Update on endocrine work at the US EPA

Office of Research and Development
Center for Computational Toxicology and Exposure

June 17, 2021

Presentation to the Endocrine Policy Forum

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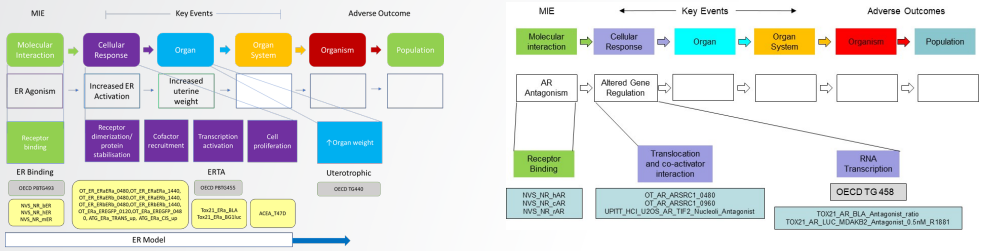
The views expressed in this presentation are those of the authors and do not necessarily reflect the views or policies of the U.S. EPA

Outline

- Human Health
  - ER/AR
    - *in vitro* models – subset analysis
    - Retrofit of ER assays for metabolic competence
  - Steroidogenesis
    - Androgen steroidogenesis (5α-reductase) *in vitro* assay
    - High throughput steroidogenesis *in vitro* assay evaluation and QSAR development
  - Thyroid
    - Thyrotropin-releasing hormone (TRH) receptor (TRHR) *in vitro* assay
    - 3D thyroid microtissue *in vitro* assay
- Ecology Focused
  - ED-related Adverse Outcome Pathway development
  - Thyroid focused *in vivo, in vitro, in silico* research
  - Computational & Experimental cross-species analyses of ED-related targets

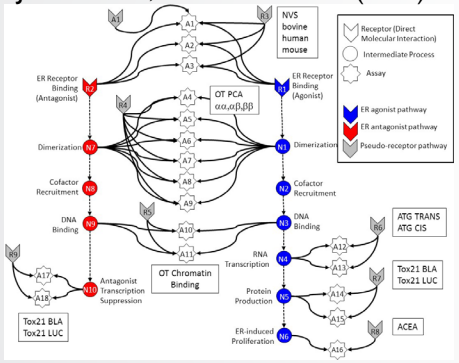
Estrogen and Androgen Receptor In Vitro Models & Subsets

Full Models



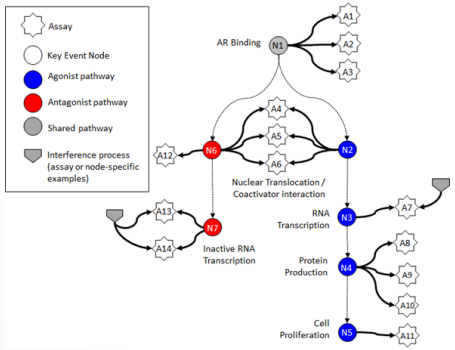
Estrogen Receptor Computational Model

Judson et al., Envi Health Pers (2015)



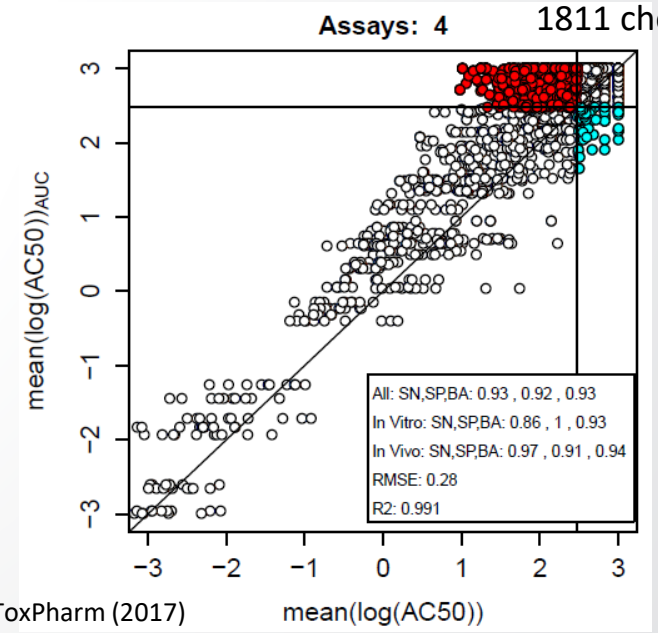
Androgen Receptor Computational Model

Kleinstreuer et al., Chem Res Toxicol (2017)



- **<u>Subset Models</u>** were developed using fewer assays and achieving similar accuracy
- Trained using results from full models and reference chemicals (in vitro and in vivo)
- Simple arithmetic replaces the original complex models
- Subsets of as few as 4 (ER agonist) or 5 (AR antagonist) assays are accurate to within uncertainty of the original model
- Chemicals that are misclassified are mostly “inactive” or “very weak”, and are ones that current tests may misclassify

1811 chemicals



- <u>Background</u>: AOPs were developed. In vitro assays were aligned to events. Computational model was developed as no single in vitro assay can accurately predict activity against ER or AR due (assay interference and noise)
- Original ER and AR computational models used large numbers of assays (18 and 11, respectively) with different cell types, assay readouts

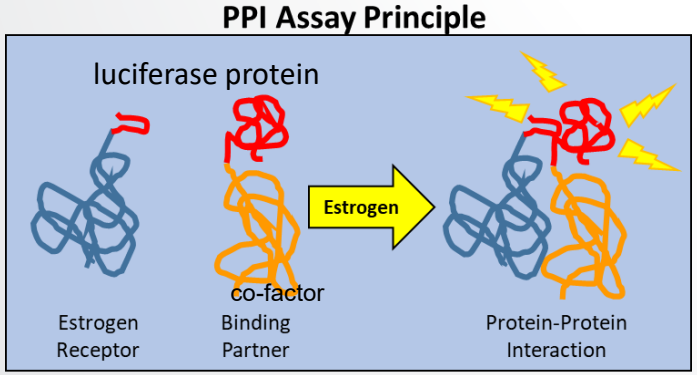
ER Subsets: Judson et al. RegToxPharm (2017)
AR Subsets: Judson et al. RegToxPharm (2020)

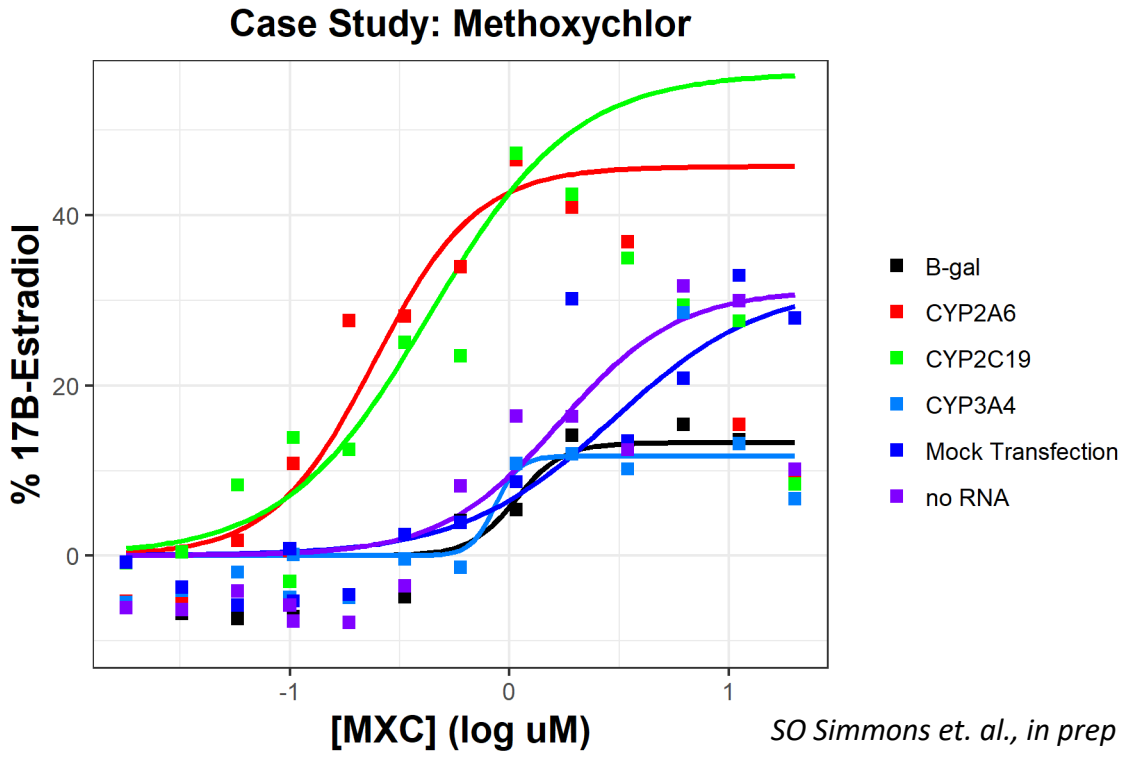
Retrofit of ER Assay for Metabolic Competence
- mRNA method

- Background: Retrofitting in vitro assays to add a component of xenobiotic metabolism into the assay

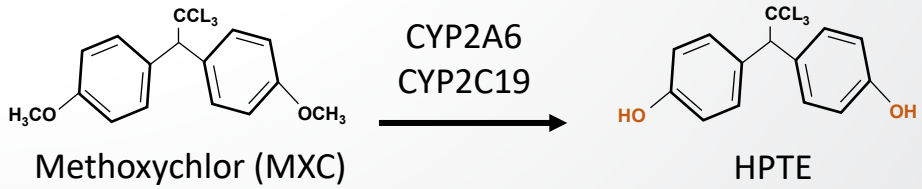
- **Concept:** Key metabolic enzyme mRNAs are introduced directly into cells *(DE DeGroot et al J Pharmacol Toxicol Methods 2018)*
  - Rapid and prolonged expression of 10 CYP metabolizing enzymes ≥ 48 hours
  - User-defined composition and ratios using multiple input mRNAs to model metabolism in multiple target tissues
  - Cost effective at $0.08 per sample in 384-well format

- **Application to ER:**
  - Retrofit novel ER co-factor recruitment assay
  - Methoxychlor with weak ER activity can be metabolized to increase ER activation after CYP expression





SO Simmons et. al., in prep



Methoxychlor (MXC) → HPTE

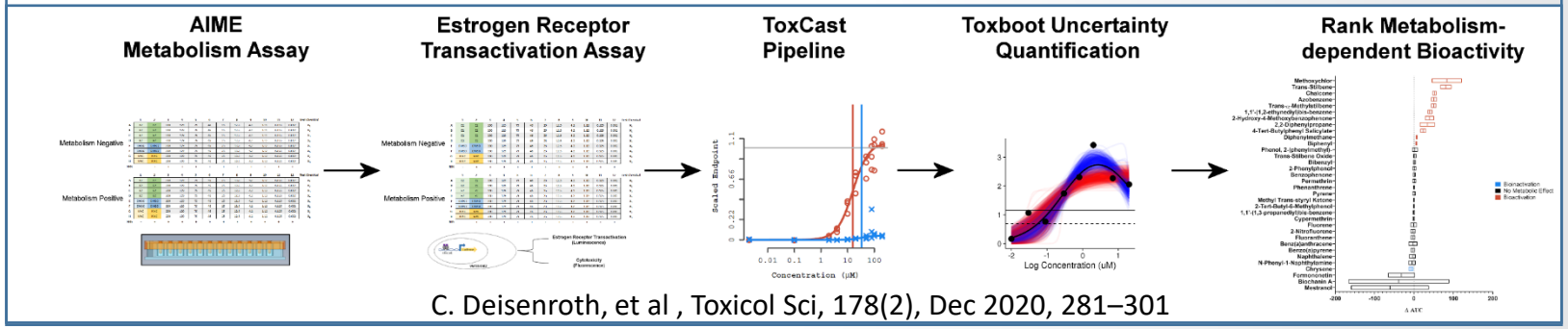
Retrofit of ER Assay for Metabolic Competence
- Alginate Immobilization of Metabolic Enzymes (AIME)

- Background: Retrofitting in vitro assays to add a component of liver metabolism into the assay – 96 well

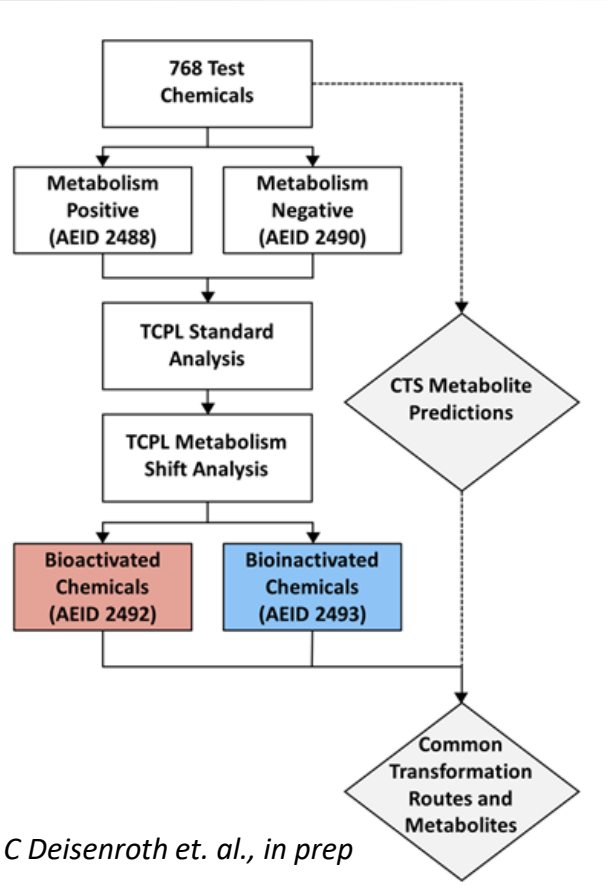
Alginate Immobilization of Metabolic Enzymes (AIME) platform encapsulates hepatic S9 fractions + custom made plate lid

Proof of Concept ER transactivation assay

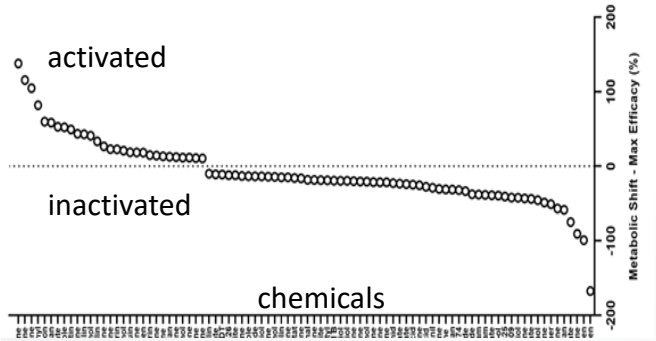
- Reprioritization of hazard based on metabolism-dependent bioactivity
- 15 reference & 48 test compounds that yield metabolites previously identified as ER +/-
- Demonstration of utility for identification of false positive and false negative target assay effects.
- Enhanced *in vivo* concordance with the rodent uterotrophic bioassay



C. Deisenroth, et al , Toxicol Sci, 178(2), Dec 2020, 281–301



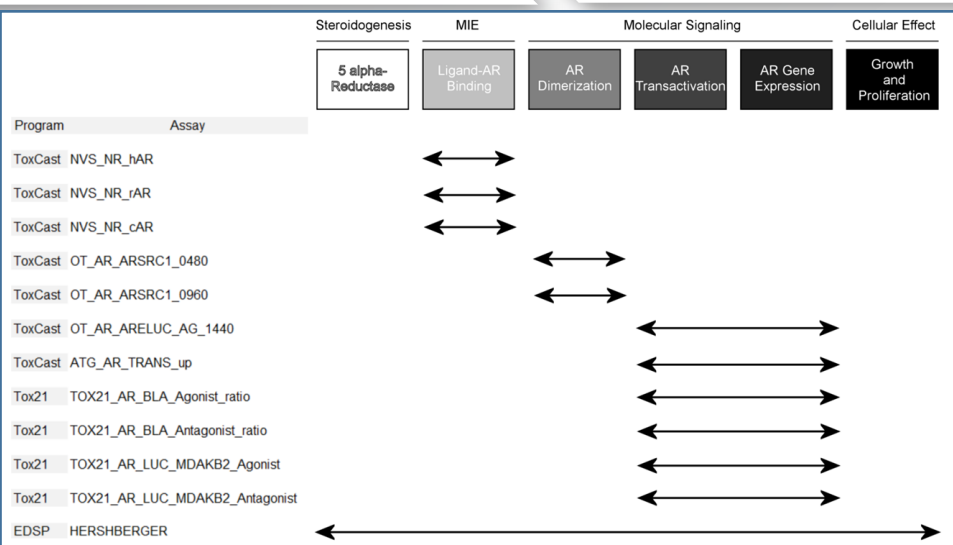
C Deisenroth et. al., in prep



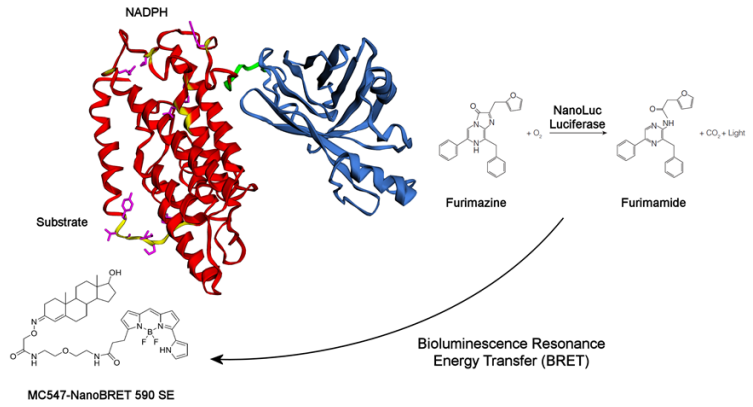
Current study (unpublished)

- 768 chemicals screened for metabolism-dependent ER effects
- Potency shift vs inactive/active
- Integration of predicted routes of biotransformation and potential metabolites

Development and Application of a 5α-Reductase High-Throughput Screening Assay for Androgen Steroidogenesis

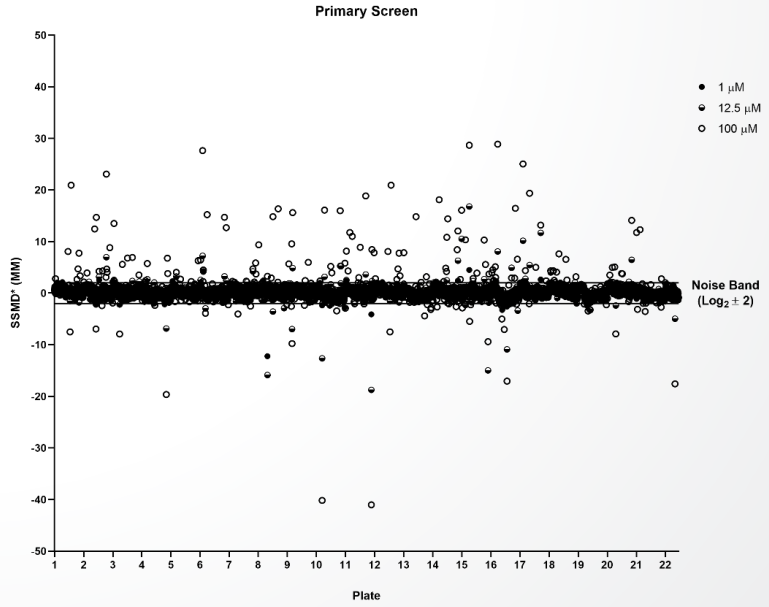


Data gaps for a comprehensive *in vitro* AR assay battery include 5α-reductase function



Developed a custom 5α-reductase NanoBRET assay

- **Problem:** Androgen steroidogenesis (Testosterone to Dihydrotestosterone) represents an *in vitro* testing gap relative to *in vivo* testing
- **Assay Development:** Custom cell-based NanoBRET assay demonstrates selectivity for 5$^{\alpha}$-reductase substrates and inhibitors
- **Screening:** Evaluation of ToxCast chemical library identifies putative inhibitors of enzyme activity
- **Limitations:** High specificity, low sensitivity & dynamic range



Screened 1803 ToxCast and E1K Chemicals

C Deisenroth et. al., in prep

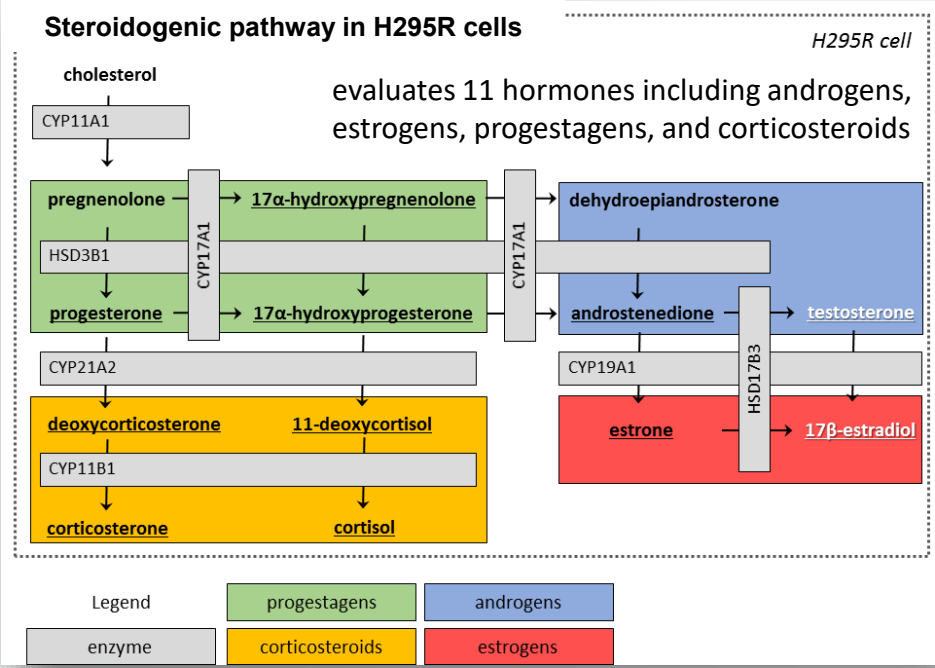
ToxCast HT-HT295R Assay and Model: Evolution of Steroidogenesis Testing for Potential Regulatory Applications

Assay development
24 to 96 well format
(Karmaus et al., 2016)

Model and comparison to OECD validation study results
(Haggard et al., 2018)

Further evaluation of the model (reproducibility, stability)
(Haggard et al., 2019)

QSAR approaches for HT-H295R bioactivity prediction
(Foster et al., in prep)



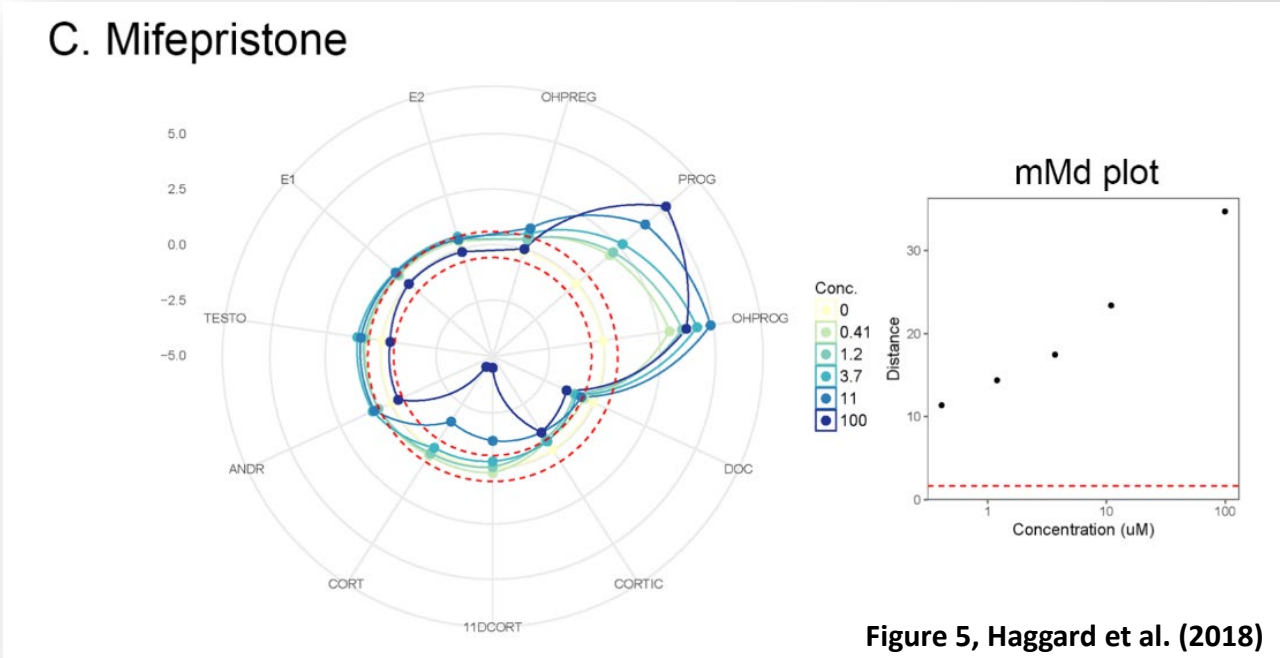


Figure 5, Haggard et al. (2018)

HT-H295R assay implementation in ToxCast, and the model (using Mahalanobis distance), with comparison to OECD H295R assay validation study, were presented to a FIFRA SAP in November 2017.
https://www.regulations.gov/docket/EPA-HQ-OPP-2017-0214

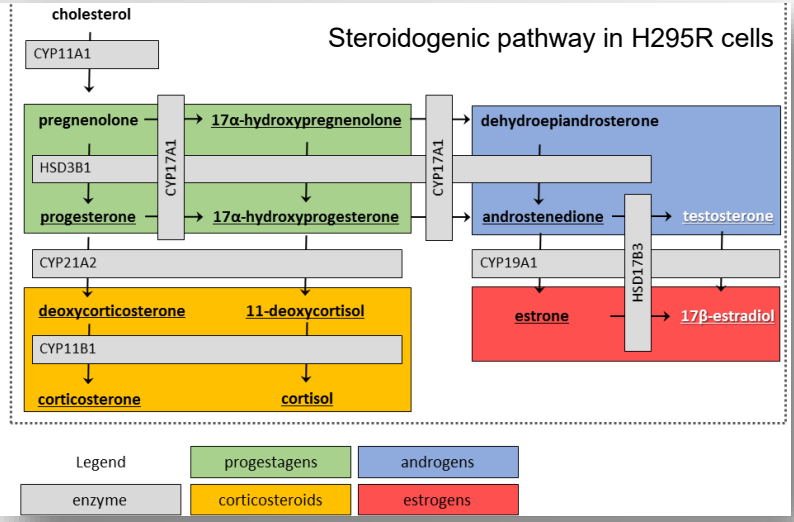
Research Status

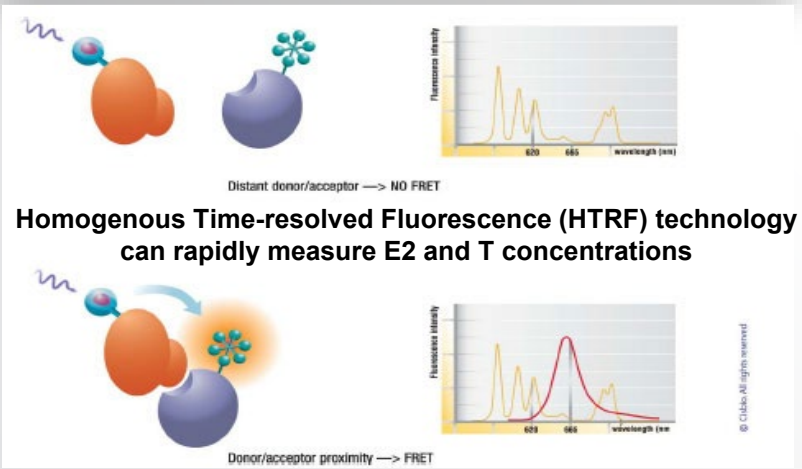
Manuscript in preparation on how to apply structure-activity relationships, including a machine learning approach and nearest neighbor approaches, to predict HT-H295R bioactivity for the rest of the EDSP Universe of chemicals
EPA Lead: Katie Paul Friedman

Increasing High Throughput of H295R Steroidogenesis Screening

Original Assay
(Karmaus et al., 2016)

Adapt the H295R Cell Line to 384-well HTS using Homogenous Time Resolved Fluorescence (HTRF) Technology to evaluate E2 and T endpoints. (*C Deisenroth* et al., in prep)

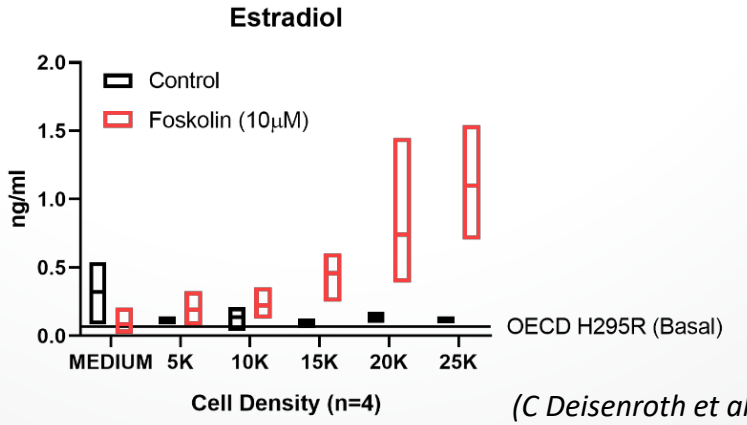


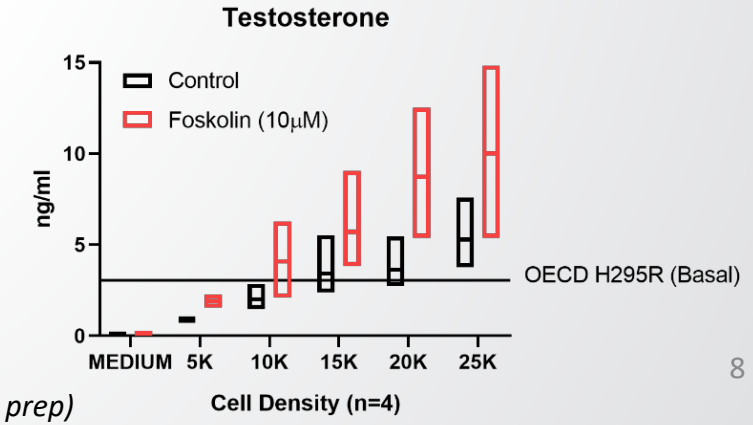


- **OCSPP 890.1550/OECD TG 456:** H295R steroidogenesis assay evaluates 17β-estradiol (E2) and testosterone (T) synthesis
- **Limitations:** Most ToxCast chemicals screened at single concentration, analytical methods are expensive and require significant expertise

Developing a 384-well format:

- Using commercial reagents and performing experiments in-house
- Assay is cost effect to preform full concentration response in replicate
- Initial basal and induced analyte levels in 384-well format are consistent with the guideline study parameters



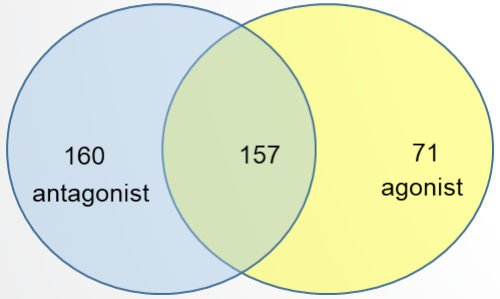


(C Deisenroth et al., in prep)

Thyrotropin-Releasing Hormone (TRH) Receptor (TRHR)
- Evaluating results from existing TOX21 assay endpoints using chemistry

- Hits from the primary screen need to be confirmed or evaluated. View these hits as putative until additional confirmation can be developed.
- Potential interference: auto-fluorescence, nonspecific Ca2+ interference, nonspecific GPCR activity, etc.

Aeid	Assay endpoint name
2364	TOX21_TRHR_HEK293_Agonist
2365	TOX21_TRHR_HEK293_Antagonist



388 Total Hits

Analysis based on reference dataset

Generation of 3D conformations

Molecular alignment of binders

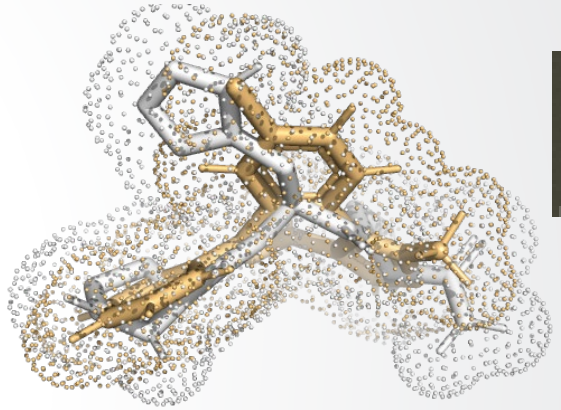
Generation & validation of pharmacophore models

Apply to all tested chemicals

Querying structure database

Analysis of model results

Uses MOE software to analyze possible 3D binding



Based on conformation of the TRHR when bound to TRH or benzodiazepines (known ligand)

Unpublished; adapted from Shobair et al., in prep

- Ongoing work to contextualize bioactivity results using chemistry: 2D chemotype and 3D molecular docking approaches will be applied to prioritize substances for any additional screening.

Research Status

Manuscript preparation (Shobair *et al.*) of the in silico approaches (2D and 3D) to evaluate TRHR screening results is ongoing and led by Ann Richard

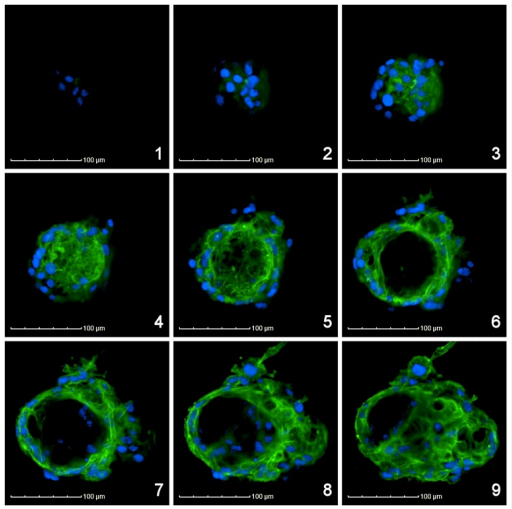
EPA Contacts: Ann Richard and Katie Paul Friedman

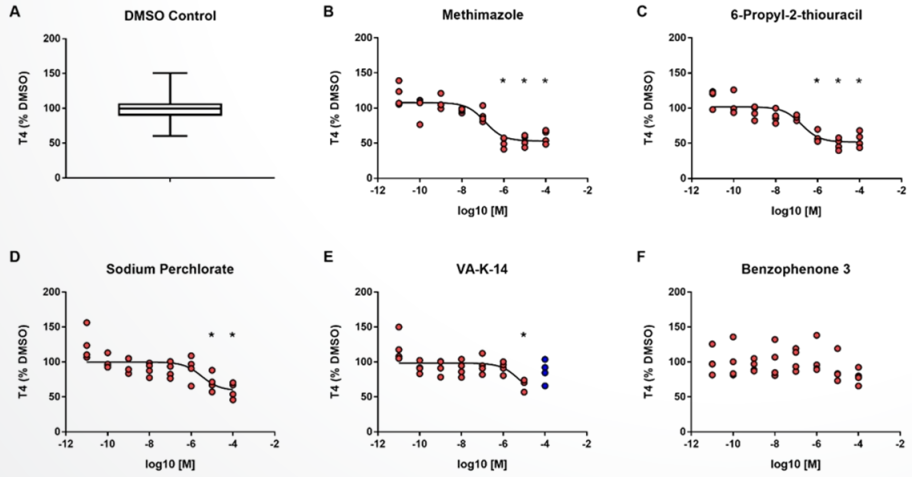
Developing Thyroid Organotypic Culture Model to Identify Thyroid Hormone Disruption

EPA POC: Chad Deisenroth

- **Problem**: Thyroid HTS assays do not directly measure thyroid hormone disruption. Need 3D architecture to simulate follicle structure and produce thyroid hormone. Primary cells have limited life span.

- Developed a human thyroid microtissue assay to evaluate chemical effects on thyroid hormone synthesis and secretion
- Developed and characterized novel human immortalized thyrocyte cell lines
- Work ongoing to screen chemicals prioritized from thyroid HTS assays

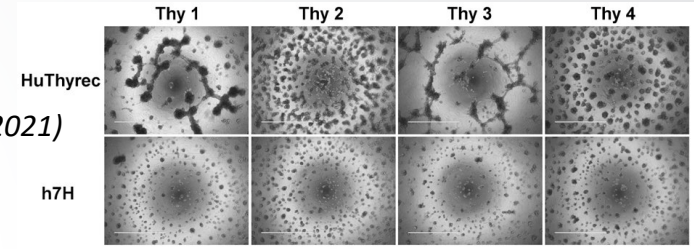


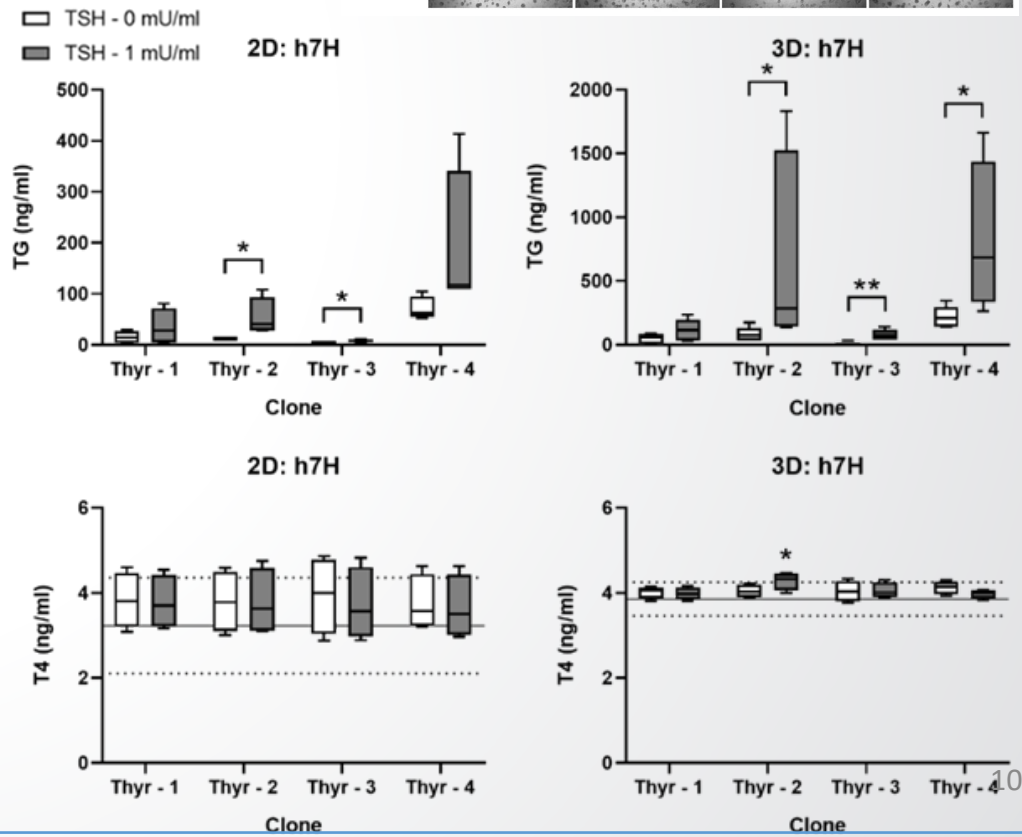


(C Deisenroth et al., Toxicol Sci 2019)

Thyroid follicular epithelial cell lines

(K Hopperstad et al., Applied In Vitro Toxicol 2021)





Ecology Focus

Endocrine-Related AOP Development (ecological)

EPA POC: Dan Villeneuve, Gerald Ankley

Goal is in developing AOPs to translate NAMs to reproductive effects (ER, AR, aromatase inhibition) and developmental effects (thyroid)

	Completed	On-going
Estrogen	• Putative AOP proposed by Ankley et al. 2010 [13] • Putative AOP 29 created in wiki – no WoE assembly	• Formal AOP **linking ER agonism to reproductive dysfunction in fish** including weight of evidence assembly (replacing AOP 29) • Evaluation of in vivo ER-mediated activity for 3 data poor PFAS identified as ER active through in vitro HTS.
Androgen	• AOP 23 linking AR agonism to reproductive dysfunction in repeat-spawning fish reviewed and endorsed by OECD WPHA/WNT • AOP network based assessment of AR agonism + arom. Inhib. [2]	• AOP 376 **linking AR agonism to male-biased sex ratio**, under preparation for review in fall 2021 • **Multi-dimensional population model** linking male-biased sex ratio to population impacts (Miller et al. 2021 – submitted)
Steroido-genesis	• AOP 25 **linking aromatase inhibition to reproductive dysfunction** endorsed by OECD WPHA/WNT • Quantitative models/understanding for AOP 25. [10] • Use of in vitro sensitivity to adjust quantitative models/relationships for cross-species applications [6,9]. • Evaluate AOP 25 relevance for fishes with synchronous oocyte development [1].	• Evaluation of quantitative AOP 25 for predicting short-term and reproductive in vivo outcomes in fish. • Updates to AOP 25 incorporating additional weight of evidence and quantitative understanding. • AOP 346 **linking aromatase inhibition to male-biased sex ratio**, in preparation for technical review in fall 2021
Thyroid	• Collaborated with University of Antwerp on **AOPs linking TPO or DIO inhibition to impaired swim bladder inflation in fish** (AOPs 155-159). [4,7,8,11,12] • AOP network-based tiered testing strategy for assessment of thyroid disruption using fish models. [3]	• AOPs 155-159 under OECD-EAGMST technical review • Coaching development of AOP 271 linking TPO inhibition to reproductive impairment in fish. • Providing AOP training and guidance to European Cluster to Improve Identification of EDs (EURION)

Integrated Thyroid Ecological Toxicity Research

EPA POC: Michael Hornung, Jon Haselman, Sigmund Degitz

Adverse Outcome Pathway

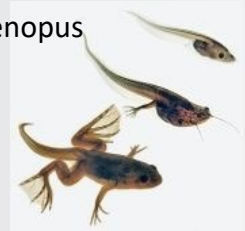
In vivo bioassays

Identified MIEs

High-Throughput Toxicology

In vitro assays

xenopus



Metamorphosis is thyroid mediated

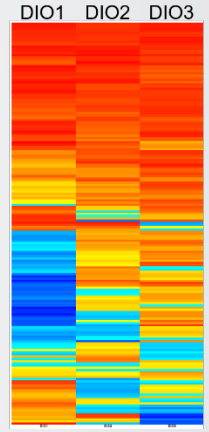
- Targeted in vivo data generation
- Quantitatively link biochemical endpoints to apical effects/phenotypes
- Computational model parameterization

JH Olker et al Toxicol Sci 2018
JT Haselman Toxicol Sci 2020
SA Mayasich et al Toxicol In Vitro 2021

Thyroid-related in vitro assays (8):
TPO, IYD, DIO1-3, TTR, TBG, NIS

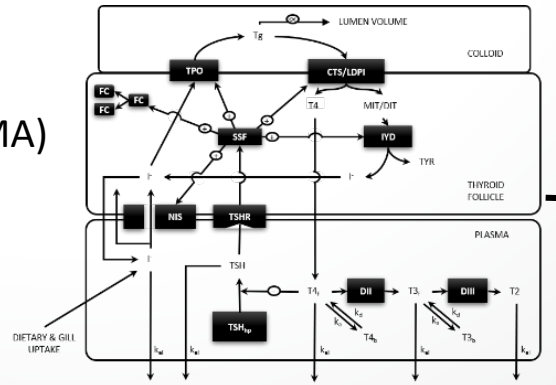
Quantitative Structure Activity Relationships (QSAR)

MW Hornung et al Toxicol Sci 2018
J Wang et al ES&T 2018
JH Olker et al Toxicol Sci 2019
JH Olker et al Toxicol In Vitro 2021
J Wang et al Arch of Toxicol 2021

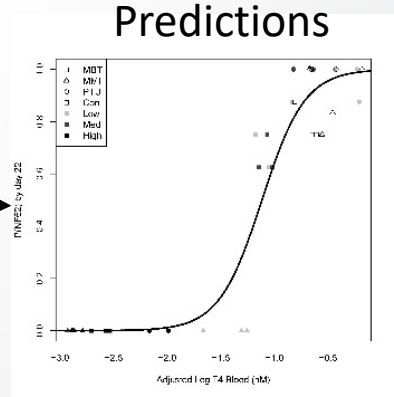


Computational Model Development

- In silico simulation of regulatory guideline study (EDSP Tier I AMA)
- Integrate MIEs represented by thyroid-related in vitro assay
- Data stream integration (e.g., physicochem. properties, fish biotransformation rates)



Predictions



Computational Cross-Species Analysis of ED-Related Targets

EPA POC: Carlie LaLone

EATS targets: Conserved across most vertebrates

Mammalian-based prioritization protective of other vertebrates

Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS) Tool:
https://seqapass.epa.gov/seqapass/

CA LaLone et al, Toxicol Sci 2016

Predict Susceptibility to 100s-1000s of species

Do mammalian-based HTS methods reasonably reflect potential impacts on other untested vertebrates?

Tiered Approach to Evaluate Structural and Functional Conservation for Species Extrapolation

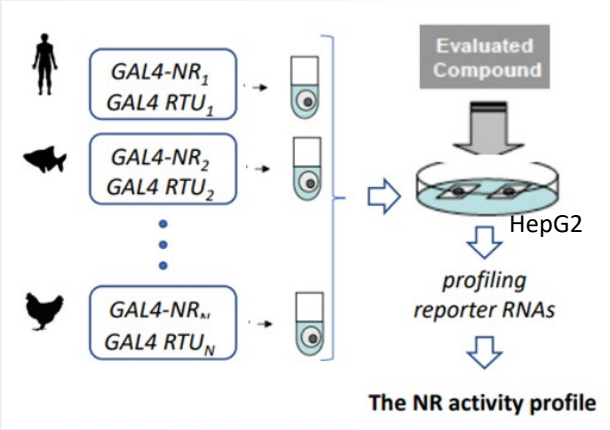
	Structure	Function
Estrogen receptor-α	✓	✓
Androgen Receptor	✓	On-Going
Thyroid axis	✓	On-Going

CA LaLone et al, ES&T 2018

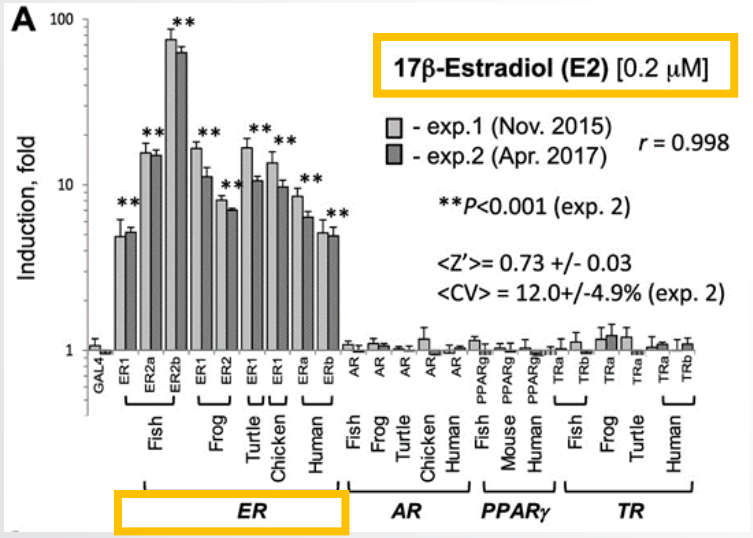
Experimental Cross-Species Analysis of ED-Related Targets

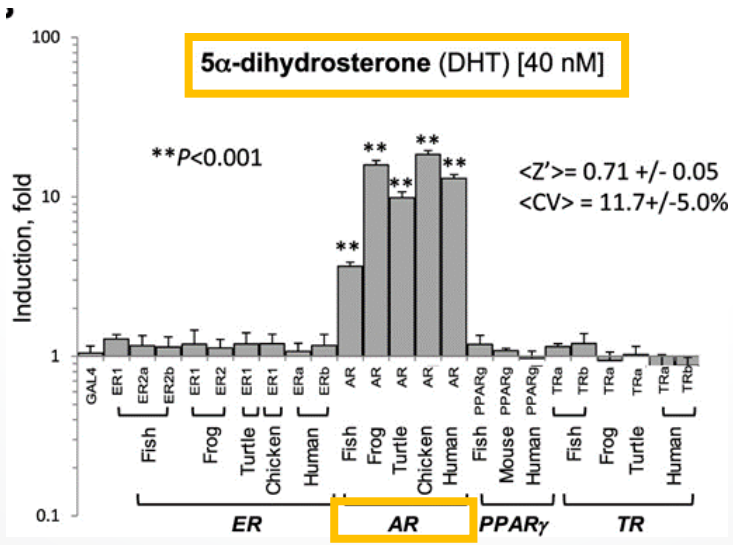
EPA POC: Dan Villeneuve

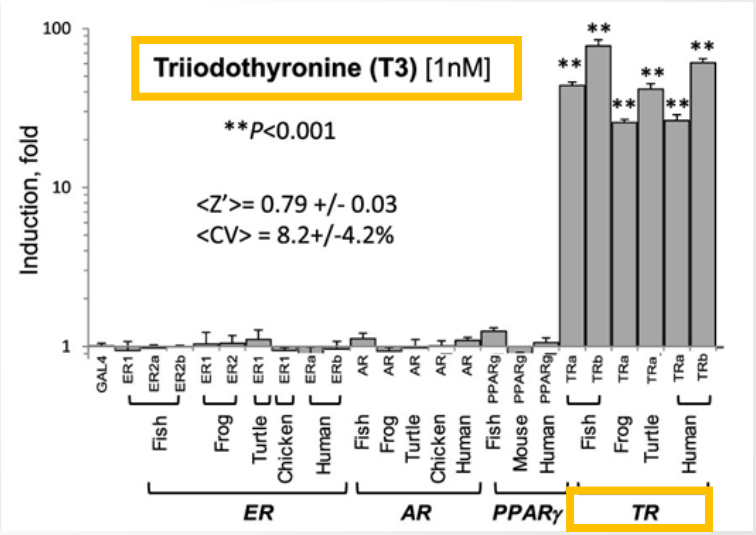
Do the human receptors adequately represent sensitivity of aquatic vertebrate receptors?



- Multiplexed reporter assay enabled parallel assessment of compound effects on ER, AR, TR, and PPARγ receptors of representative mammals, birds, reptiles, amphibians, and fish
- Prototypical NR agonists specifically induced all corresponding NR reporter constructs across species, with a minimal cross-reactivity of irrelevant reporters
- The EC50 values for NR ligands were consistent with those reported for conventional assays







Medvedev et al. ES&T 2020