Use of the ToxCast and Tox21 Screening Strategies in Support of Chemical Prioritization for Risk Assessment

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June 18th, 2018

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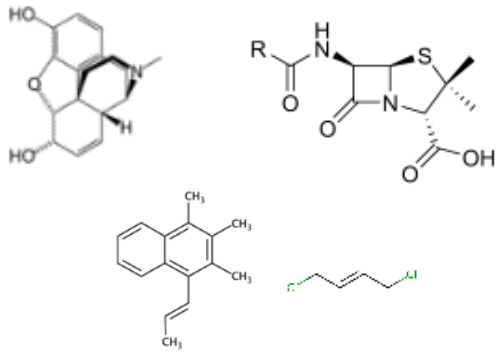
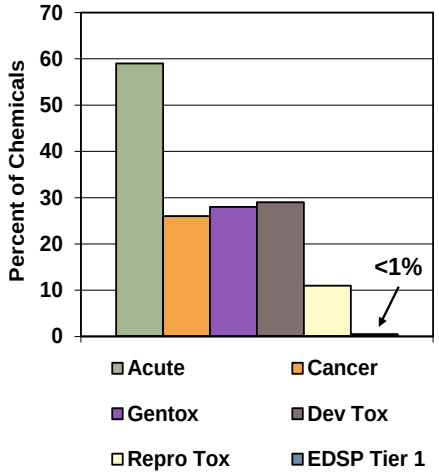
Regulatory Agencies Make a Broad Range of Decisions on Chemicals…

Number of Chemicals /Combinations



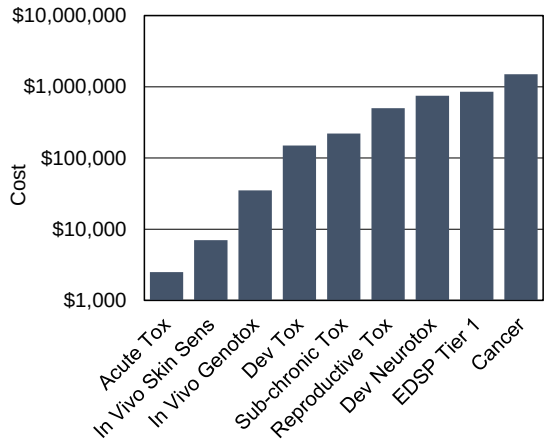
Lack of Data



Modified from Judson *et al.*, EHP 2010

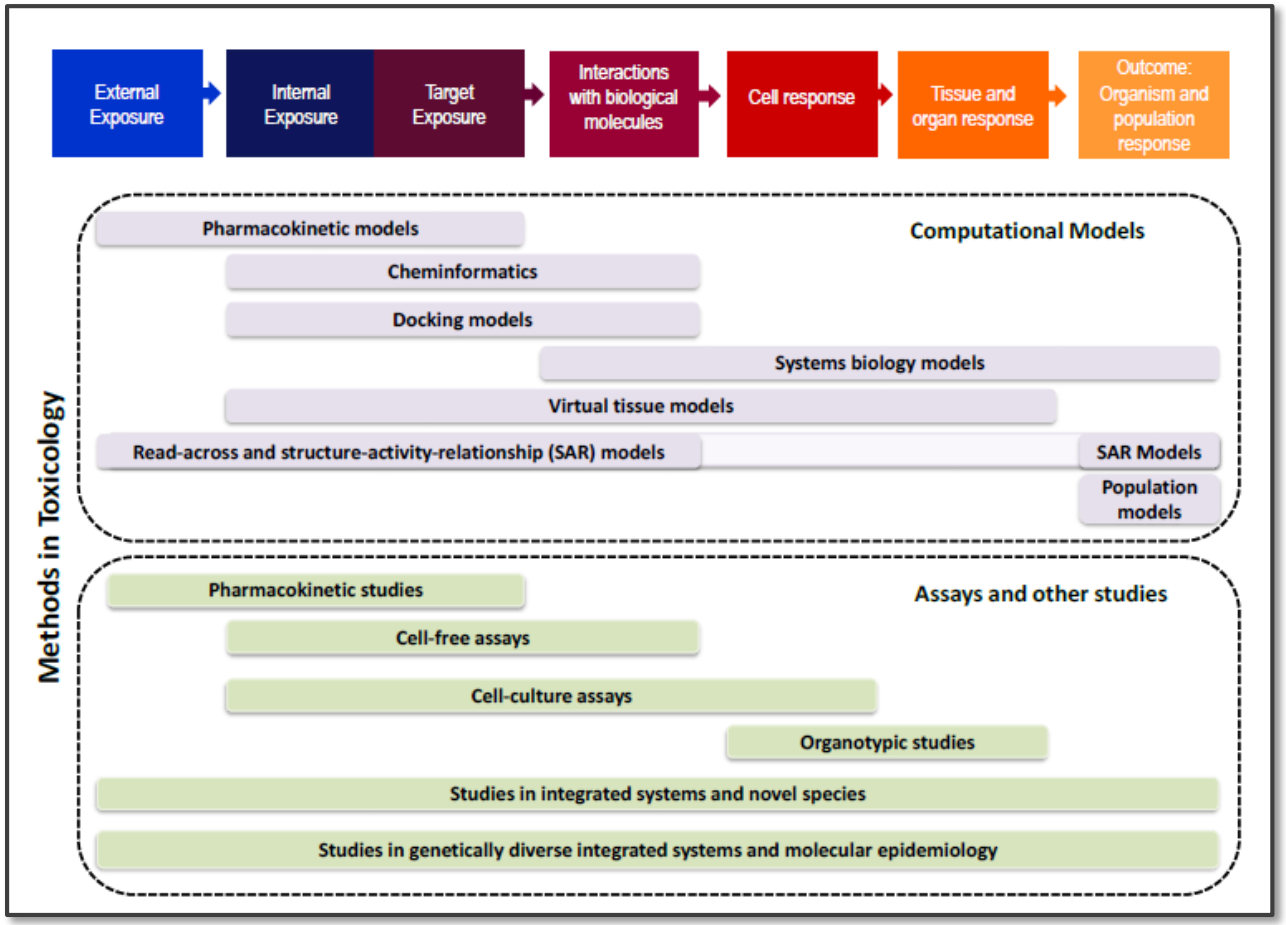
Ethics/Relevance Concerns

Economics

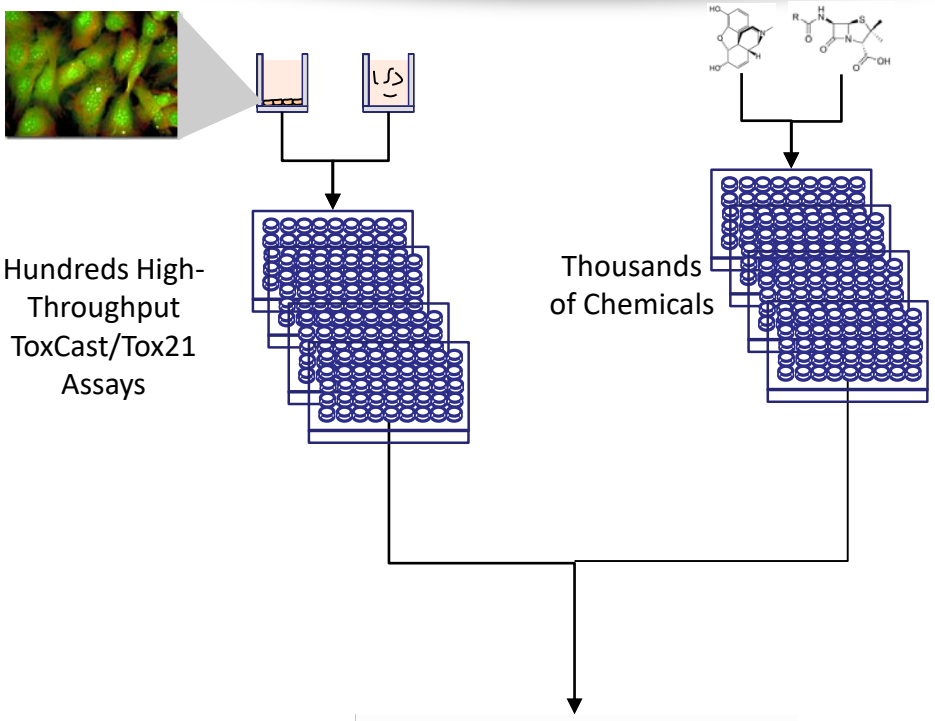


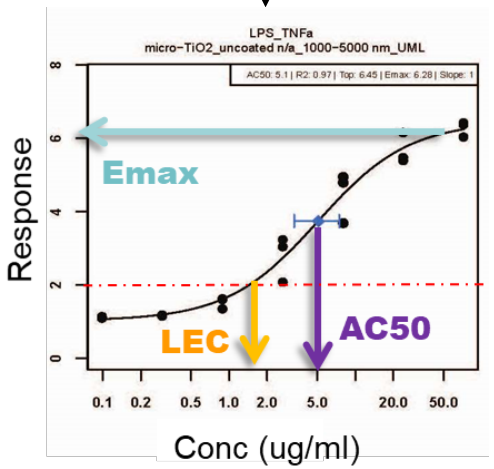
- Number of chemicals and combinations of chemicals is extremely large (>20,000 substances on active TSCA inventory)
- Due to historical regulatory requirements, most chemicals lack traditional toxicity testing data
- Traditional toxicology testing is expensive and time consuming
- Traditional animal-based testing has issues related to ethics and relevance

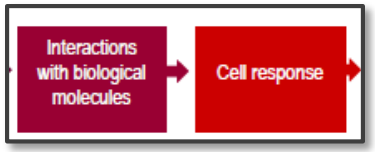
Toxicology Moving to Embrace 21st Century Methods



High-Throughput Assays Used to Screen Chemicals for Potential Toxicity



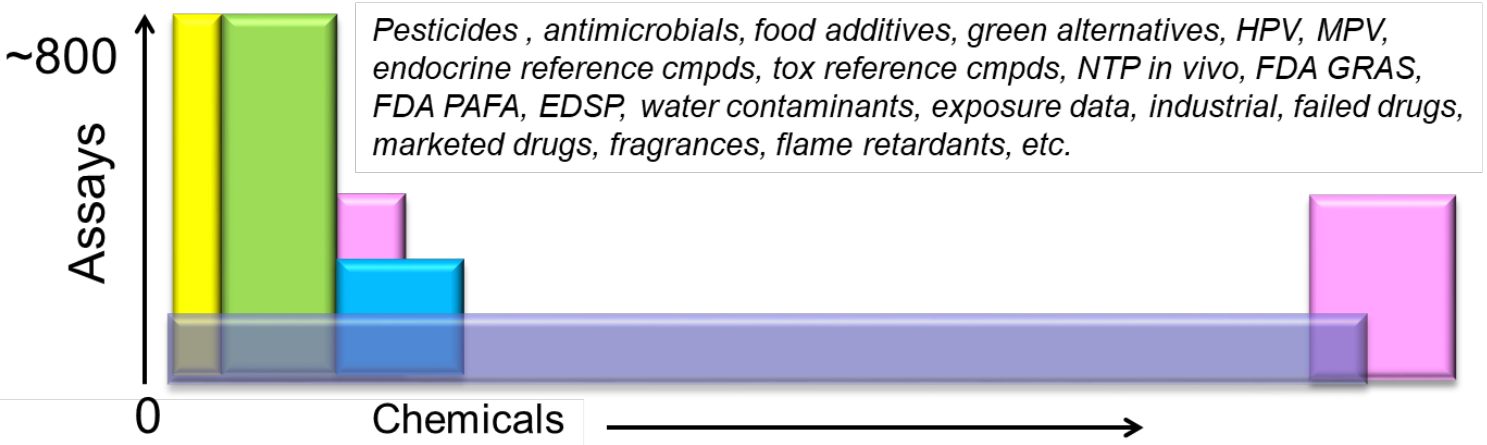




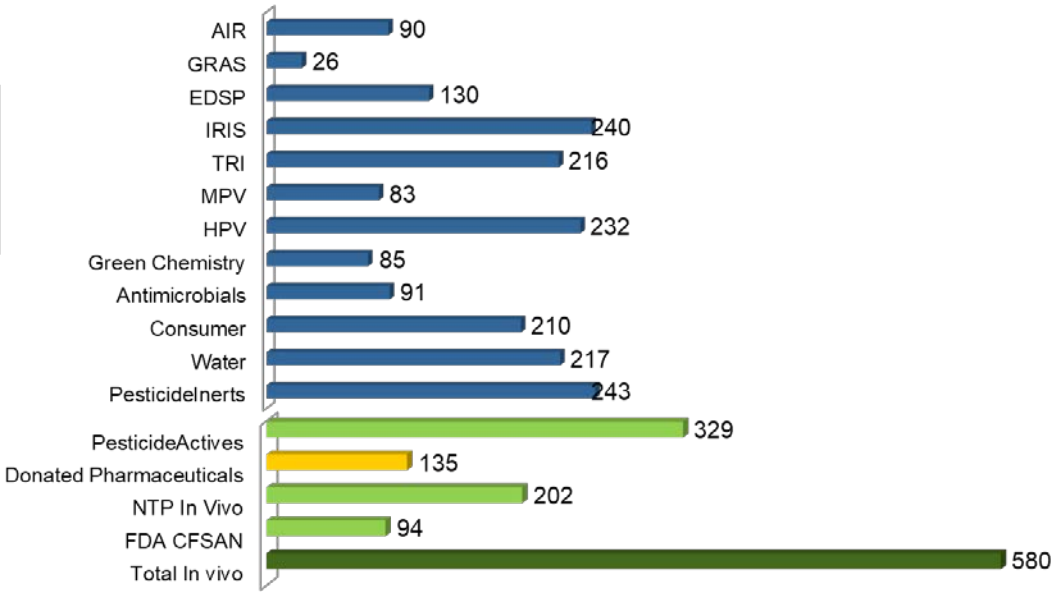
- Understanding of what cellular processes/pathways may be perturbed by a chemical
- Understanding of what amount of a chemical causes these perturbations

ToxCast & Tox21: Chemicals and Assays

Set	Chemicals	Assays	Endpoints	Completion	Available
ToxCast Phase I	293	~600	~700	2011	Now
ToxCast Phase II	767	~600	~700	03/2013	Now
ToxCast E1K	800	~50	~120	03/2013	Now
Tox21	~8300	~80	~150	In progress	Ongoing
ToxCast Phase III	~900	~300	~300	Beginning	2015-2016

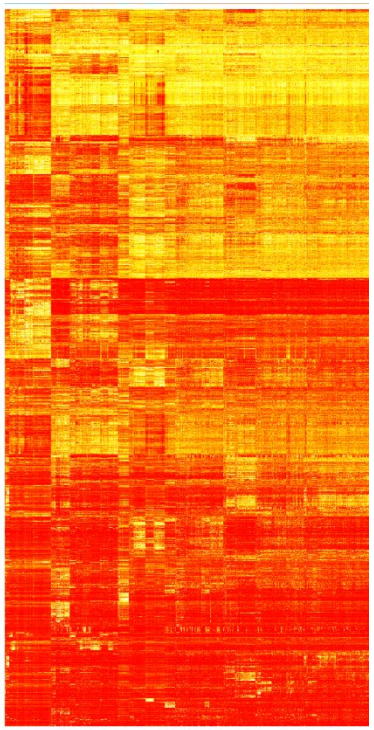


Pesticides , antimicrobials, food additives, green alternatives, HPV, MPV, endocrine reference cmpds, tox reference cmpds, NTP in vivo, FDA GRAS, FDA PAFA, EDSP, water contaminants, exposure data, industrial, failed drugs, marketed drugs, fragrances, flame retardants, etc.

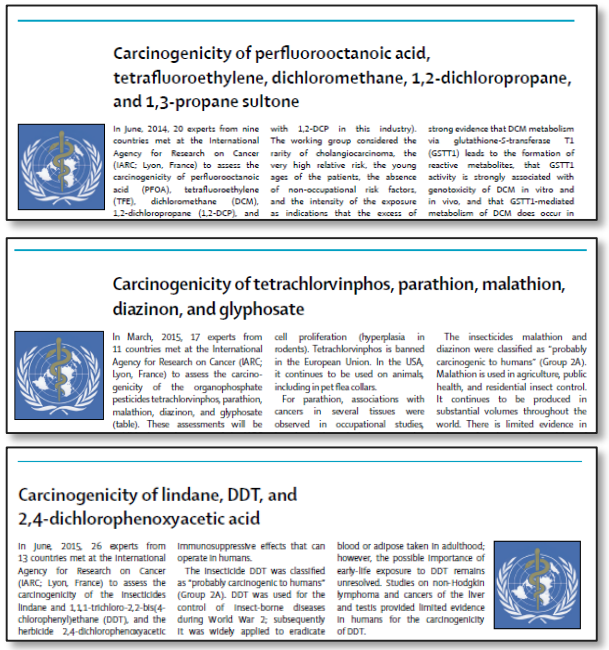


Broad Success Derived from High-Throughput Screening Approaches

Group Chemicals by Similar Bioactivity and Predictive Modeling

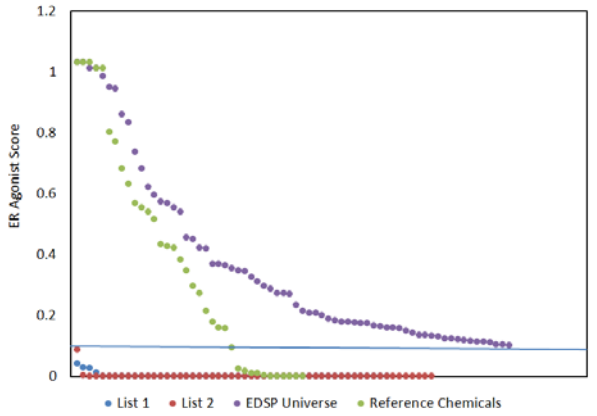


Provide Mechanistic Support for Hazard ID



IARC Monographs

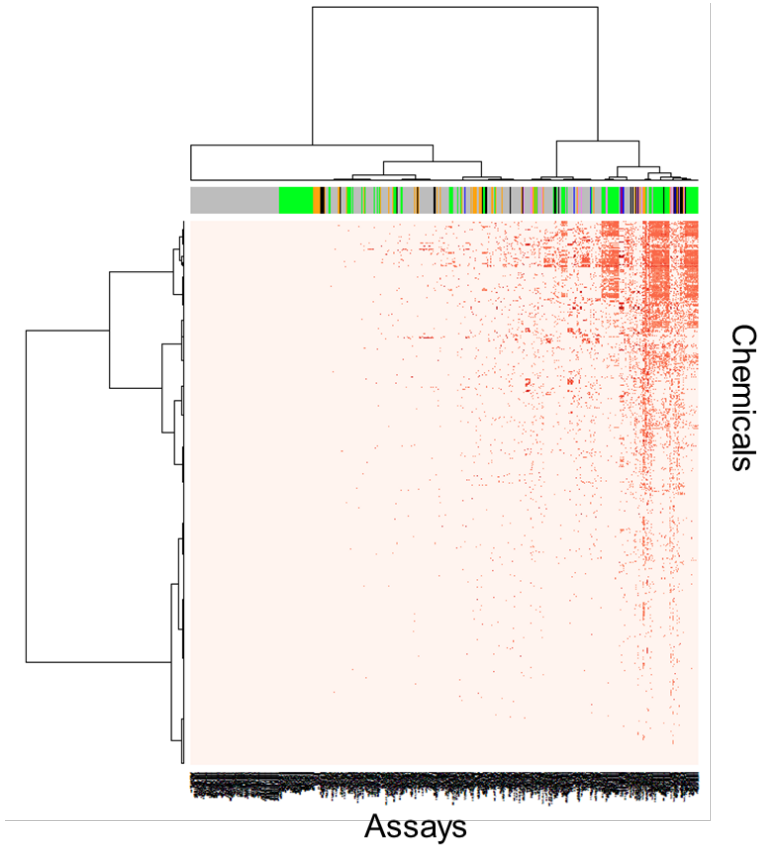
Prioritization of Chemicals for Further Testing

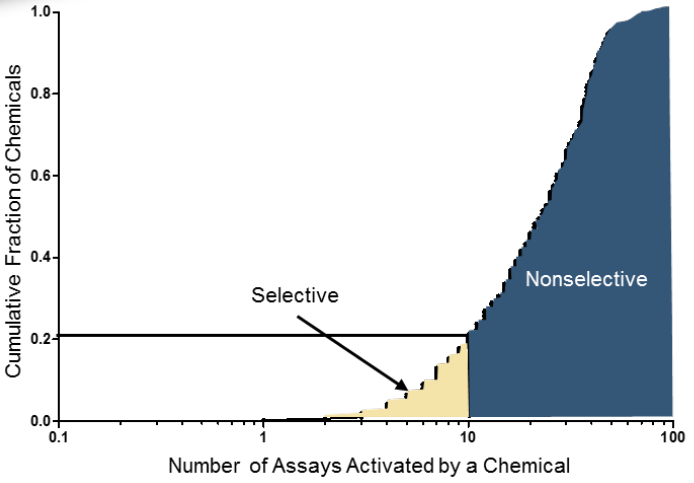


FIFRA SAP, Dec 2014

Promiscuous Chemical Response is the Rule

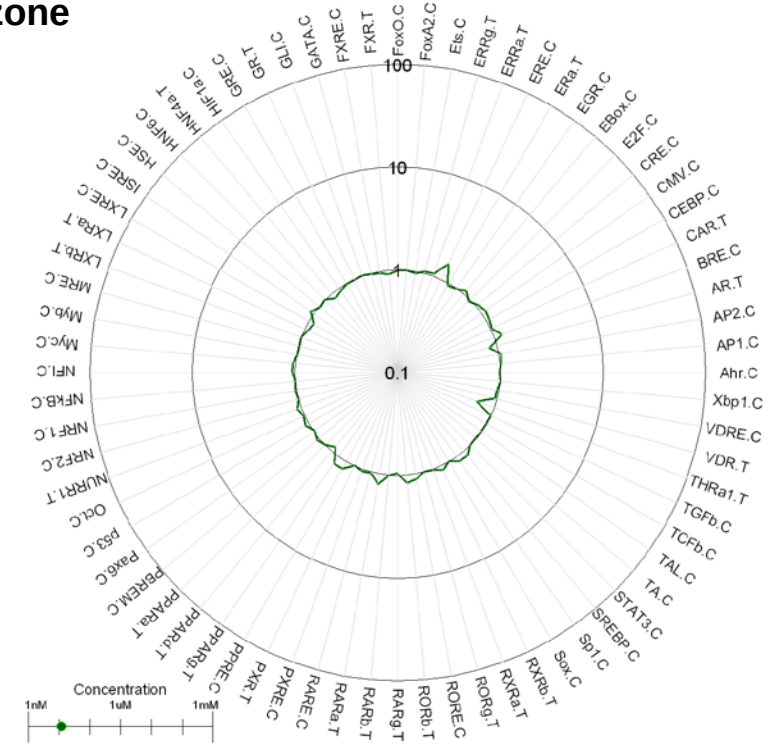
1000 chemicals/
800 assay endpoints





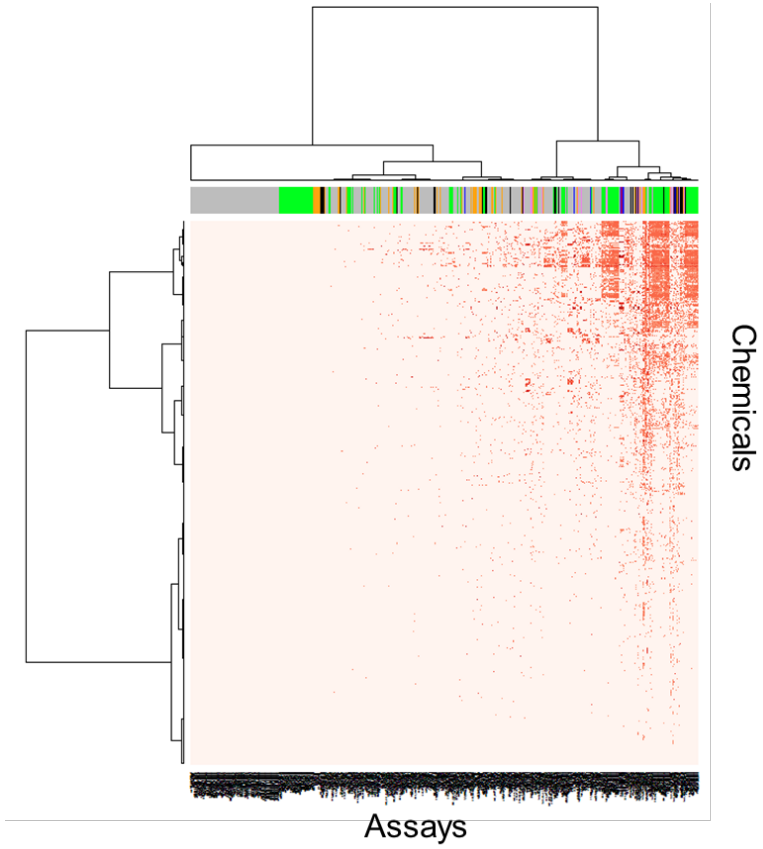
Thomas et al., 2013

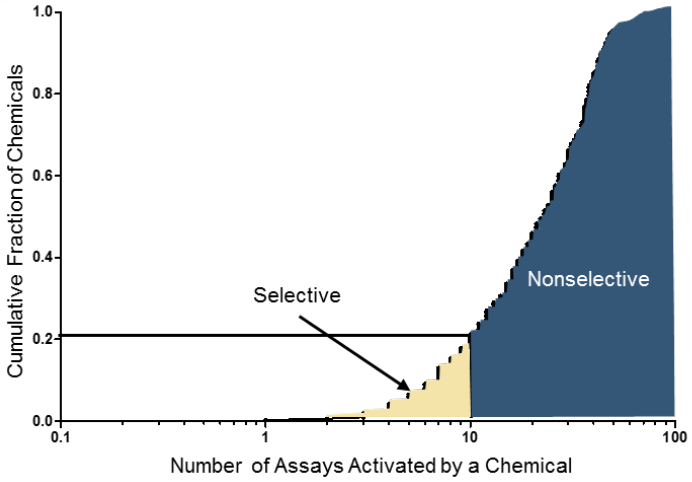
Troglitazone



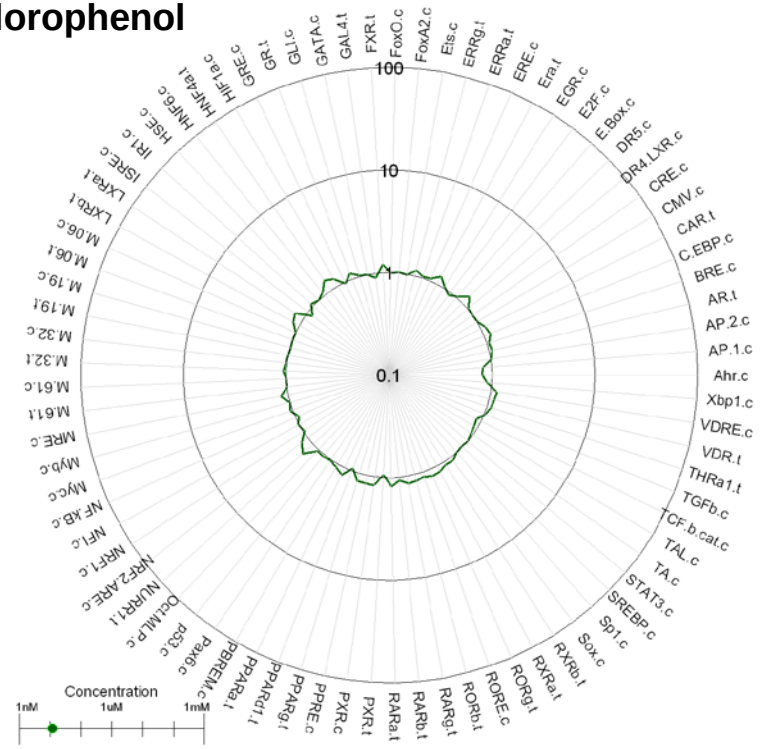
Promiscuous Chemical Response is the Rule

1000 chemicals/
800 assay endpoints

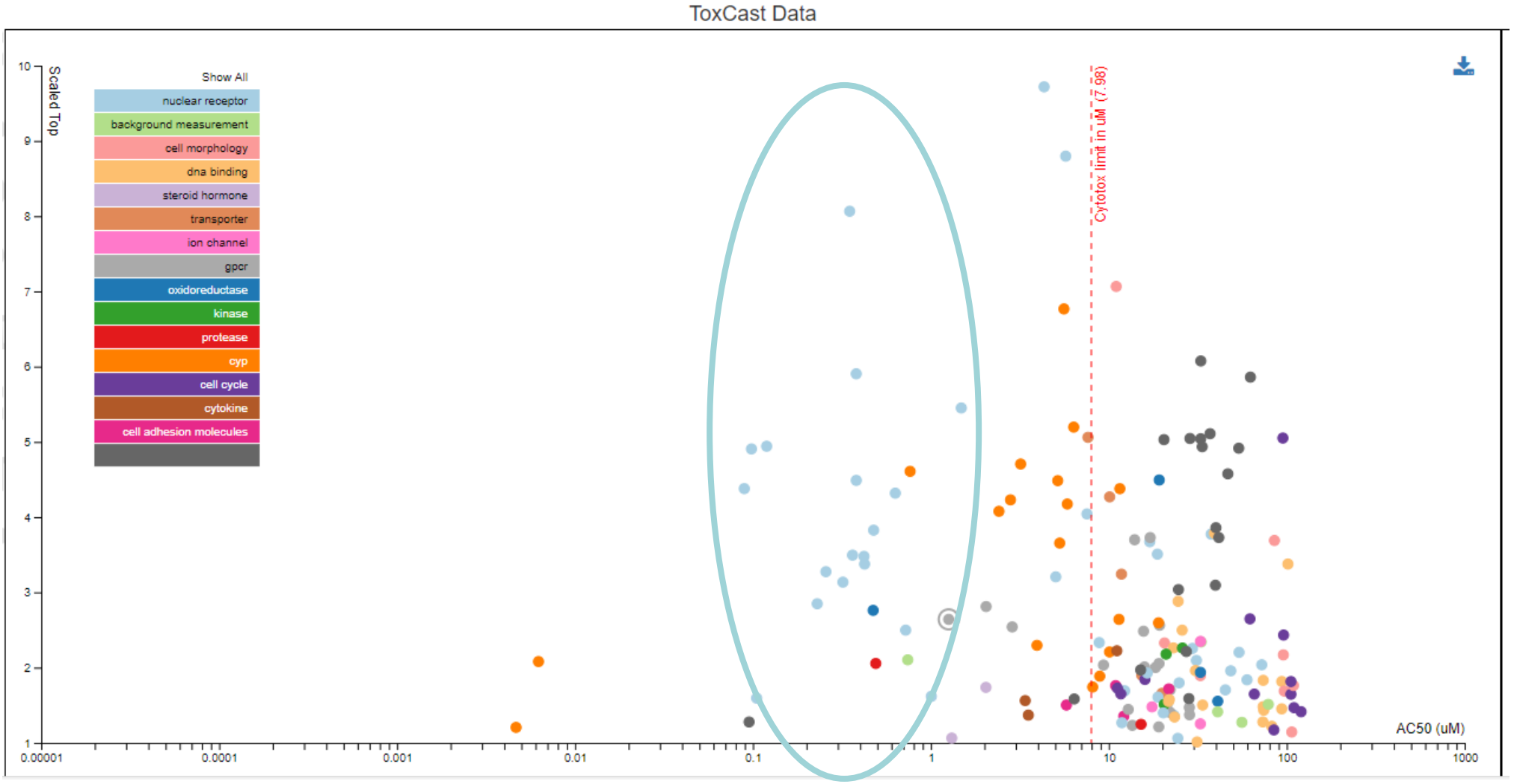




Pentachlorophenol



Bisphenol A ToxCast actives



Nuclear Receptors and Xenobiotics

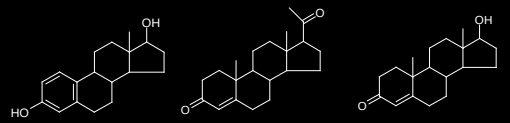
- Family of ligand-regulated nuclear transcription factors
- Conserved, modular domains
- Ligand-binding domain
  - Bind lipophilic small molecules
  - Steroid hormones, fatty acids
- Endogenous ligand physiochemical properties consistent with cell permeable qualities
- Gene regulation of key physiological processes: endocrine system, growth and differentiation, metabolism
- Good focus for **selective** xenobiotic effects

By Boghog2 [Public domain], from Wikimedia Commons

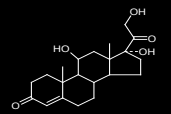
Ligands for Nuclear Hormone Receptors

Sex Steroids — *ER*, *PR*, *AR*

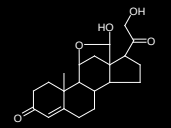
Estrogens, Progestins, Androgens



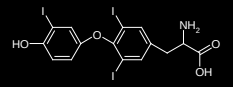
Glucocorticoids — *GR*



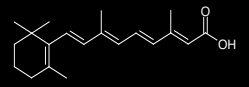
Mineralocorticoids — *MR*



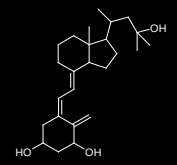
Thyroid Hormones — *TR*



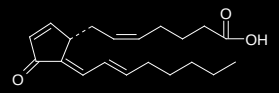
Retinoids — *RAR*, *RXR*



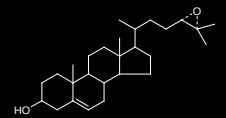
Vitamin D — *VDR*



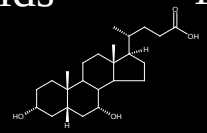
Lipids — *PPAR*



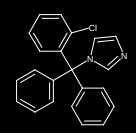
Oxysterols — *LXR*



Bile Acids — *FXR*

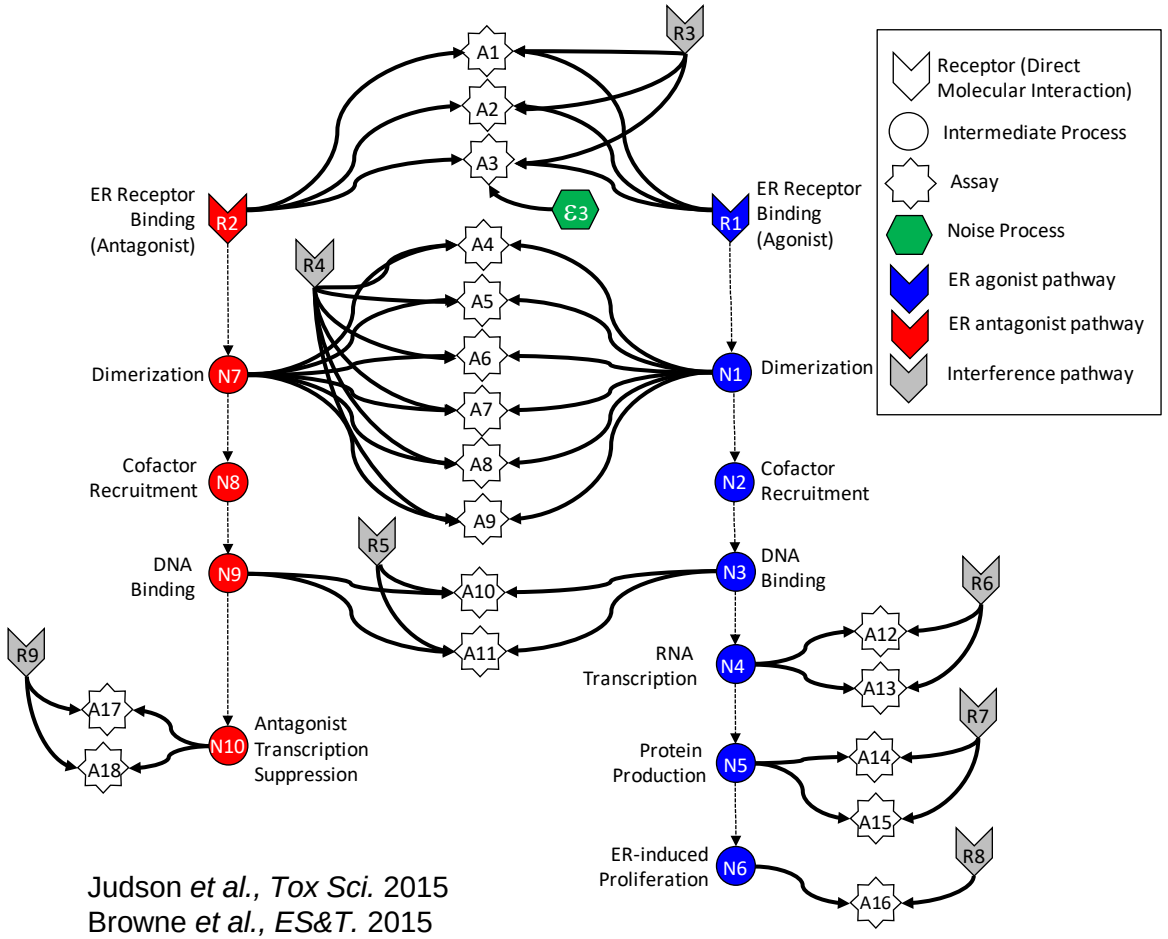


Xenobiotics — *PXR*, *CAR*

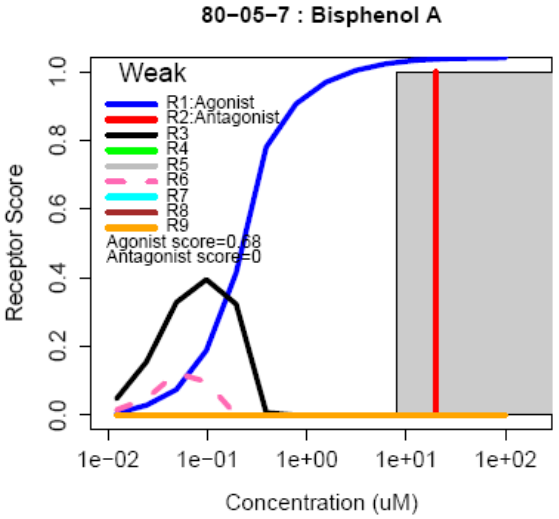


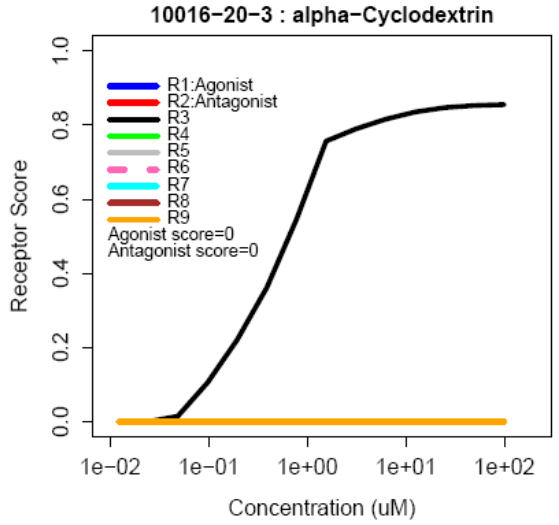
Targeted Pathways (AOP Approach)

18 *In Vitro* Assays Measure ER-Related Activity

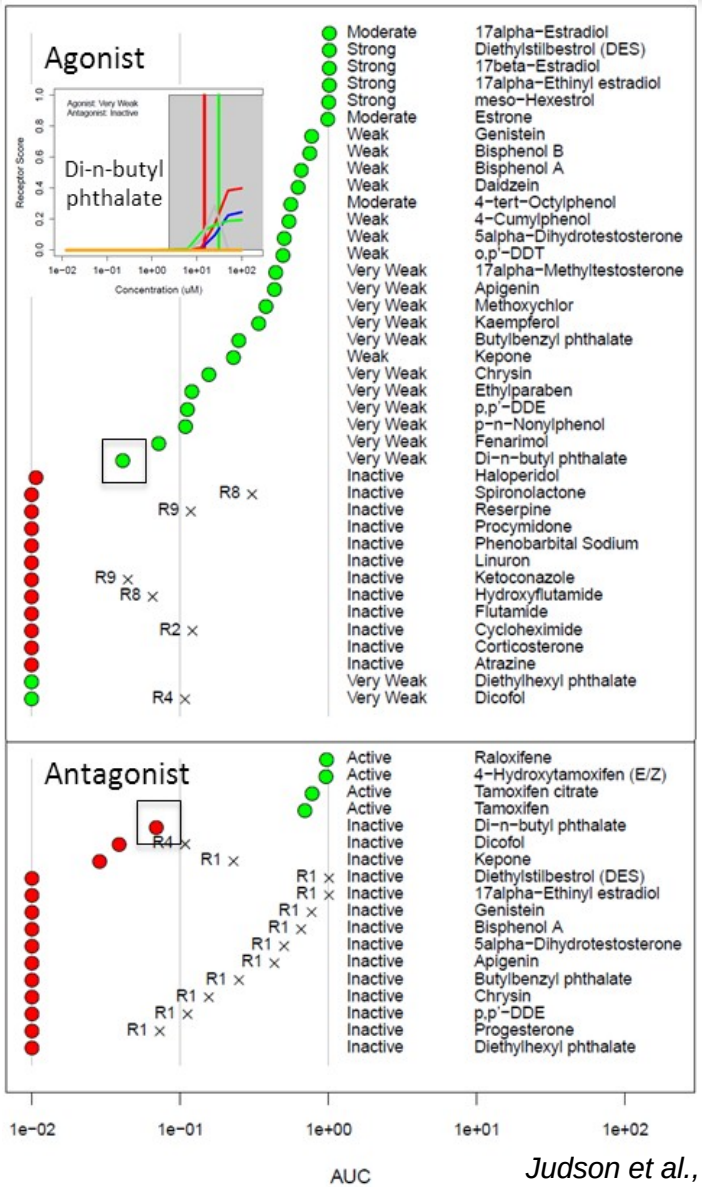


Judson *et al., Tox Sci.* 2015
Browne *et al., ES&T.* 2015
Kleinstreuer *et al., EHP* 2016

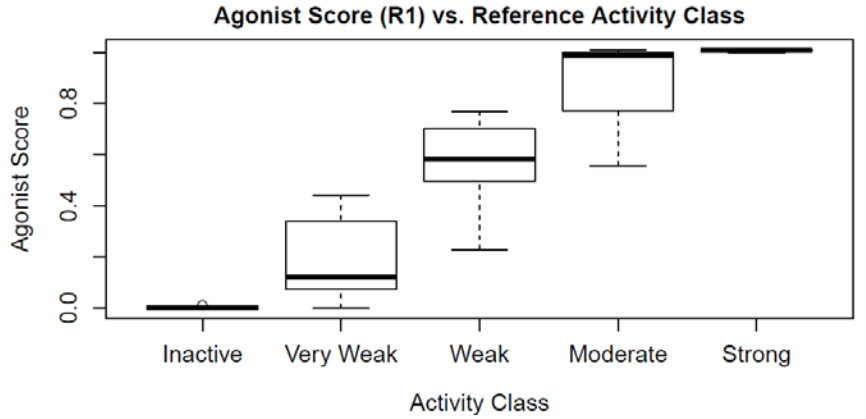


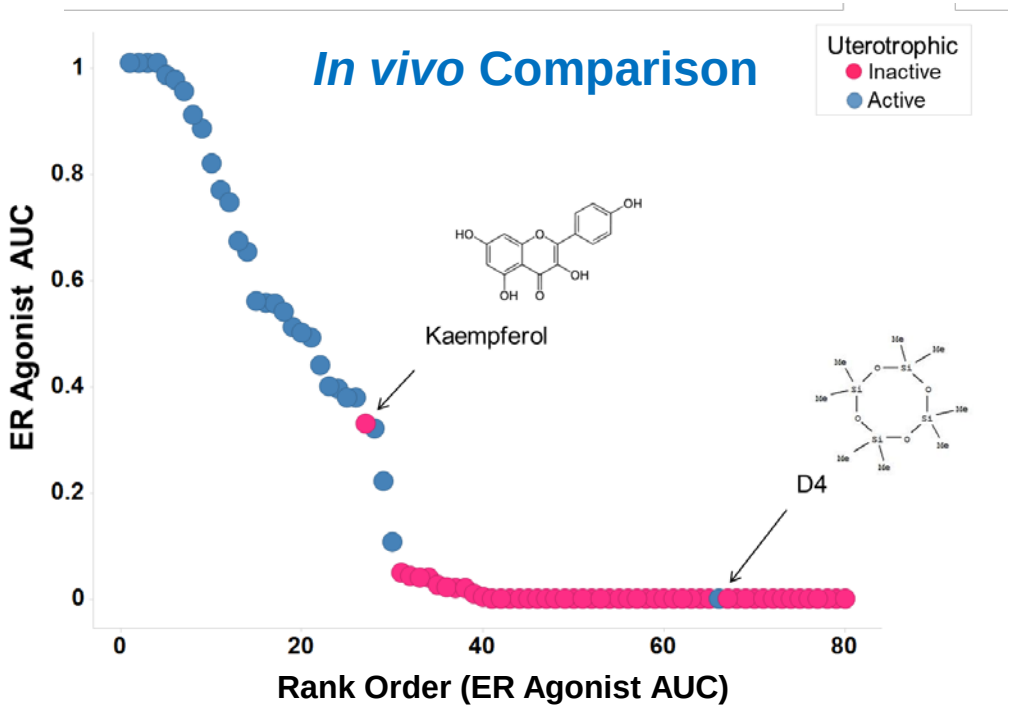


ER Model Performance



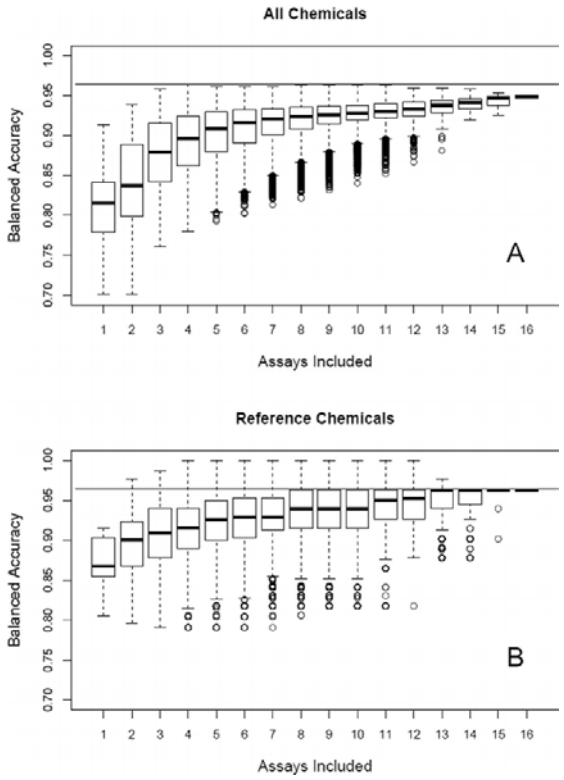
Judson et al., Tox Sci 2015

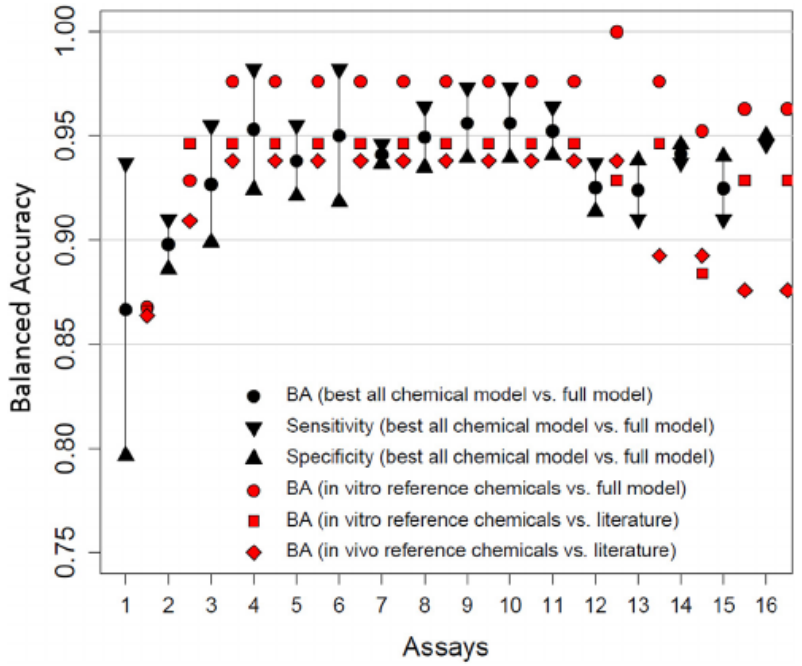




Browne et al., Environ. Sci. Technol., **2015**

ER Minimal Model





R.S. Judson et al. / Regulatory Toxicology and Pharmacology 91 (2017) 39e49

Regulatory Applications: EDSP

FEDERAL REGISTER
The Daily Journal of the United States Government

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Notice

Use of High Throughput Assays and Computational Tools; Endocrine Disruptor Screening Program; Notice of Availability and Opportunity for Comment

A Notice by the Environmental Protection Agency on 06/19/2015

This document has a comment period that ends in 53 days (08/18/2015) SUBMIT A FORMAL COMMENT

ACTION Notice.

SUMMARY

This document describes how EPA is planning to incorporate an alternative scientific approach to screen chemicals for their ability to interact with the endocrine system. This will improve the Agency's ability to fulfill its statutory mandate to screen pesticide chemicals and other substances for their ability to cause adverse effects by their interaction with the endocrine system. The approach incorporates validated high throughput assays and a computational model and, based on current research, can serve as an alternative for some of the current assays in the Endocrine Disruptor Screening Program (EDSP) Tier 1 battery. EPA has partial screening results for over 1800 chemicals that have been evaluated using high throughput assays and a computational model for the estrogen receptor pathway. In the future, EPA anticipates that additional alternative methods will be available for EDSP chemical screening based on further advancements of high throughput assays and computational models for other endocrine pathways. Use of these alternative methods will accelerate the pace of screening, decrease costs, and reduce animal testing. In addition, this approach advances the goal of providing sensitive, specific, quantitative, and efficient screening using alternative test methods to some assays in the Tier 1 battery to protect human health and the environment.

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Publication Date: Friday, June 19, 2015

Agency: Environmental Protection Agency

Dates: Comments must be received on or before August 18, 2015.

Comments Close: 08/18/2015

Entry Type: Notice

Action: Notice.

Document Citation: 80 FR 35350

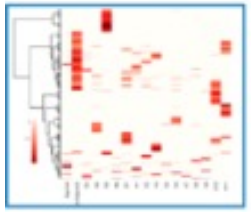
Page: 35350 -35355 (6 pages)

Agency/Docket Numbers: EPA-HQ-OPPT-2015-0305 FRL-9928-69

Document Number: 2015-15182

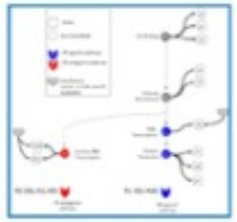
"The approach incorporates validated high-throughput assays and a computational model and, based on current research, can serve as an alternative for some of the current assays in the Endocrine Disruptor Screening Program (EDSP) Tier 1 battery."

AR Model



Internal

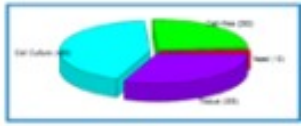
AR High-Throughput Screening Data



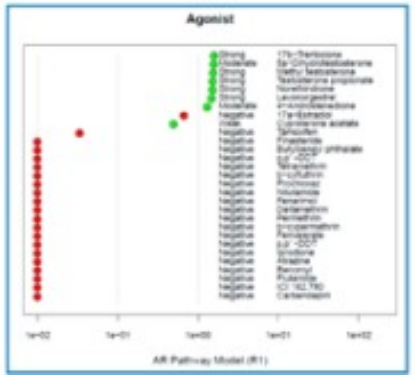
AR Pathway Computational Model

External

Reference Data Literature Review

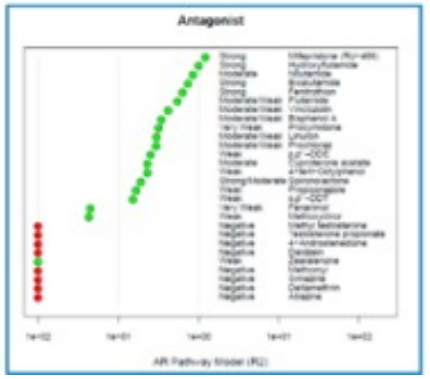


Reference Chemical Classifications



Model Performance Evaluation

Validated Model for Chemical Screening



Chemical Research in Toxicology

Development and Validation of a Computational Model for Androgen Receptor Activity

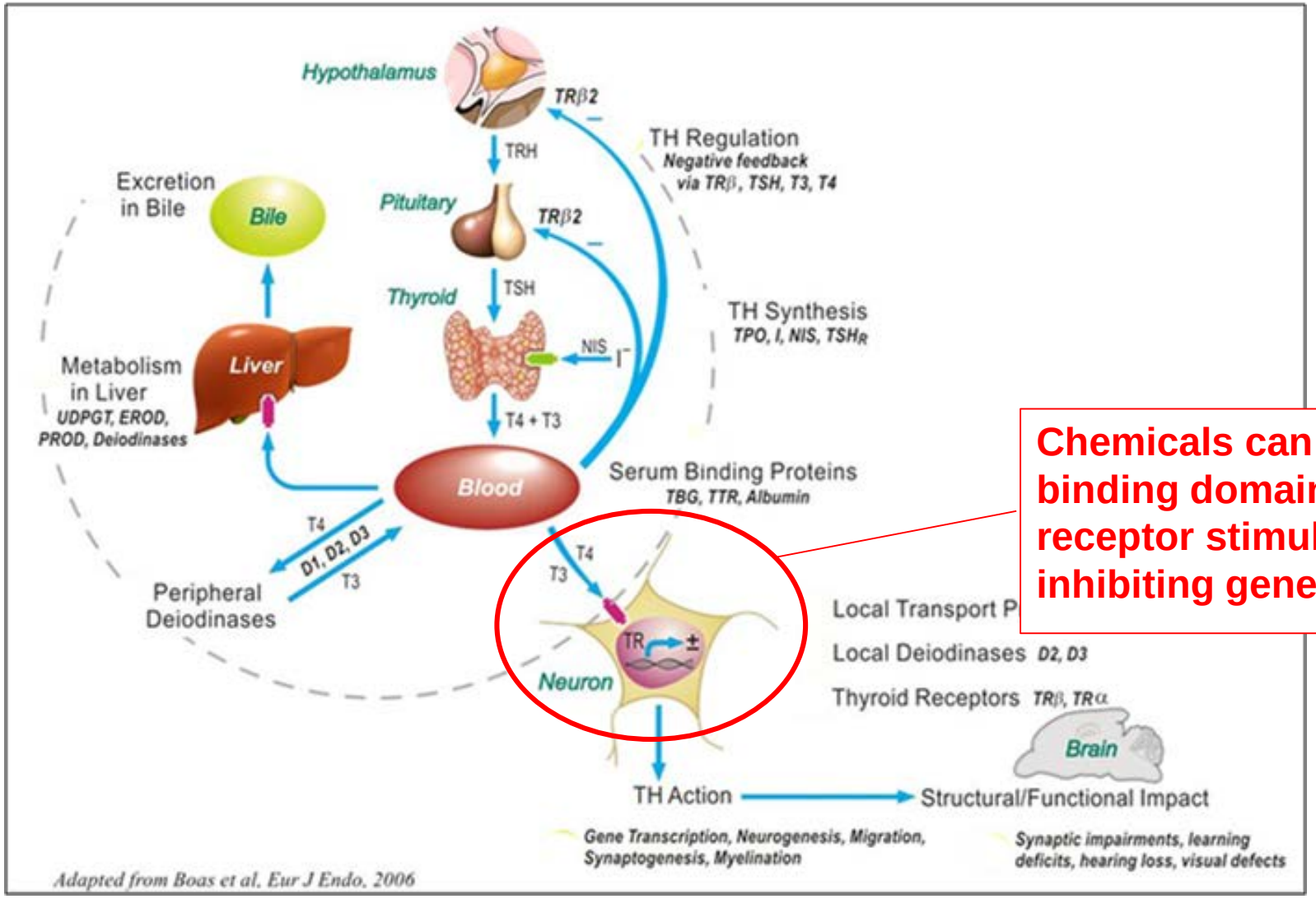
Nicole C. Kleinstreuer,*,† Patricia Ceger,‡ Eric D. Watt,§ Matthew Martin,§ Keith Houck,§ Patience Browne,‖ Russell S. Thomas,§ Warren M. Casey,† David J. Dix,⊥ David Allen,‡ Srilatha Sakamuru,# Menghang Xia,# Ruili Huang,# and Richard Judson§

Chem Res Toxicol. 30:946-964, 2017.

AR Performance

- Applying model outside reference chemical set identified likely false positives for AR antagonists due to cytotoxicity
- Added additional characterization assays (analysis in progress)
  - Coregulator recruitment (functional biochemical)
  - Coactivator recruitment (high-content imaging)

Thyroid Axis Overview



Chemicals can bind to ligand-binding domain of thyroid receptor stimulating or inhibiting gene transcription

Thyroid Receptor

Agonist

Antagonist

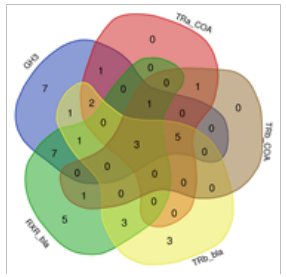
Viability

tcpl

5300 total

Assay	# Tested
gh3-tre-agonist-followup	84
tra-coa-agonist	84
trb-coa-agonist	84
trb-bla-agonist	84
tr-followup-rxr-bla	84
tr-followup-rxr-bla	84
trb-bla-agonist	84

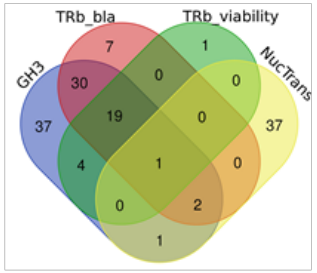
Actives



5300 total
800 >10X Viability

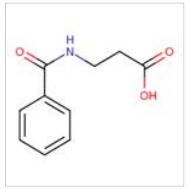
Assay	Tested
GH3-Luc-followup	1010
TRb_bla	100
TRb_bla_viability	100
TR_GR_GFP_nuctrans	263

Actives

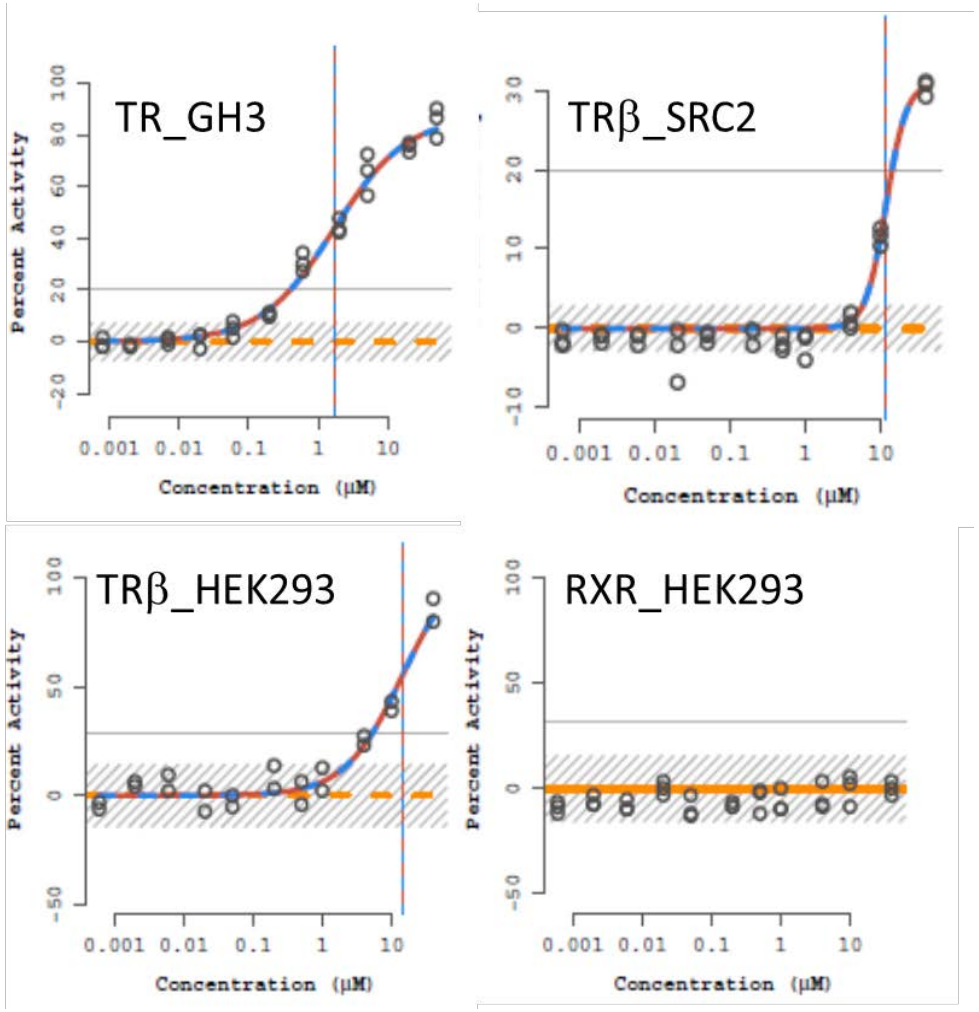


Pharmacology of TR Actives

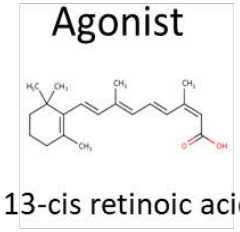
Direct TR Agonist



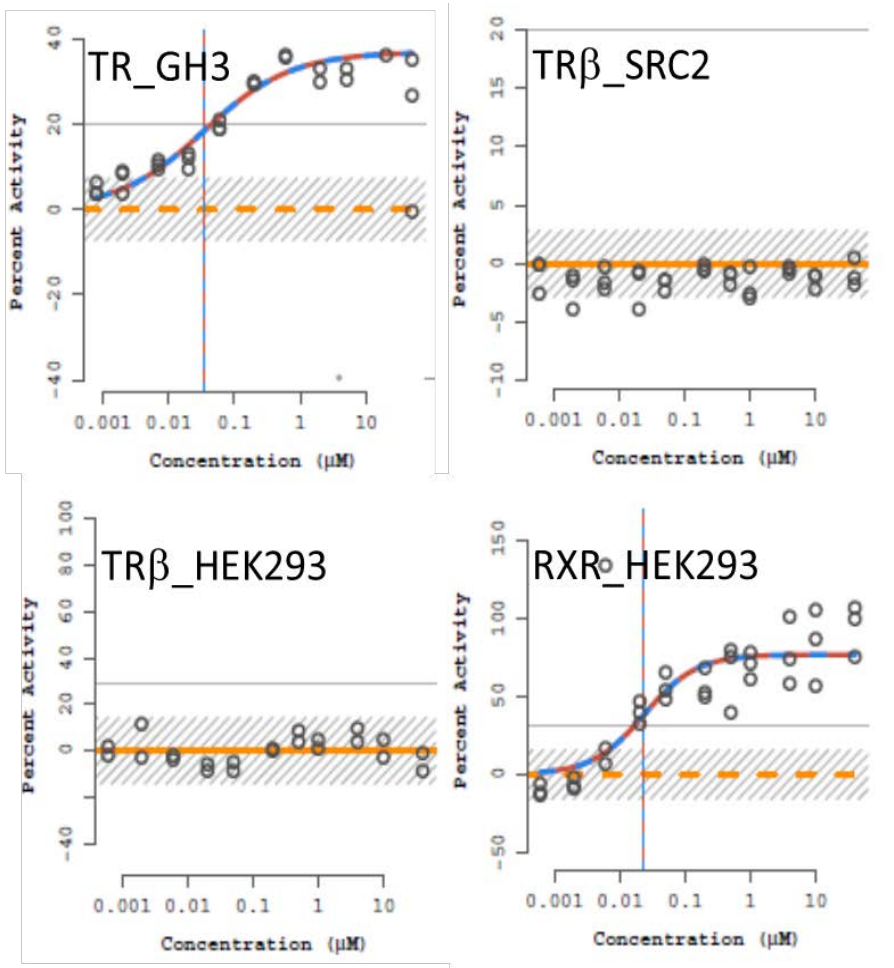
Betamipron



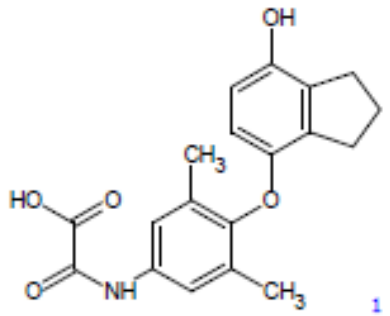
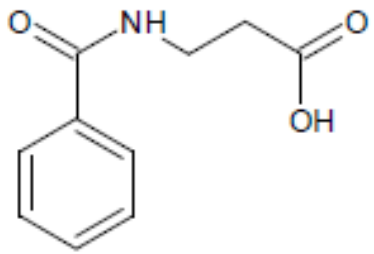
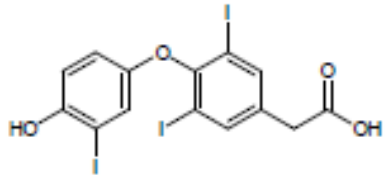
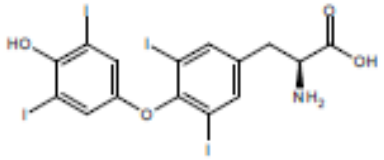
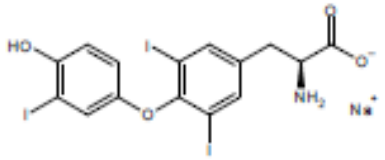
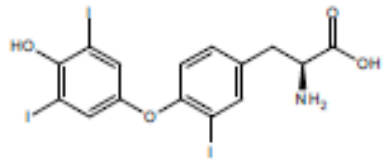
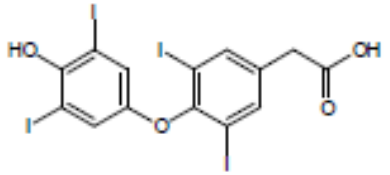
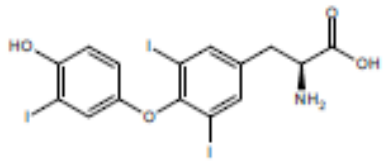
Indirect TR Agonist/RXR Agonist



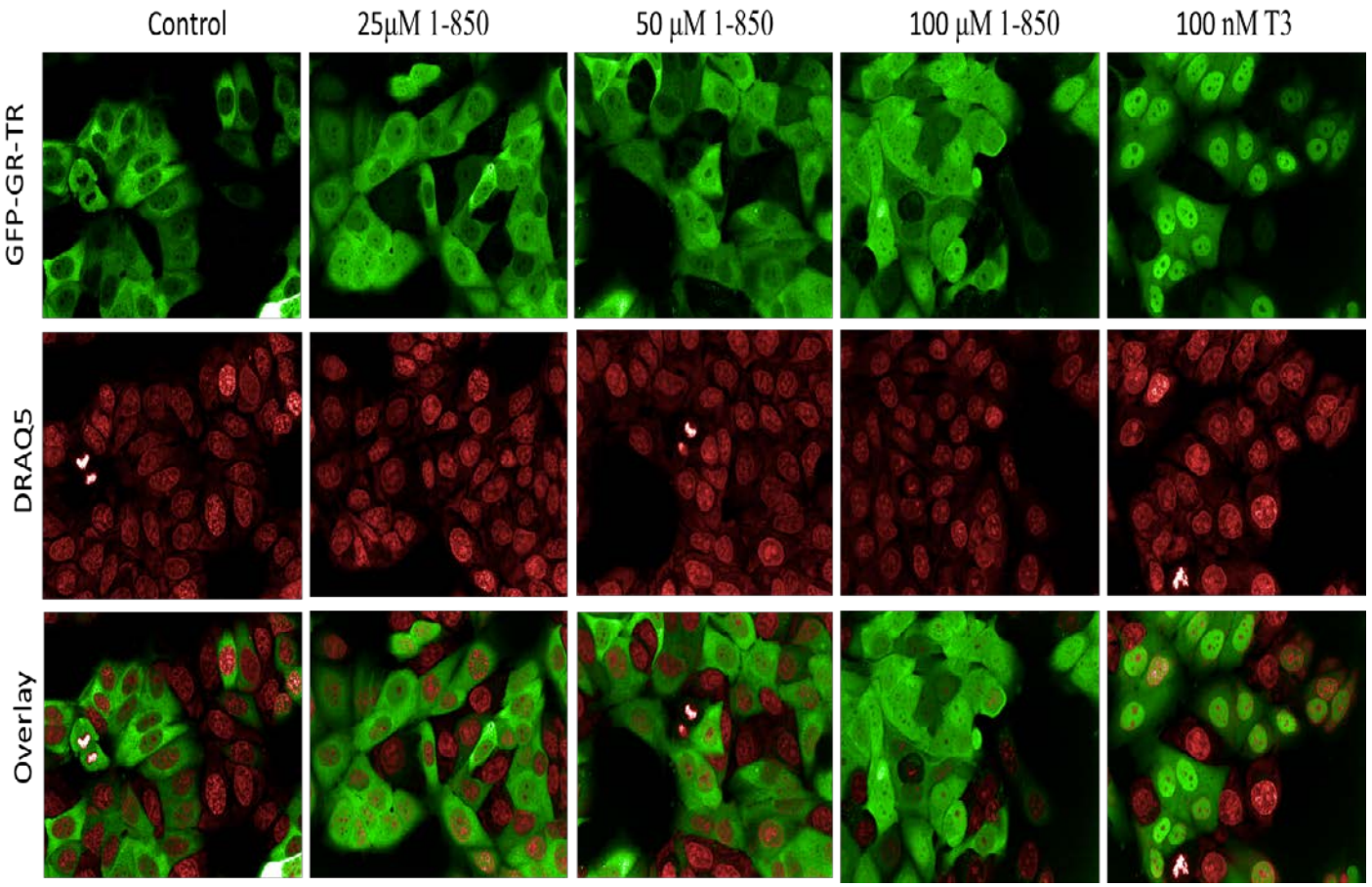
13-cis retinoic acid

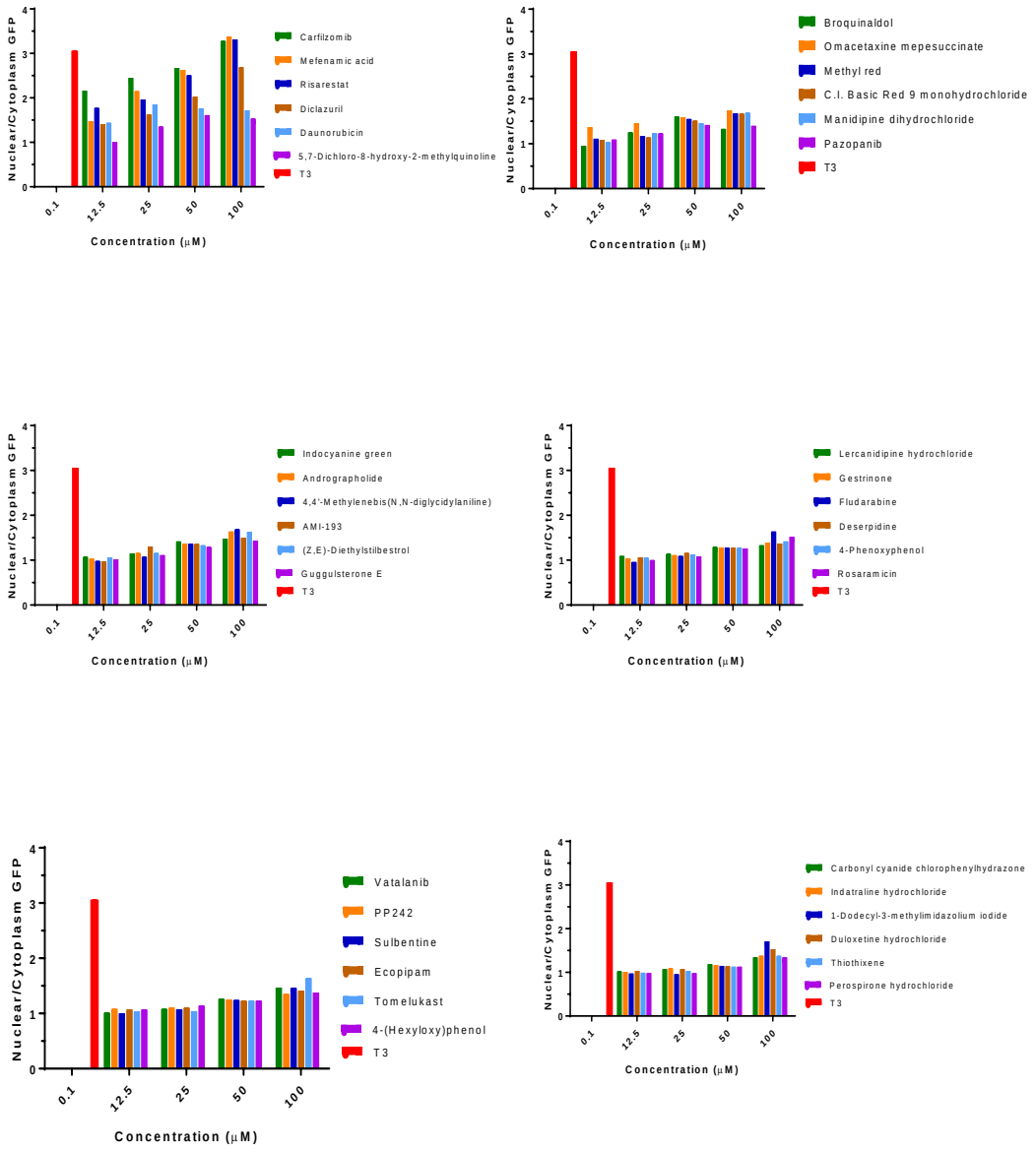


Structures assigned to TR agonist class

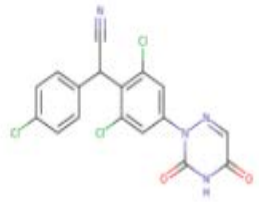
1 	2 	3 	4 	5 
CP-634384 290352-28-2	Betamipron 3440-28-6	Tiratricol 51-24-1	Levothyroxine 51-48-9	3,3',5-Triiodo-l-thyronine sod 55-06-1
6 	7 	8 		
3,3',5'-Triiodo-L-thyronine 5817-39-0	Tetrac 67-30-1	3,5,3'-Triiodothyronine 6893-02-3		

TR Nuclear Translocation Assay

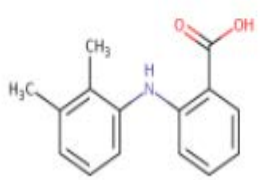




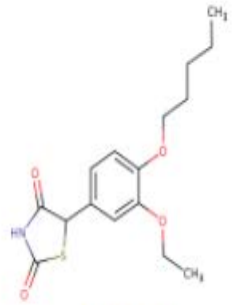
Structure Assigned to TR Antagonist Class



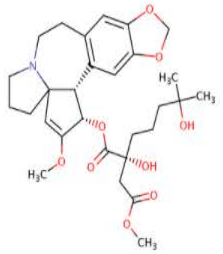
Diclazuril
101831-37-2



Mefenamic acid
61-68-7



Risarestat
79714-31-1



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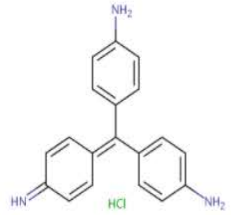
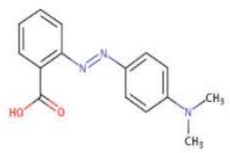
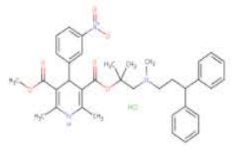
26833-87-4



C.I. Basic Red 9 monohydrochloride
569-61-9

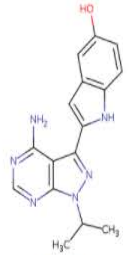


Methyl red
493-52-7

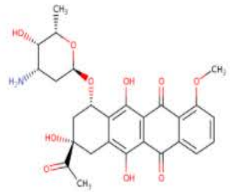


Lercanidipine hydrochloride
132866-11-6

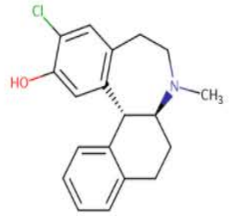
Carfilzomib
868540-17-4



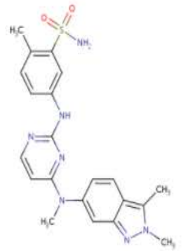
PP242
1092351-67-1



Daunorubicin
20830-81-3



Ecopipam
112108-01-7



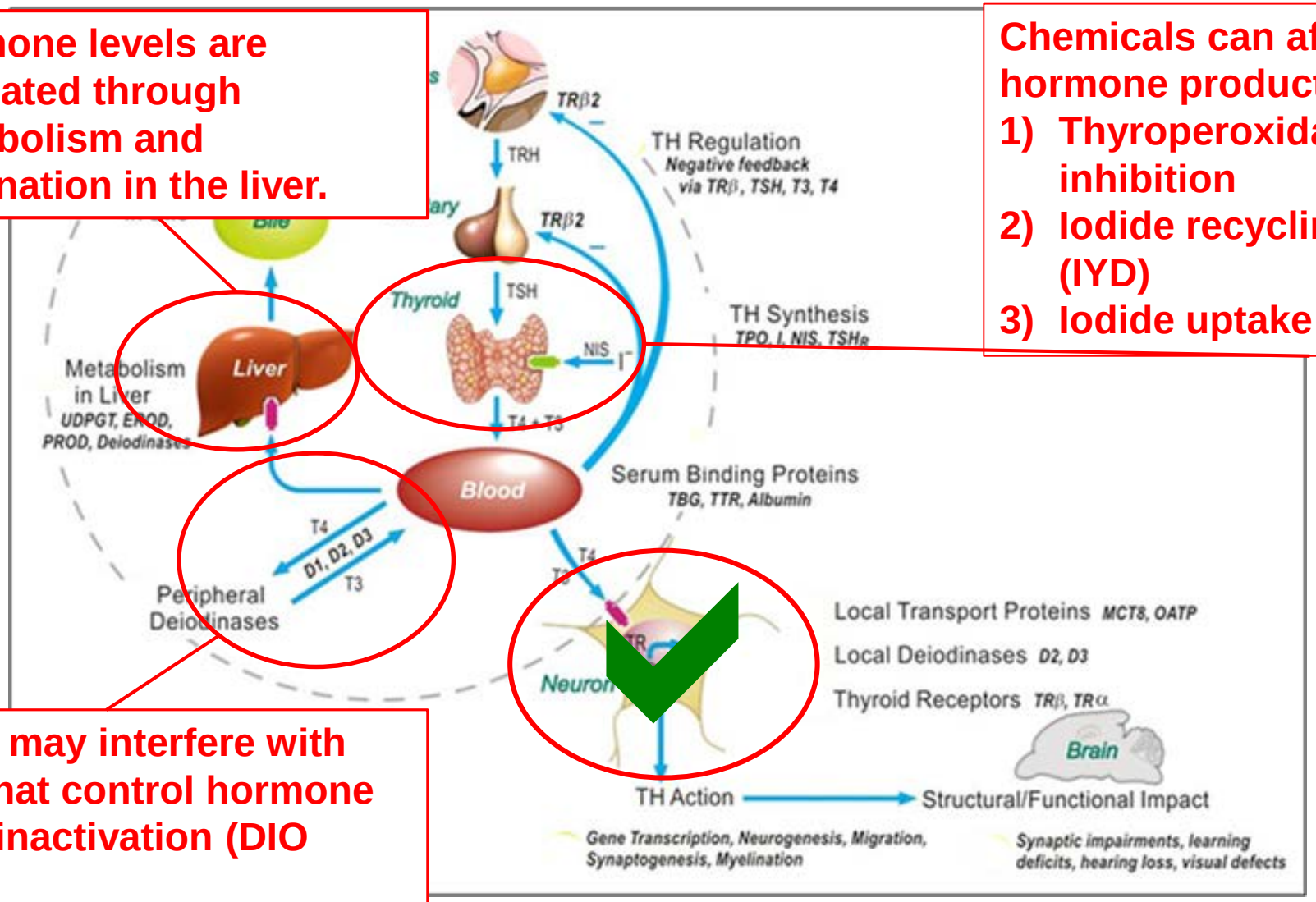
Pazopanib
444731-52-6

Thyroid Axis Overview

Hormone levels are regulated through metabolism and elimination in the liver.

Chemicals can affect thyroid hormone production via

1) **Thyroperoxidase (TPO) inhibition**
2) **Iodide recycling inhibition (IYD)**
3) **Iodide uptake inhibition (NIS)**



Chemicals may interfere with enzymes that control hormone activation/inactivation (DIO 1,2,3)

Thyroid-Axis Targets for *In Vitro* Assays

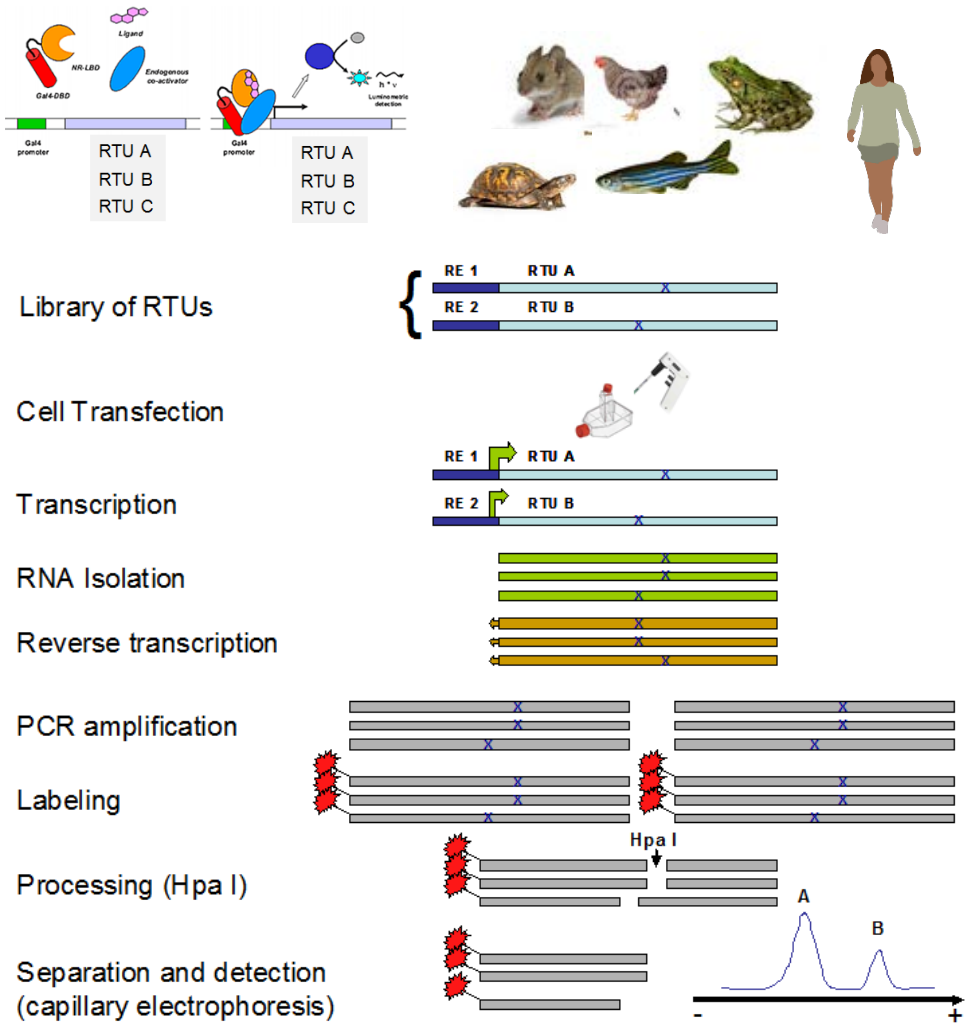
Molecular Target	Screening Assay Status	
	Existing	In Development
TRH Receptor	X (ToxCast)	
TSH Receptor	X	
Sodium-Iodide Symporter (NIS)	X	
Pendrin		
Dual Oxidase (DUOX)		
Thyroperoxidase (TPO)	X	
TH Serum Transport Proteins	X	
TH Membrane Transporters		
Iodothyronine Deiodinase Type I	X	
Iodothyronine Deiodinase Type II	X	
Iodothyronine Deiodinase Type III	X	
Iodotyrosine Deiodinase		X
Nuclear Receptors	X (ToxCast)	
Sulfation and Glucuronidation		
Alanine Side Chain Activation		
TH Receptor Binding		
TH Transcription (Agonist/Antagonist)	X (ToxCast)	

What is New with the ToxCast Strategy?

- Nuclear receptor multispecies screening
- High-throughput transcriptomics
- Phenotypic screening cell painting assay
- Use of metabolic activation systems in vitro

Assessing Cross-Species Differences in Response

Multispecies Attagene *Trans* Reporter Assay

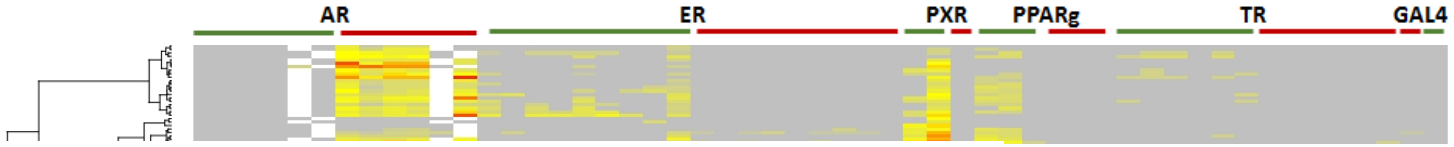


Houck et al., Unpublished

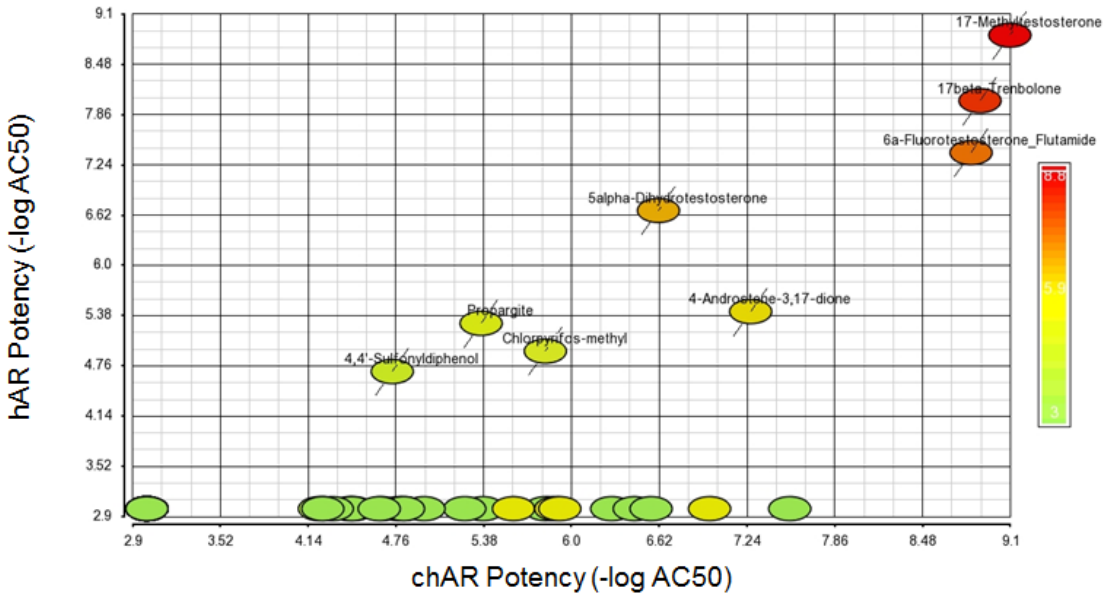
Receptor Family	Receptor Name	Species
Estrogen Receptor	ERa	Human
Estrogen Receptor	ERb	Human
Estrogen Receptor	ER1	Zebrafish
Estrogen Receptor	ER2a	Zebrafish
Estrogen Receptor	ER2b	Zebrafish
Estrogen Receptor	ERa	Chicken
Estrogen Receptor	ER1	Frog
Estrogen Receptor	ER2	Frog
Estrogen Receptor	ERa	Turtle
Estrogen Receptor	AR	Human
Estrogen Receptor	AR	Chicken
Estrogen Receptor	AR	Turtle
Estrogen Receptor	AR	Frog
Estrogen Receptor	AR	Zebrafish
Peroxisome Proliferator Activated Receptor γ	PPARg	Mouse
Peroxisome Proliferator Activated Receptor γ	PPARg	Zebrafish
Peroxisome Proliferator Activated Receptor γ	PPARg	Human
Pregnane X Receptor	PXR	Mouse
Thyroid Receptor	TRa	Turtle
Thyroid Receptor	TRb	Zebrafish
Thyroid Receptor	TRb	Zebrafish
Thyroid Receptor	TRa	Frog
Thyroid Receptor	TRa	Human
Thyroid Receptor	TRb	Human
Controls	M-06	NA
Controls	GAL4	NA
Controls	M-19	NA
Controls	m-32	NA
Controls	m-61	NA

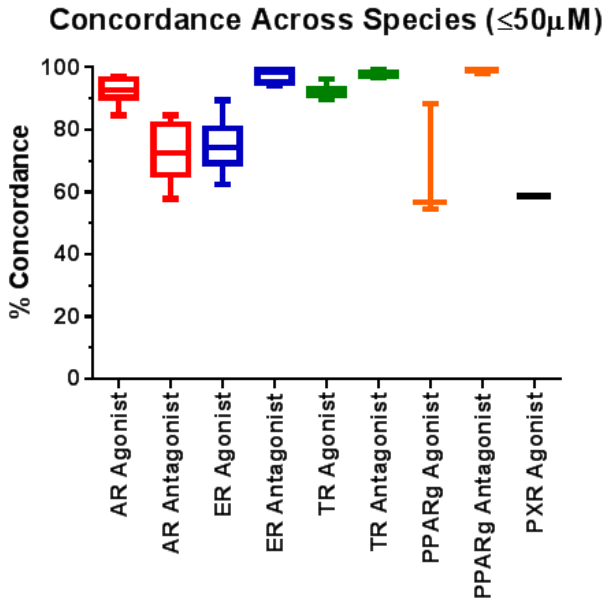
- Host cell: human HepG2
- Stimulation with EC20 of 6a-fluorotestosterone for detection of androgen receptor antagonists
- 100 chemicals with ER, AR, PPAR activity tested in concentration-response
- Data calculated as fold-change over control (6a-fluorotestosterone/DMSO)

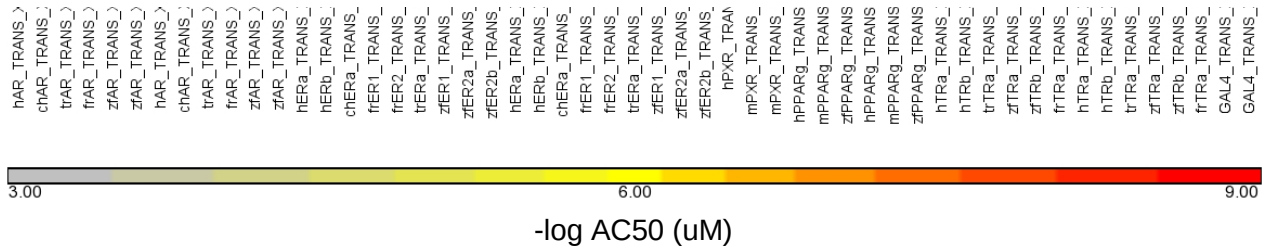
Cross-Species Differences in Nuclear Receptor Responses



= agonist
= antagonist

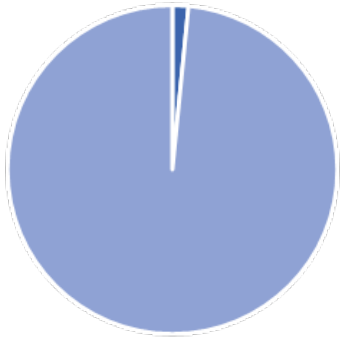






Beginning to Address Concerns for Increased Biological Coverage

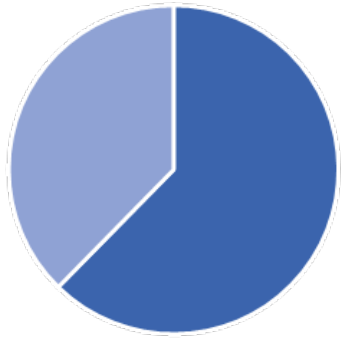
Gene Coverage



ToxCast

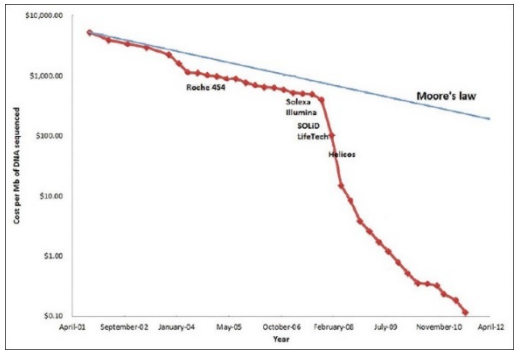
Not in ToxCast

Pathway Coverage*

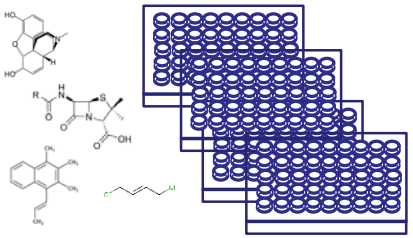


*At least one gene from pathway represented

High-throughput Genomics (HTTr)

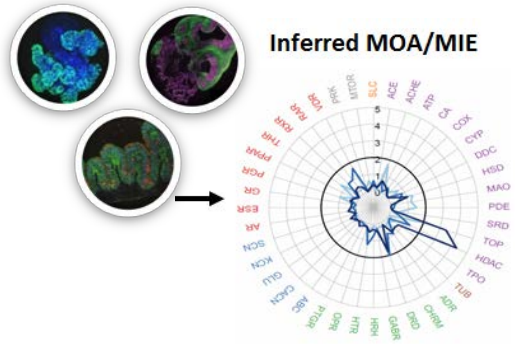


Thousands of chemicals



X

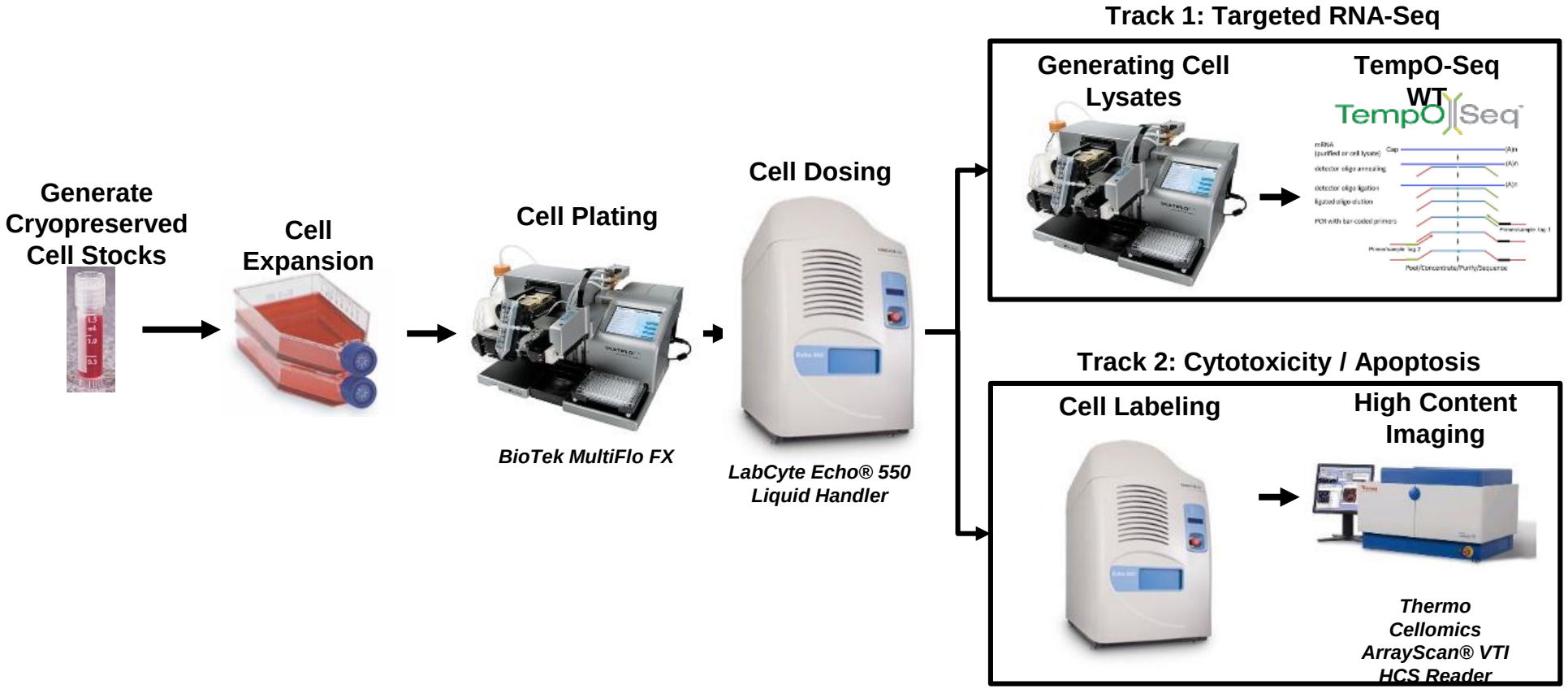
Multiple Cell Types



Requirements:

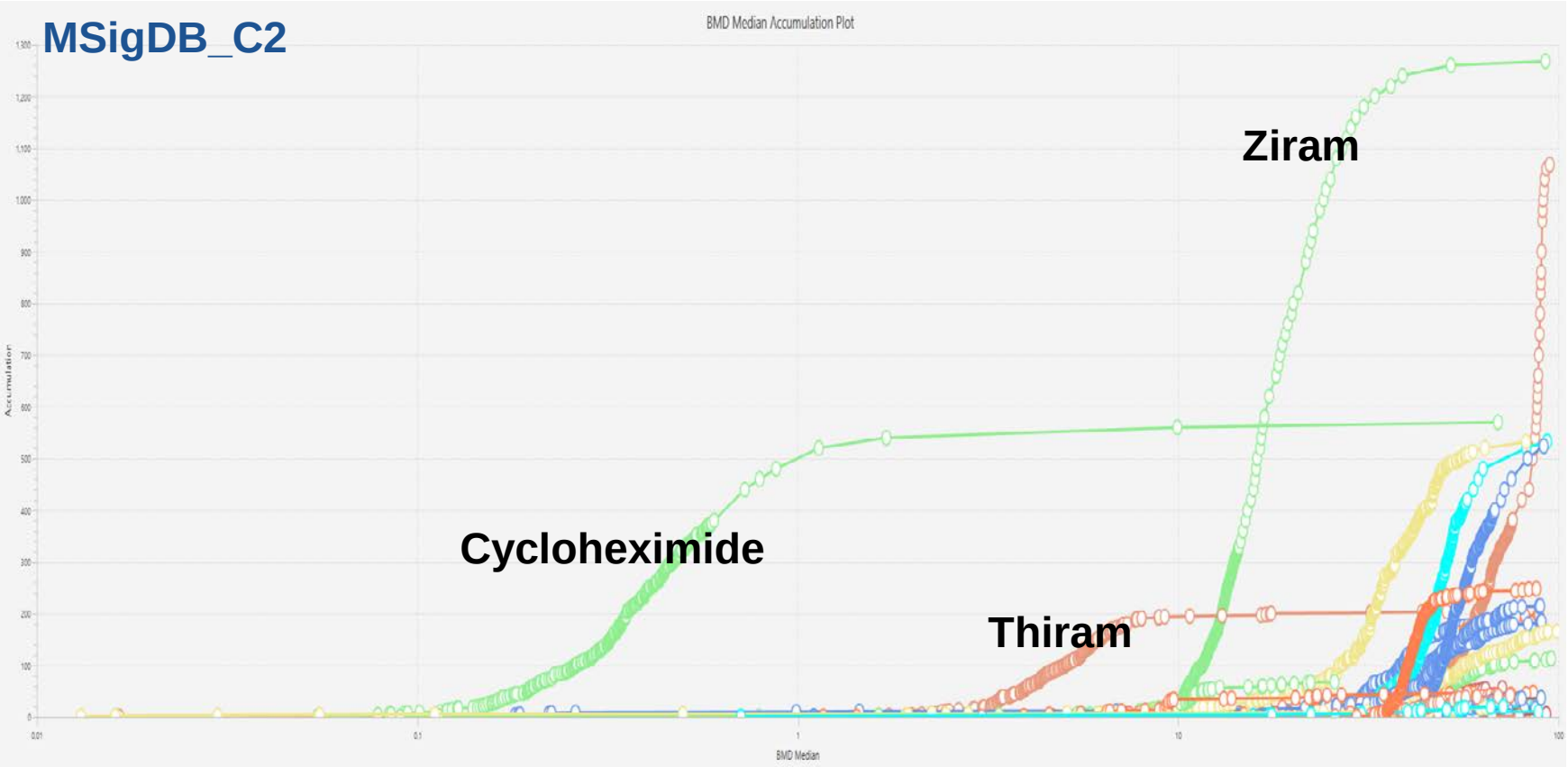
- Low cost
- Whole genome
- 384 well
- Automatable

HTTr Pilot: Workflow



Josh Harrill/NCCT

Pathway Potencies by Benchmark Dose Analysis

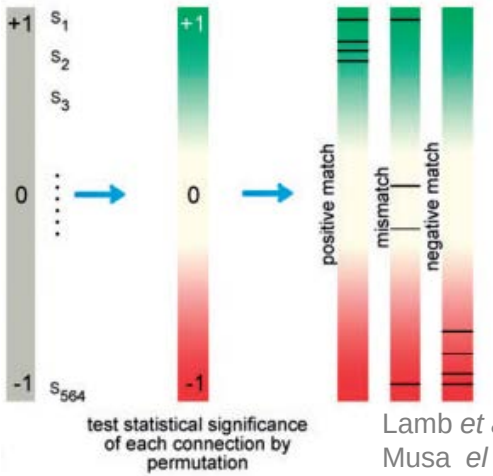


- Broad range of pathway level potency estimates and number of pathways affected across chemicals.

Josh Harrill, NCCT, unpublished

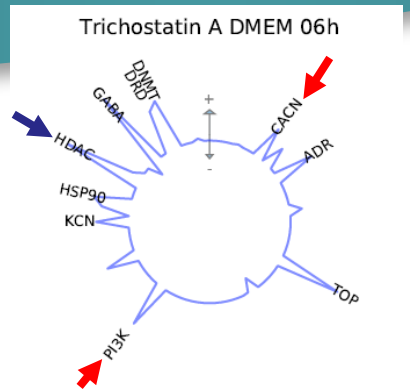
Predicting MoA by Connectivity Mapping

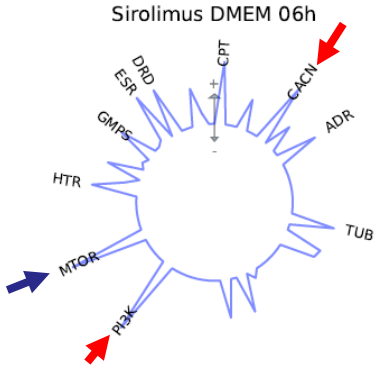
Connectivty mapping

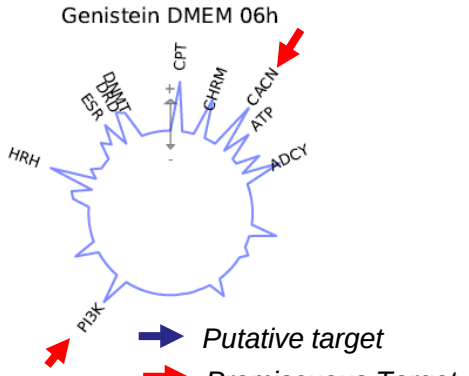


Lamb *et al* (2006)
Musa *el al* (2017)

- Connectivity Mapping
  - Use DEGs / CRGs to define "signatures" for each chemical or treatment
  - Search signature database annotated with MoA
  - Infer MoA using pair-wise similarity between signature







- Differential gene expression observed with reference chemicals
- Putative targets identified using Connectivity Mapping
- Large degree of promiscuity of predicted targets observed
- Currently evaluating additional methods for MIE prediction

Imran Shah, NCCT, unpublished

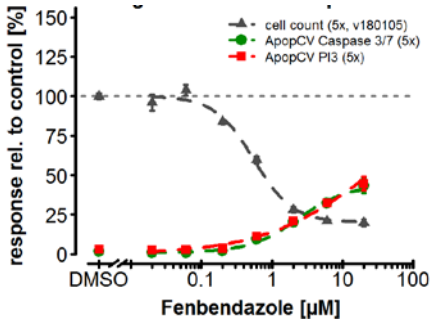
Cell Painting Phenotypic Screen Background

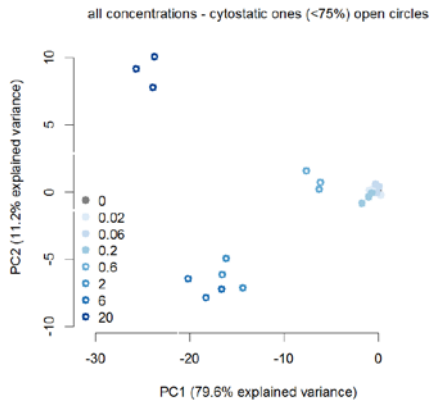
- Cell Painting (Bray et al., 2016, *Nature Protocols*): A cell morphology-based phenotypic profiling assay multiplexing six fluorescent "non-antibody" labels, imaged in five channels, to evaluate multiple cellular compartments and organelles.
- Key Features:
  - Non-targeted screening (i.e. target agnostic)
  - Tractable across different adherent cell lines
  - High content 100s – 1000s of features measured at the cell level
  - Concentration-response analysis
  - Fingerprinting and clustering

Marker	Cellular Component	Labeling Chemistry	Labeling Phase	Opera Phenix	
				Excitation	Emission
Hoechst 33342	Nucleus	Bisbenzamide probe that binds to dsDNA	Fixed	405	480
Concanavalin A – AlexaFluor 488	Endoplasmic reticulum	Lectin that selectively binds to α-mannopyranosyl and α-glucopyranosyl residues enriched in rough endoplasmic reticulum		435	550
SYTO 14 nucleic acid stain	Nucleoli	Cyanine probe that binds to ssRNA		435	550
Wheat germ agglutinin (WGA) – AlexaFluor 555	Golgi Apparatus and Plasma Membrane	Lectin that selectively binds to sialic acid and N-acetylglucosaminyl residues enriched in the trans-Golgi network and plasma membrane		570	630
Phalloidin – AlexaFluor 568	F-actin (cytoskeleton)	Phallotoxin (bicyclic heptapeptide) that binds filamentous actin			
MitoTracker Deep Red	Mitochondria	Accumulates in active mitochondria	Live	650	760

Example Results

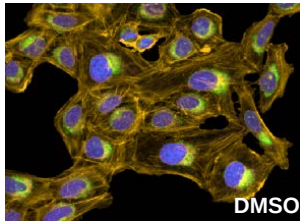
U-2 OS (-SYTO)

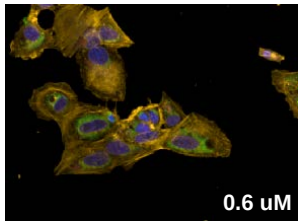


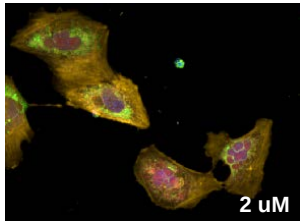


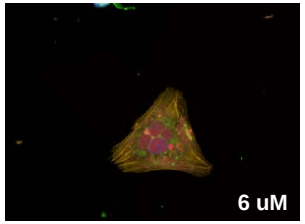
- multinucleated cells!

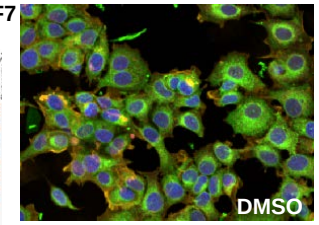
Expected phenotype: Giant, multi-nucleated cells

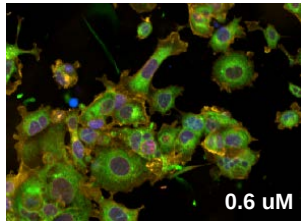


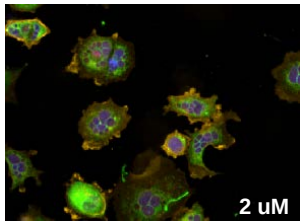


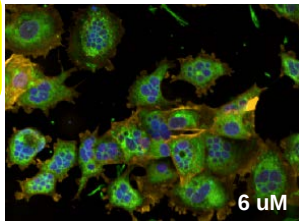




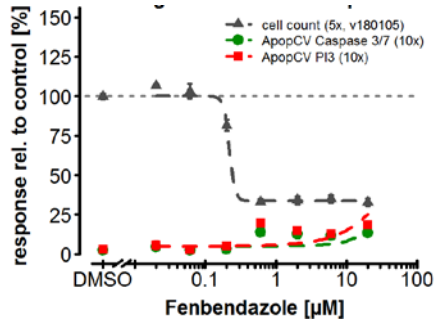


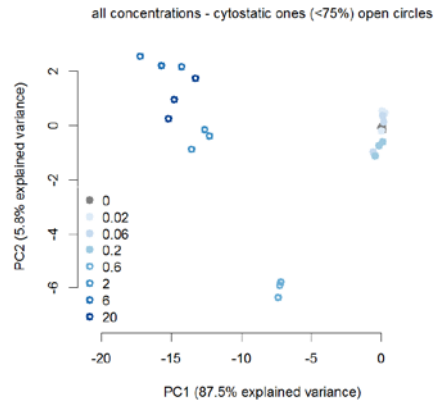






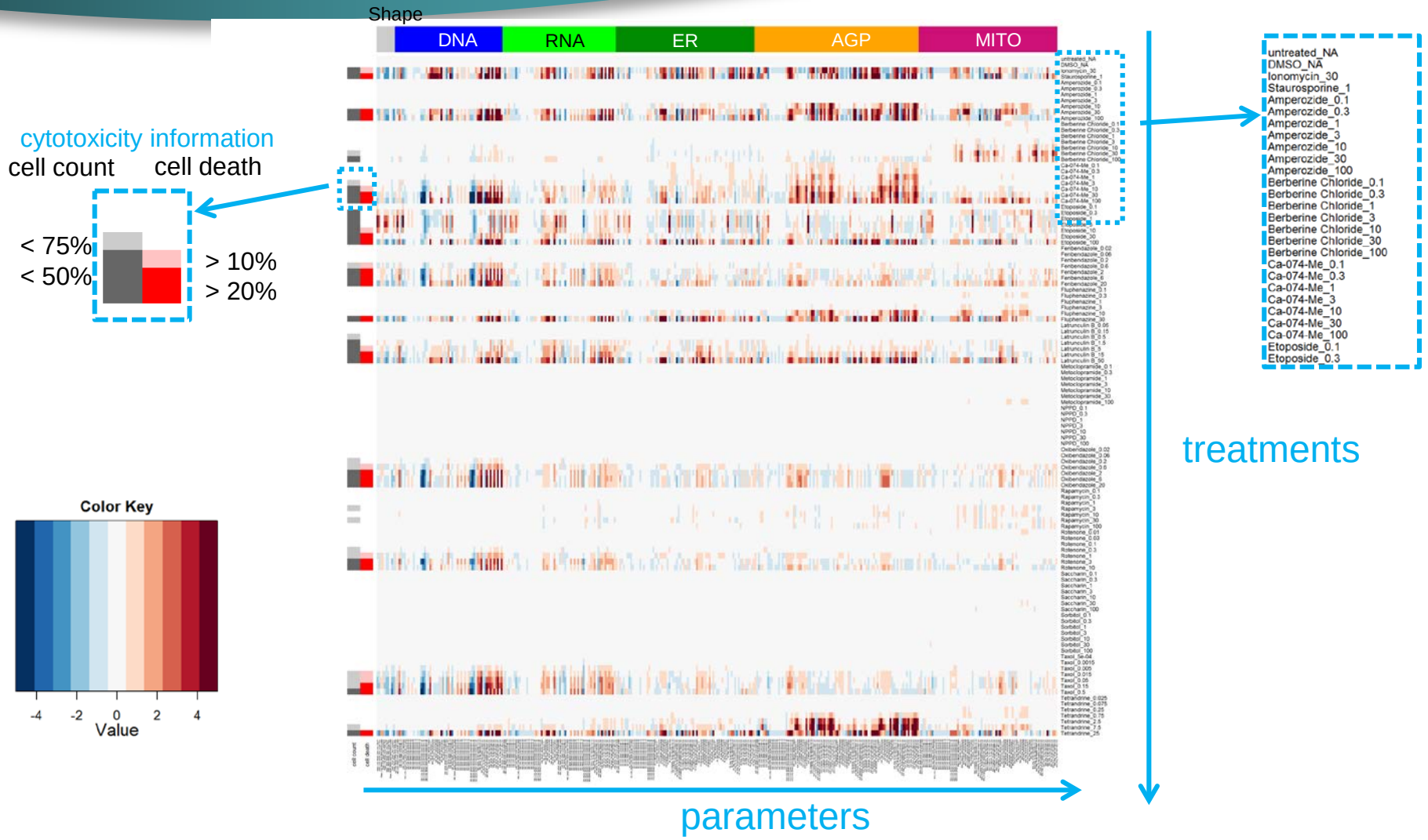
MCF7 (-SYTO)





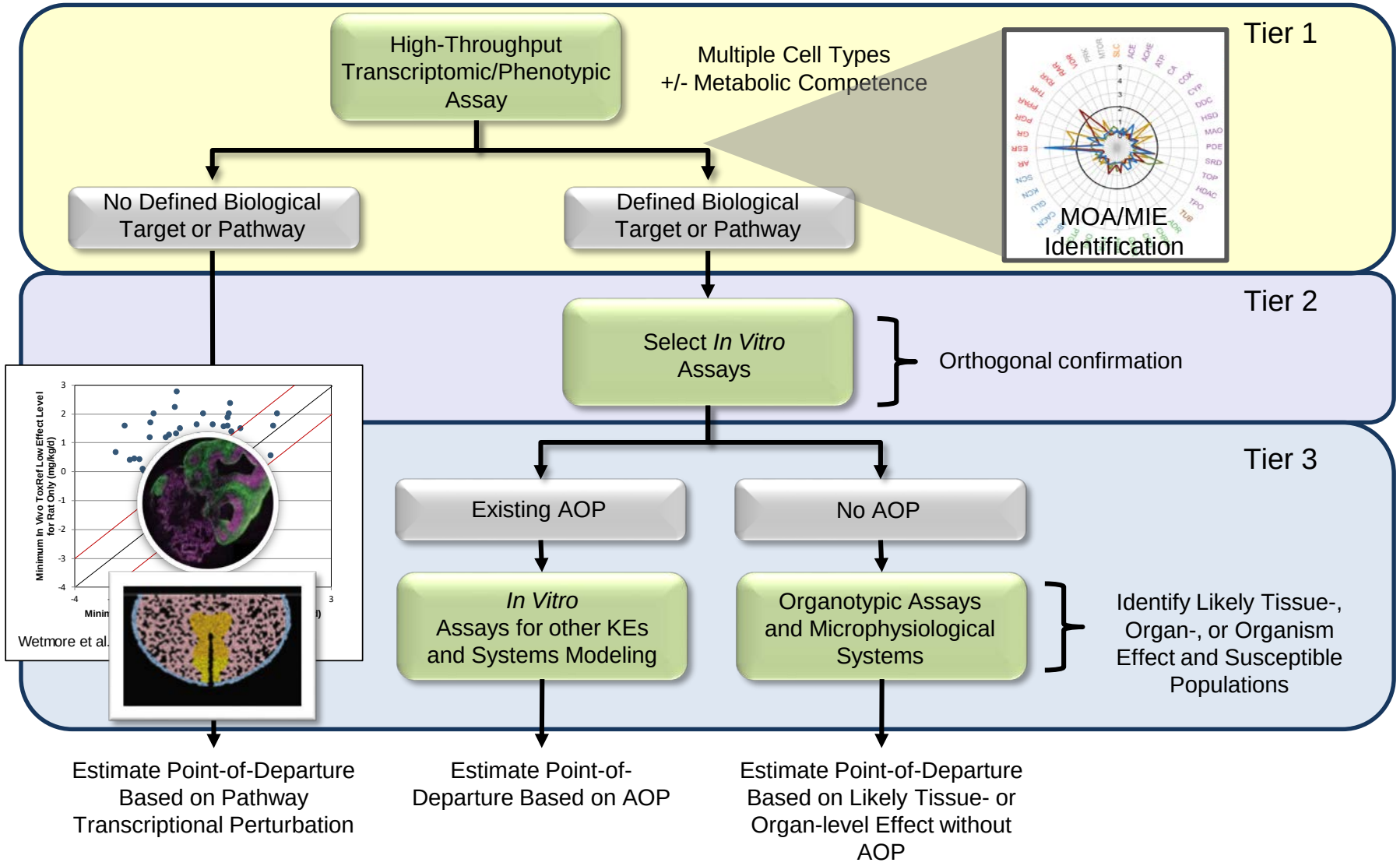
Josh Harrill, NCCT, unpublished

Preliminary Results



Josh Harrill, NCCT, unpublished

Framework for Integrating Hazard Components…



Moving Towards Risk: Adding a High-Throughput Toxicokinetic Component

EPA ToxCast Phase I and II Chemicals

Human Liver Metabolism

Human Plasma Protein Binding

Population-Based IVIVE Model

Upper 95th Percentile Css Among 100 Healthy Individuals of Both Sexes from 20 to 50 Yrs Old

- Currently evaluated ~700 ToxCast Phase I and II chemicals
- Models available through "“httk” R package (https://cran.r-project.org/web/packages/httk/)

In Vitro Potency Value

Plasma Concentration

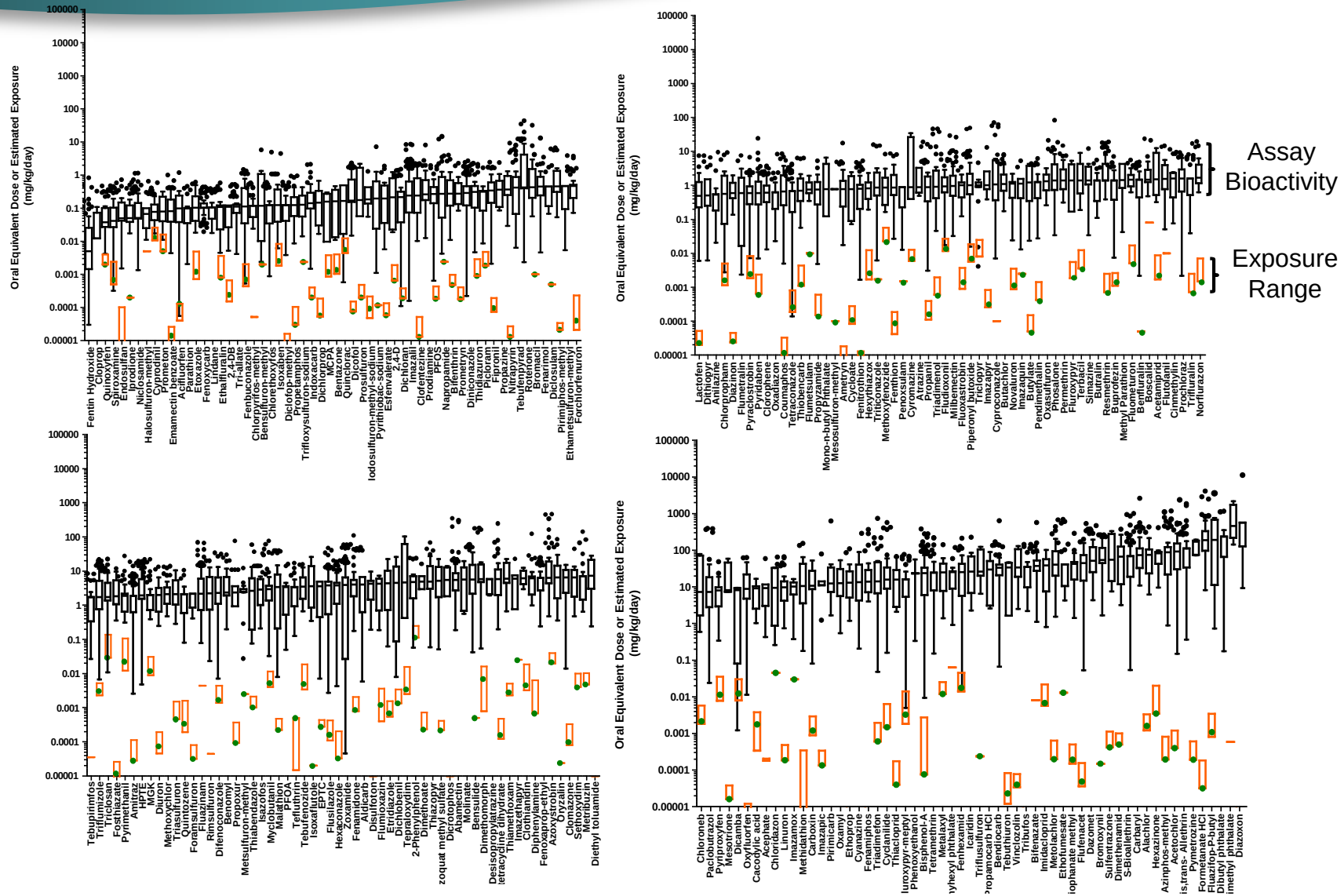
Oral Exposure

Reverse Dosimetry

Oral Dose Required to Achieve Steady State Plasma Concentrations Equivalent to *In Vitro* Bioactivity

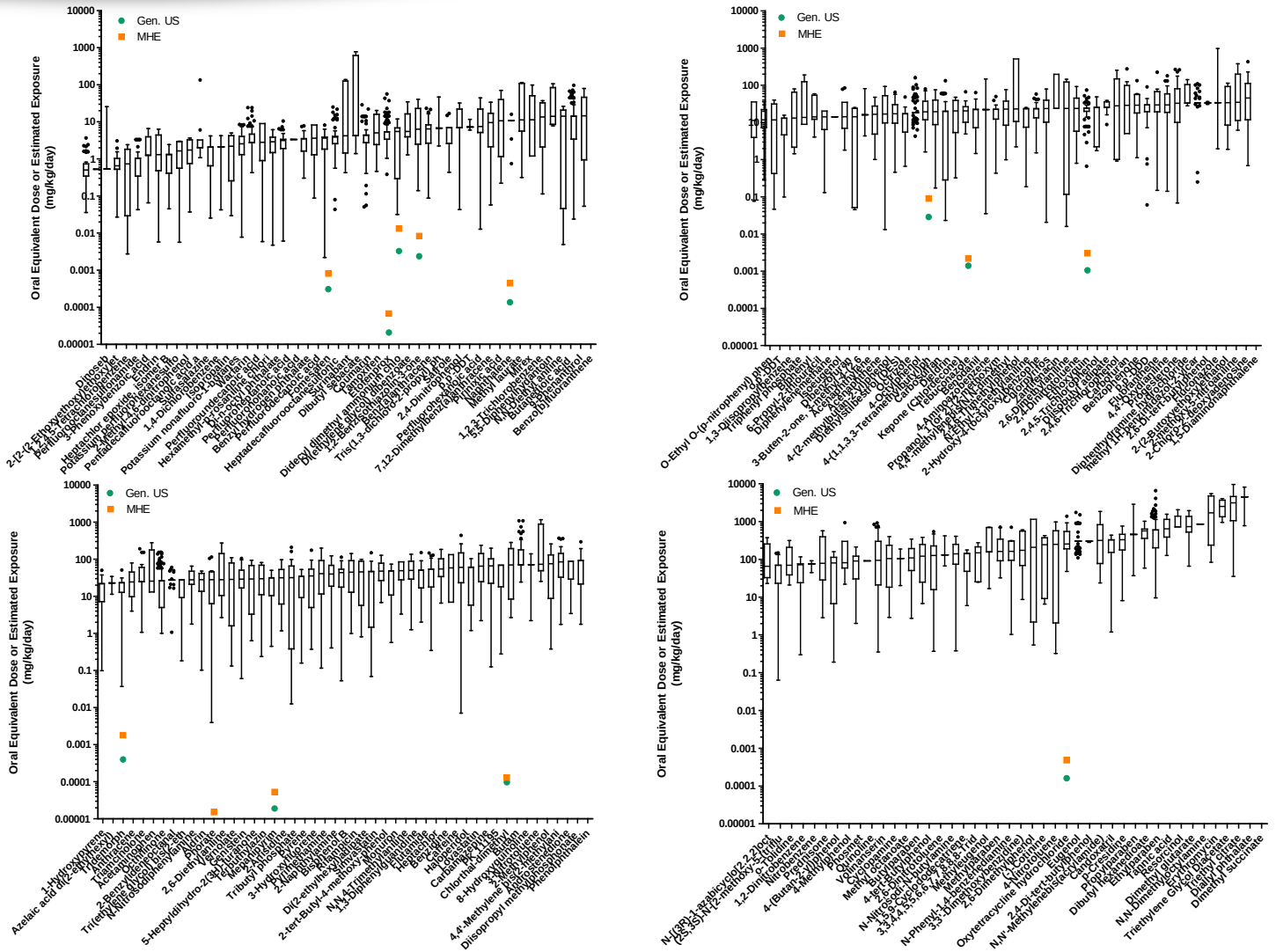
Rotroff *et al., Tox Sci.*, 2010
Wetmore *et al., Tox Sci.,* 2012

Comparing Dosimetry Adjusted Bioactivity with Exposure



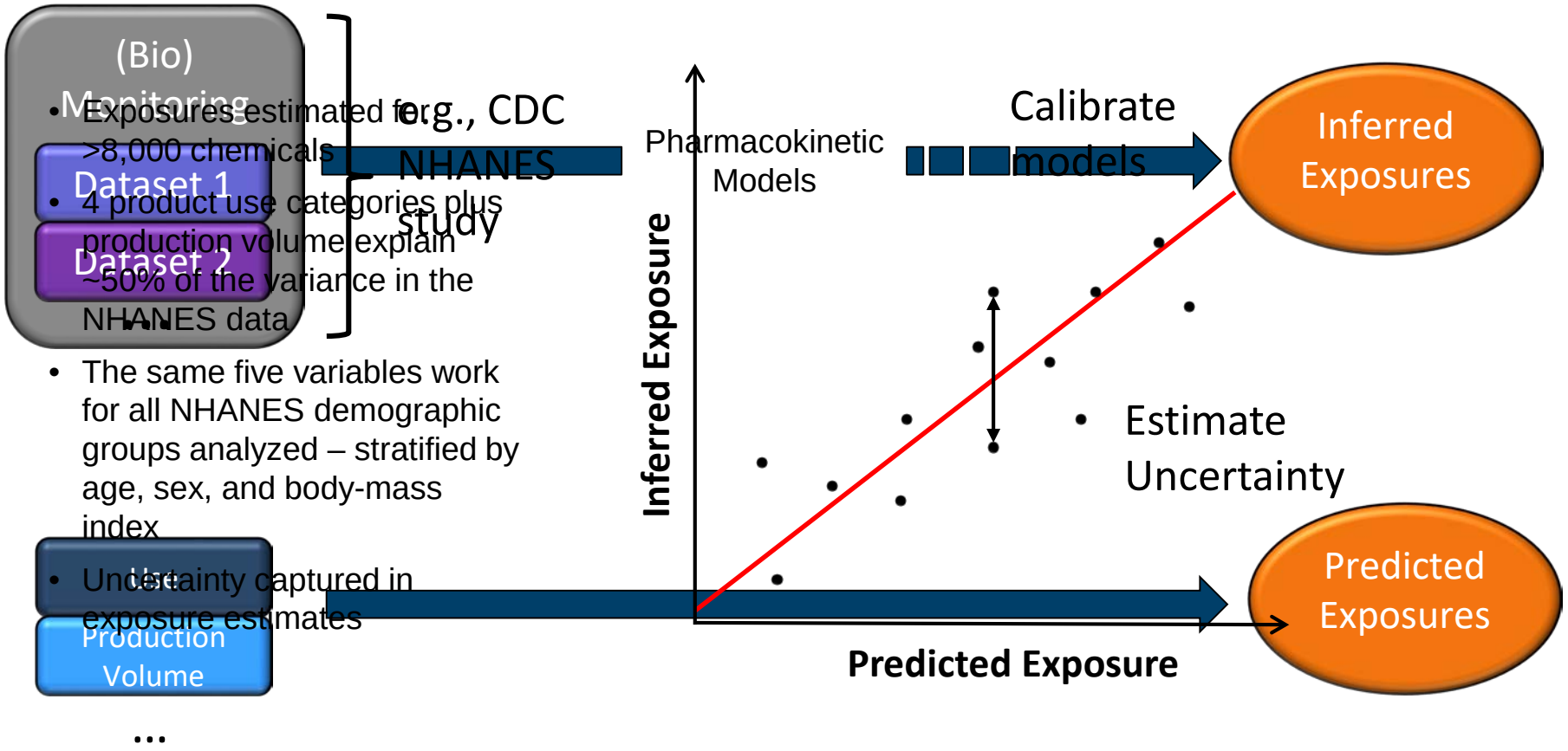
Wetmore *et al.*, *Tox Sci.*, 2012

But, Exposure Information is Lacking on Most Chemicals



Bioactivity from Wetmore *et al., Tox Sci.,* 2015

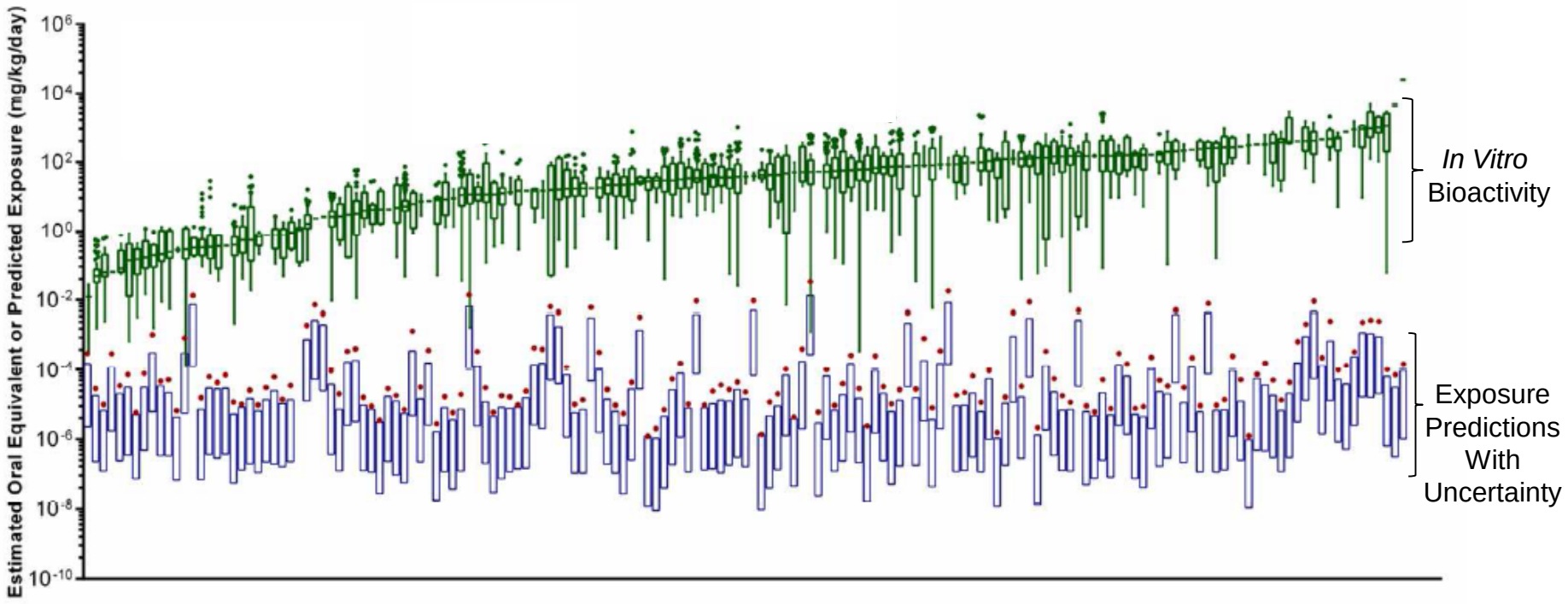
Adding a High-Throughput Exposure Component



- Exposures estimated for >8,000 chemicals
- 4 product use categories plus production volume explain ~50% of the variance in the NHANES data
- The same five variables work for all NHANES demographic groups analyzed – stratified by age, sex, and body-mass index
- Uncertainty captured in exposure estimates

Wambaugh *et al.*, 2014

Comparing Bioactivity with Exposure Predictions for Risk Context



Wetmore *et al., Tox Sci.,* 2015

Concluding Remarks

- In vitro/alternative approaches valuable for chemical prioritization, especially where we understand the toxicity pathways
- May also be useful in conservative point-of-departure approaches for unknown/non-selective effects
- Capable of data generation for thousands of chemicals
- *In vitro* assays usually require orthogonal assays to distinguish **true** from **false** positives
- Combining with exposure predictions allows prioritization for risk

Thank You for Your Attention!

EPA's National Center for Computational Toxicology