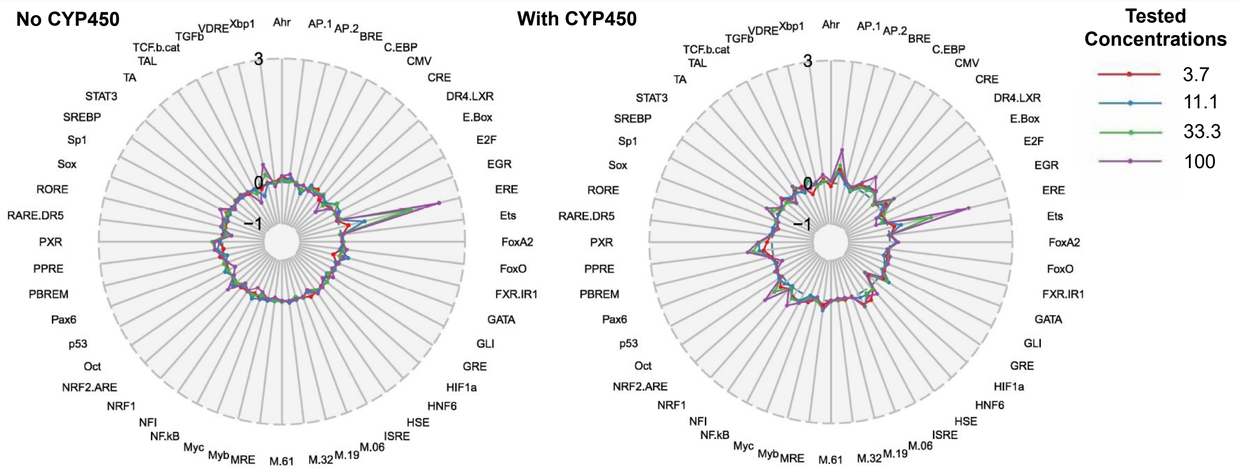
Day 3
- Cell collection

Day 3-8
- Sample processing (RNA isolation, PCR, labeling)

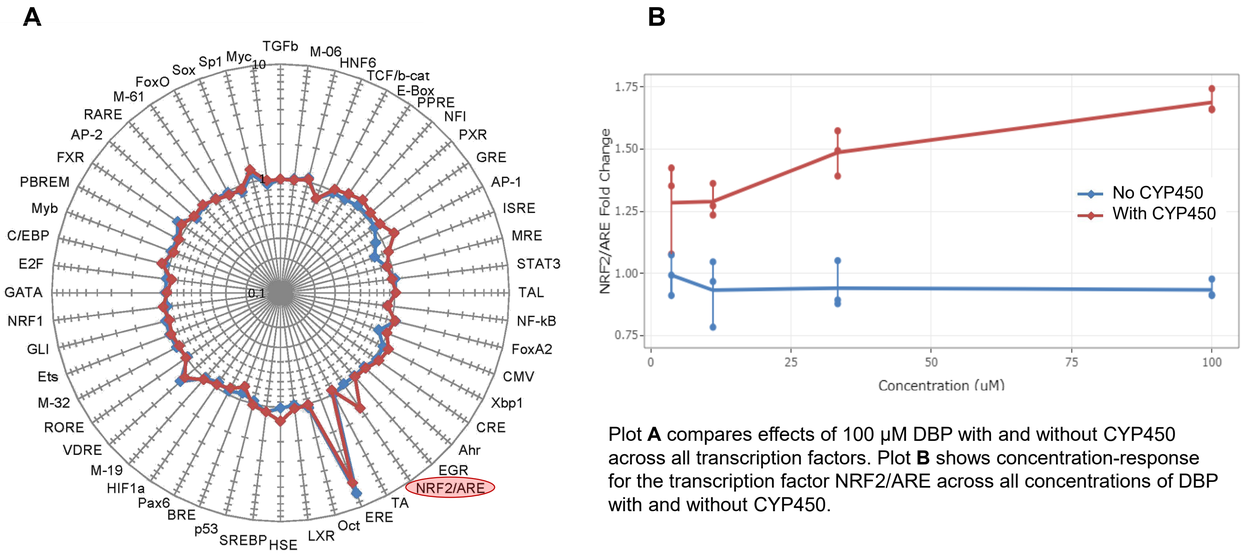
Day 9-12
- Capillary electrophoresis
- Data collection and analysis

Experimental design for the cis-FACTORIAL and the CYP-factorial assay.

Evaluation of Dibutyl Phthalate (DBP)

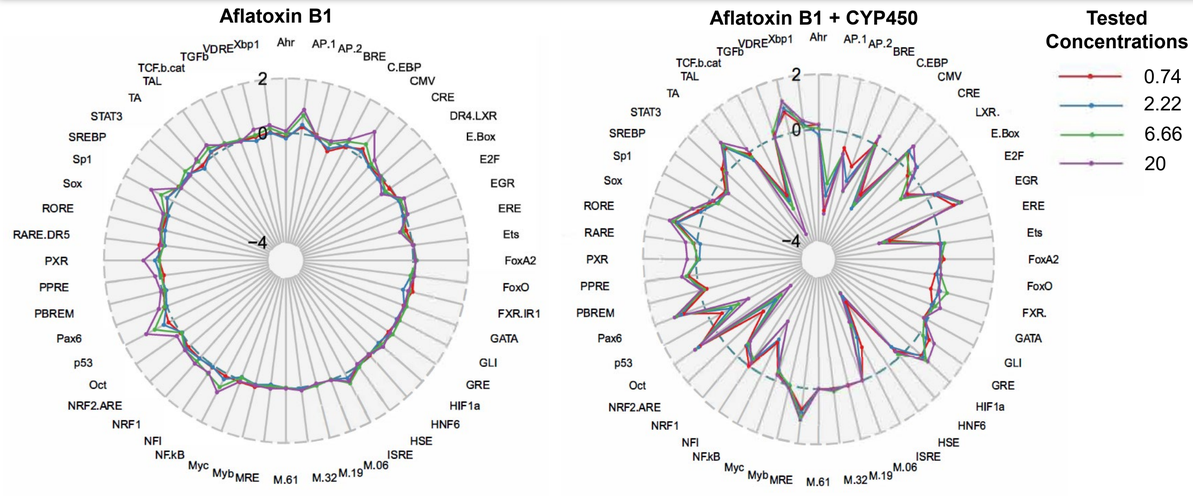


Plots illustrate the transactivation profiles of 46 transcription factors across four testing concentrations of DBP with and without CYP450.



Plot **A** compares effects of 100 µM DBP with and without CYP450 across all transcription factors. Plot **B** shows concentration-response for the transcription factor NRF2/ARE across all concentrations of DBP with and without CYP450.

Aflatoxin B1 Positive Control



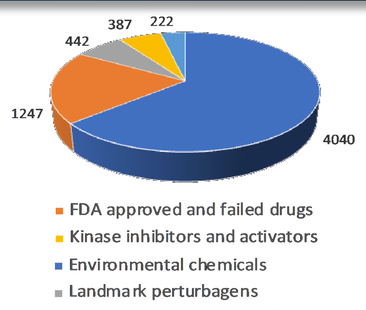
Aflatoxin B1 was used as a positive control to confirm CYP450 activity. Plots show fold-induction mean values (n=3) of four test concentrations vs. vehicle (DMSO) plotted in log2 scale, illustrating the impact of Phase I metabolism on the transcription factor activation profile.

Profiling to Support Mechanistic Interpretation

Attagene cis-FACTORIAL Database of Cell Responses

Data from a comprehensive database of transcription factor transactivation profiles generated by all samples run in these assays were compared in a "biological read-across" approach to identify chemicals that have similar effects.

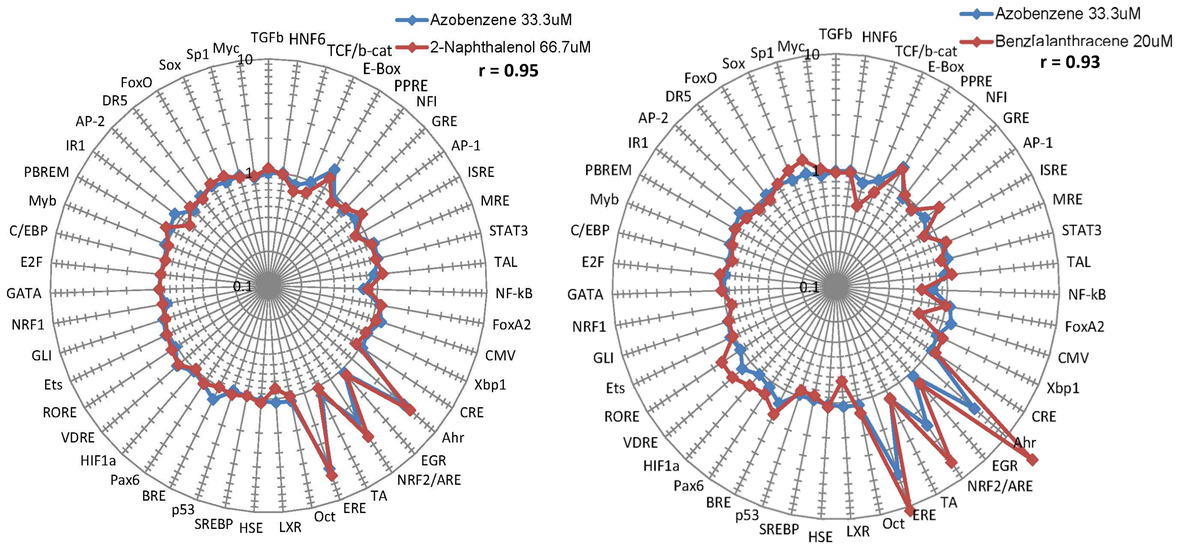
Database size: 36,824 profiles (September 2022)
Number of compounds: 6,338
Data type: Profiles of fold-induction values vs. vehicle



Substances Identified as Similar to Azobenzene Using Profile Comparisons

Test Compound	Conc	Similarity*	Test Compound	Conc	Similarity*	Test Compound	Conc	Similarity*
Azobenzene+CYPs	33.3uM	0.95	2-Naphthalenol	66.67uM	0.92	Benz[a]anthracene	200uM	0.90
2-Naphthalenol	66.7uM	0.95	Benz[a]anthracene	60uM	0.92	Cupferron	66.7uM	0.90
Benz[a]anthracene	20uM	0.93	Benz[a]anthracene	2.2uM	0.92	Benz[a]anthracene	7.4uM	0.90
Trans-Stilbene	33.3uM	0.93	Trans-alpha-Methylstilbene+CYPs	33.3uM	0.92	Benzo[a]pyrene	60uM	0.90
Benz[a]anthracene	6.7uM	0.93	3-Phenyl-2-propen-1-ol	200uM	0.91	Cyclohexylphenylketone	66.7uM	0.90
Trans-Stilbene+CYPs	33.3uM	0.92	4-Nitro-1,2-phenylenediamine	200uM	0.91	Benz[a]anthracene	22.2uM	0.90
4-Pentylphenol	22.2uM	0.92	4-Nitro-1,2-phenylenediamine	66.7uM	0.91			

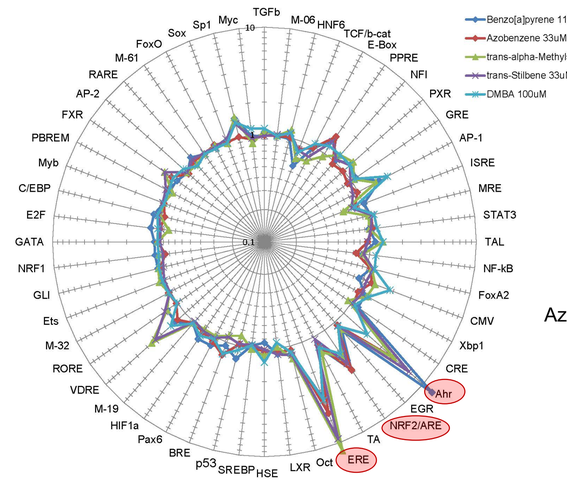
*Similarity to PAH compounds is computed using a custom algorithm developed for this database and analysis by Attagene.

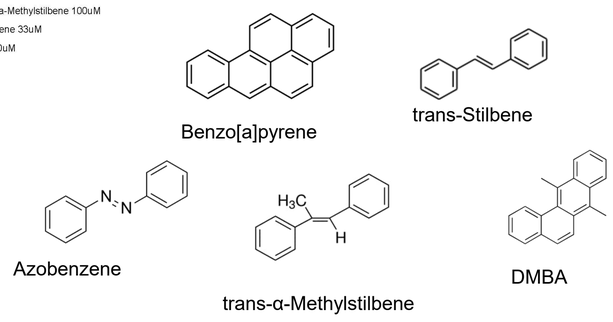


The transcription factor activation profile for 33 uM azobenzene was compared to all other profiles in the database, revealing a similarity to polyaromatic hydrocarbon (PAH) compounds. The two most similar were 2-naphthalenol and benz[a]anthracene, highlighted in the table above in blue and brown shading, respectively. Plots show the similarity to azobenzene in how these chemicals affect transcription factors.

Transcription Factors as Molecular Targets for Toxicity

Common PAH-like Cell Response Profiles Include AhR, NRF2, and ERE

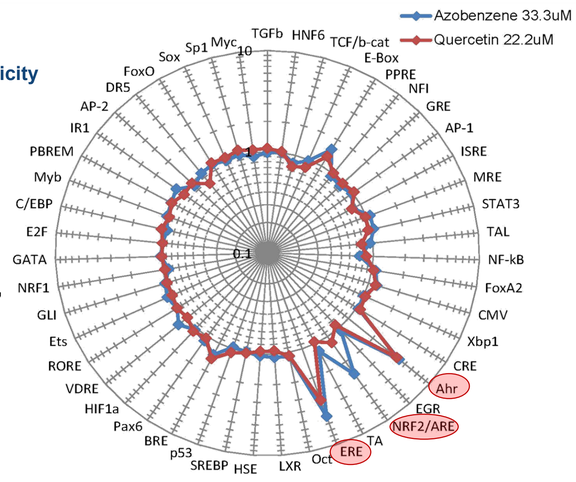




Identifying Profile Characteristics Associated with Predicting Toxicity

Azobenzene's transcription factor activation profile is similar to that of non-toxic compounds such as quercetin. The most notable difference between the non-toxic quercetin profile and the the PAH profile is that the NRF2 pathway is **not** activated by the non-toxic quercetin.

This profiling approach can identify mechanistic pathways, such as the role of NRF2 activation in the toxicity of PAH profiles, for distinguishing toxic from nontoxic profiles.



References and Acknowledgements

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