Evaluation of an *In Vitro* Multi-Receptor and Multi-Species Assay for Potential Endocrine Disruptor Targets

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OVERVIEW

- A multiplexed, multi-receptor transactivation assay was developed to target nuclear receptors from diverse ecological species.
- Screening a targeted library showed high correlation between receptor sequence similarity and high potency chemical response.

INTRODUCTION

Screening technologies have been developed to identify xenobiotic chemicals that bind nuclear receptors and thus have potential for adverse health effects through disruption of endocrine function. However, the focus has been on human receptors despite environmental exposure to a huge diversity of other species. We evaluated a multiplexed transactivation assay providing the ability to screen for effects across multiple species and receptors.

METHODS

Assay: The Attagene trans Factorial! Assay[1], which targeted the human nuclear receptor family using a multiplexed mammalian one-hybrid platform, was adapted to additional species by swapping nuclear receptor ligand binding domains. Each species/receptor had a unique reporter gene (RTU) which generates an RNA transcript with a Hpa I restriction site at a specific location. Reporter genes and GAL4-DNA-binding domain/NR ligand-binding domain fusion proteins constructs were cotransfected in to HepG2 cells and pools of cells containing all receptors generated and plated for screening. Pooled cells were treated for 24 hr with test chemicals in concentration-response format followed by RNA isolation, cDNA generation, restriction digest (Hpa I) and quantitation by gel electrophoresis.

Chemical Library: Compounds were selected from the ToxCast chemical library based on observed activity from ToxCast testing against the human forms of the receptors in the trans Factorial! Assay. Estrogen (ER) and androgen receptor (AR) agonists and antagonists; along with thyroid receptor (TR), peroxisome proliferator activated-receptor gamma (PPARγ), and pregnane X receptor (PXR) agonists were included.

Data Analysis: Response values were normalized by converting to fold change over solvent (DMSO) controls. Values were run through the ToxCast tcpl pipeline to fit to a constant, Hill or Gain-Loss model and the model with the lowest Akaike information criterion value is selected as the 'winning' model[2]. Hit criteria required at least a two-fold change in activity.

Sequence Comparison: Sequence similarity between species was calculated with the SeqAPASS tool[3] or directly through NCBI BLAST[4].

References:
1. Martin et al., (2010) Chem. Res. Toxicol. 23:578-90.
2. Filer et al. (2017) Bioinformatics 33 (4): 618-620.
3. LaLone, C. A., et al. (2016). Tox. Sci 153(2): 228-245.
4. https://blast.ncbi.nlm.nih.gov/Blast.cgi

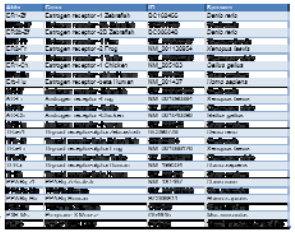

Table 1. Receptors & Species

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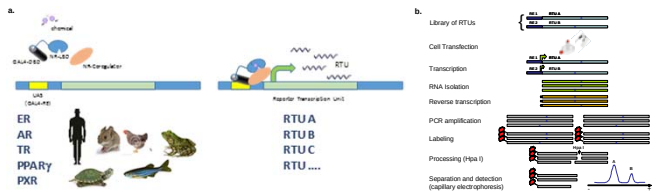

Fig 1a. The ligand-binding domains of nuclear receptors shown in Table 1 were cloned into a mammalian one-hybrid system. Receptors were co-transfected with unique RTU reporter genes in to HepG2 cells, treated with chemicals for 24 hr, and reporter expression quantitated as shown in **b)**.

RESULTS

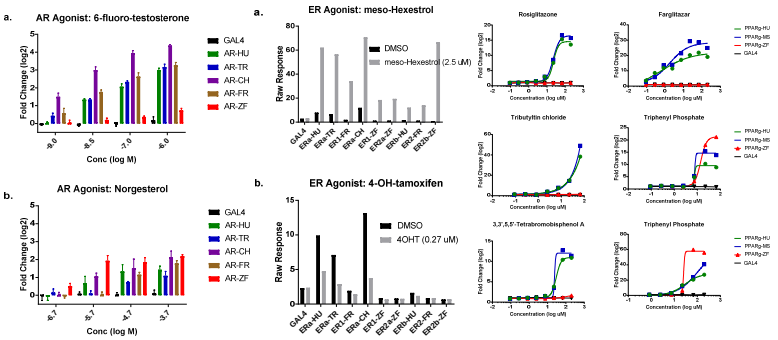

Fig. 2 AR Response. a) Initial testing included 2 nM 6-fluoro-testosterone in order to allow AR antagonist detection but zebrafish was found to be weakly responsive. **b)** Norgesterol was shown to stimulate zebrafish AR and subsequent assays included both norgestrel and 6FT.

Fig. 3 ER Response. a) All ER's responded to meso-Hexestrol as an agonist. **b)** 4OH-tamoxifen reduced basal ER activity for some but not all of the ER's permitting detection of ER antagonists without added agonist.

Fig. 4 PPARγ Response. Human and mouse receptors responded as expected to TZD's and an organotin; however the zebrafish receptor was unresponsive. All 3 were strongly activated by the flame retardant triphenyl phosphate.

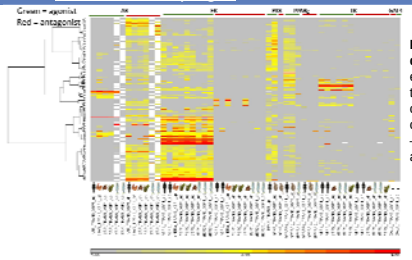

Fig. 5. Hierarchical clustering of all data. 189 chemicals selected for enrichment of receptor ligands were tested in concentration response. Hit calls were made and AC50 values calculated for actives and plotted as –logM values. Gray indicates negative activity call. White indicates no data.

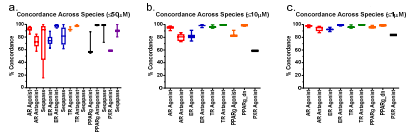

Fig. 6. Concordance across species was calculated by % agreement (hit or inactive) at the designated potency cutoff **a)** 50 μM, **b)** 10 μM and **c)** 1μM.

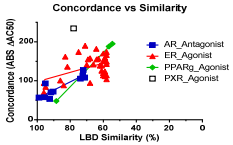

Fig. 7. Amino acid sequence similarity between species was calculated and compared to Concordance Value calculated by summing the absolute difference of AC50 values for each chemical.

CONCLUSIONS

- All receptors responded to reference chemicals indicating the human host cell was competent for NRs from diverse species
- High potency compounds were similar across species; less so for lower potency compounds
- Distinct differences found for some potent compounds, particularly for zebrafish
- LBD similarity correlates with compound sensitivity
- Flexible platform readily adaptable to screen multiple receptors/species of interest

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