National Institute of Environmental Health Sciences
*Division of Translational Toxicology*

# Mechanistic Insights from Profiling Chemical-Mediated Transcription Factor Transactivation with the Integration of Cytochrome P450 Metabolism

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## Developing Assays for Specific Context of Use

- Context of Use: A clearly articulated description delineating the manner and purpose of use for a particular method or approach.
  - Models may try to be as close to biologically representative as possible to encompass even unknown biological effects or alternatively, models may be completely engineered to enable better mechanistic understanding by controlling all biological variables, as described herein.
- In vitro assay systems using immortalized cell lines provide a biological context in which to evaluate chemical effects on mechanistic endpoints such as transcription factor activity. Such mechanistic insight can help build confidence in characterizing how chemicals elicit toxicity.
- Immortalized cell lines are not necessarily physiologically "normal" and generally lack any metabolic activity, but they are easily engineered via transfection to enable multiplexed detection of transcription factor activity and even introduce metabolic capacity in a controlled manner. Here we present a model in which Attagene has introduced select metabolic enzymes in known quantities to 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.
- Transcription factors are key regulators of gene expression for many biological pathways that can be the molecular initiating event in chemical-mediated effects. Profiling effects on a panel of 46 transcription factors to gain mechanistic insight is the premise of the Attagene cis-FACTORIAL assay system. A new format, the Attagene CYP-Factorial assay format, enables the evaluation of chemical effects on transcription factor activity with CYP-mediated Phase 1 metabolism integrated.
- To gain better understanding of whether CYP-mediated oxidation results in an altered bioactivity profile, we have compared the results of 24 chemicals in both the cis-FACTORIAL and CYP-Factorial formats.

### Cytochrome P450 Phase I Metabolic Enzymes in the Attagene CYP-Factorial Assay Format

| | | |
|---|---|---|
| CYP1A1 | CYP2A6 | CYP2D6 |
| CYP1A | CYP2B6 | CYP2E1 |
| CYP1B1 | CYP2C9 | CYP3A4 |

## Study Design

- **Cell Line:** HepG2
- **Concentration-Response Screening:** 24 chemicals at 4 concentrations in triplicate
- **Attagene transcription factor profiling in cis-FACTORIAL and CYP-Factorial formats**

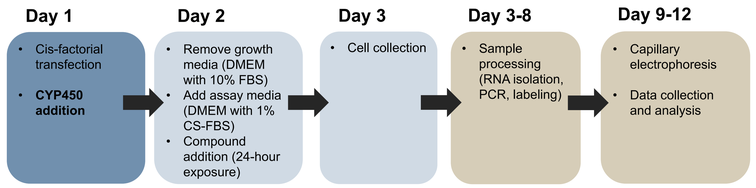



**Figure 1:** Experimental design for the cis-FACTORIAL and the CYP-factorial assay formats. When CYP450 are included in the system, they are added at the same time as the transcription factor reporter gene transfection on day 1.

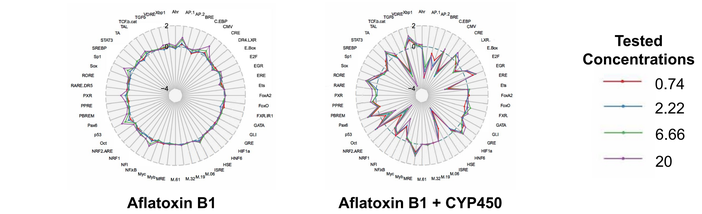



**Figure 2:** Aflatoxin B1 positive control confirmation of CYP450 activity and impact on transcription factor activation profile. Radial graphs show fold-induction mean values (n=3) of aflatoxin B1 at four tested concentrations vs. vehicle (DMSO) plotted in log2 scale.

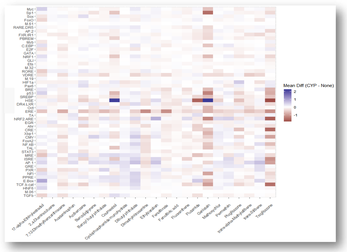
## Questions Evaluated in this Study

1. **Effect on transcription factor activation with CYP450 inclusion**
   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. **Leveraging the transcription factor profile to infer "toxicity" outcome**
   Profiling across the panel of transcription factors yields signatures that can be compared among reference chemicals to infer putative biological outcomes.

## Impact of CYP450 Integration

### Effects on Transcription Factor Activity

**Figure 3:** Heatmap shows the maximum fold-change difference between the maximum response per transcription factor at the highest testing concentration for 24 chemicals in this study in the presence or absence of nine cytochrome P450 enzymes. Colored blocks indicate a measurable difference between fold change with and without CYP450 metabolism (calculated by CYP450 – noCYP450). White indicates no difference between the two modes (suggesting parent compound and metabolites have similar effects or no effects), blue reflects more activation with CYP450 metabolism present (suggesting metabolites are eliciting more transactivation of the transcription factor), and red indicates more activation without CYP450 metabolism (suggesting parent compound is more active).




### Characterizing Effects of Dibutyl Phthalate (DBP) on Transcription Factor Targets

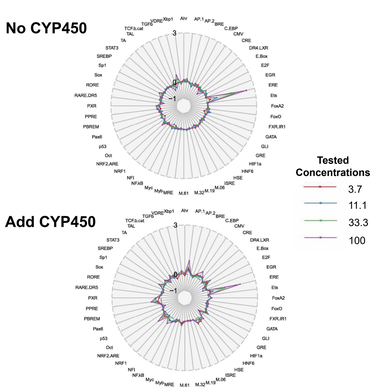



**Figure 4:** Characterizing transactivation of 46 transcription factors across four testing concentrations of DBP in both assay modes.

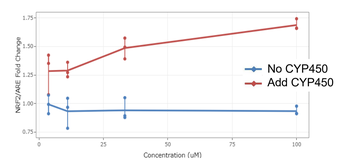


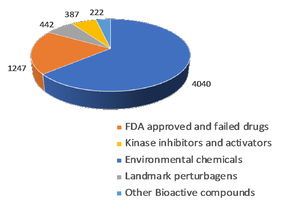

**Figure 5:** Comparing 100 µM DBP with and without CYP450 across all transcription factors to facilitate direct comparison. Concentration-response for NRF2/ARE.

## Profiling to Support Mechanistic Interpretation

### Attagene cis-FACTORIAL Database of Cell Responses

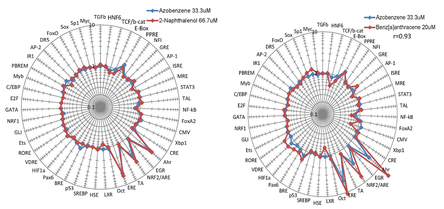
Using a comprehensive database of profiles (transactivation of 46 transcription factors) generated by all samples run in these assays, "biological read-across" can be done to compare profiles of transcription factor activation and identify which chemicals have similar effects.

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




### Using Profile Comparisons to Gain Insight on Toxicity

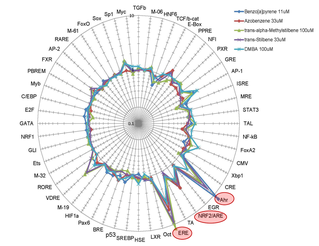
**Figure 6:** The transcription factor activation profile for azobenzene was compared to all other profiles in the database, revealing a similarity to polyaromatic hydrocarbon (PAH) compounds, namely benz[a]anthracene. The profile for 33 uM azobenzene was used as input for similarity searching against individual testing concentrations for all other chemicals in the database.

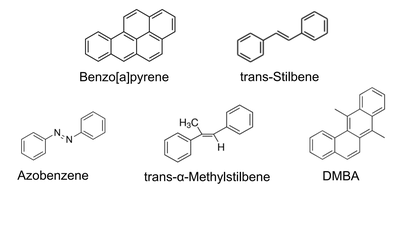



| Sample ID | Conc | Similarity |
|---|---|---|
| Azobenzene+CYPs | 33.3uM | 0.95 |
| 2-Naphthalenol | 66.7uM | 0.95 |
| Benz[a]anthracene | 20uM | 0.93 |
| trans-Stilbene | 33.3uM | 0.93 |
| Benz[a]anthracene | 6.7uM | 0.93 |
| trans-Stilbene+CYPs | 33.3uM | 0.92 |
| 4-Pentylphenol | 22.2uM | 0.92 |
| 2-Naphthalenol | 66.667 | 0.92 |
| Benz[a]anthracene | 60uM | 0.92 |
| Benz[a]anthracene | 2.2uM | 0.92 |
| Trans-alpha-Methylstilbene+CYPs | 33.3uM | 0.92 |
| 3-Phenyl-2-propen-1-ol | 200uM | 0.91 |
| 4-Nitro-1,2-phenylenediamine | 200uM | 0.91 |
| 4-Nitro-1,2-phenylenediamine | 66.7uM | 0.91 |
| Benz(a)anthracene | 200uM | 0.90 |
| Cupferron | 66.7uM | 0.90 |
| Benz(a)anthracene | 7.4uM | 0.90 |
| Benzo(a)pyrene | 60uM | 0.90 |
| Cyclohexylphenylketone | 66.7uM | 0.90 |
| Benz(a)anthracene | 22.2uM | 0.90 |

## Transcription Factors as Molecular Targets for Toxicity

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



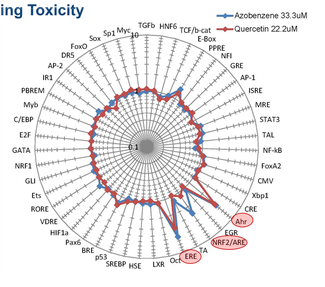




### Identifying Profile Characteristics Associated with Predicting Toxicity

Azobenzene's transcription factor activation profile also results in high similarity to non-toxic compounds such as quercetin.

The most notable difference between the non-toxic quercetin profile vs. the PAH profile is that the NRF2 pathway is **not** activated despite both ERE and AhR being activated.

**This profiling approach can identify mechanistic hypotheses for distinguishing toxic vs. non-toxic profile such as the role of NRF2 activation in the toxicity of PAH profiles.**




## Summary

**The Attagene cis-FACTORIAL and CYP-factorial assays are complementary assay platforms that can be leveraged to gain mechanistic insight into characterizing chemical-elicited effects on transcription factor transactivation and the impact of Phase I metabolism.**

- The cis-FACTORIAL assay characterizes chemical effects on a panel of 46 transcription factors, and the CYP-factorial format integrates specific human CYP450 enzymes into this human cell-based assay.
- Comparison of results between assay modes (with and without CYP450 enzymes) can identify chemicals that have active parent and/or metabolites and differences in transcription factor transactivation.
- Both modes of this assay provide mechanistic, specific, human-relevant insight into chemical (or metabolite)-elicited effects on transcription factor activation, whether evaluating transcription factors one at a time or based on profiles.

### Results from this study

- CYP450 integration by transfection successfully introduces human Phase I metabolism in the system, as confirmed with the positive control, aflatoxin B1.
- We identified chemicals (e.g., DBP) for which CYP450 metabolism alters the profile of transcription factor transactivation, confirming different activities between parent vs. metabolite compounds.
- By profiling across all 46 transcription factors, patterns for toxicity are evident and can be compared.
- "Biological read-across" can identify chemicals with similar effects to classify effect patterns.
- Profiles for "toxic" vs. "non-toxic" chemicals yield insight into the biological mechanisms underlying adversity.

## Acknowledgements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