

Heavy Metals in ToxCast: Relevance to Food Safety

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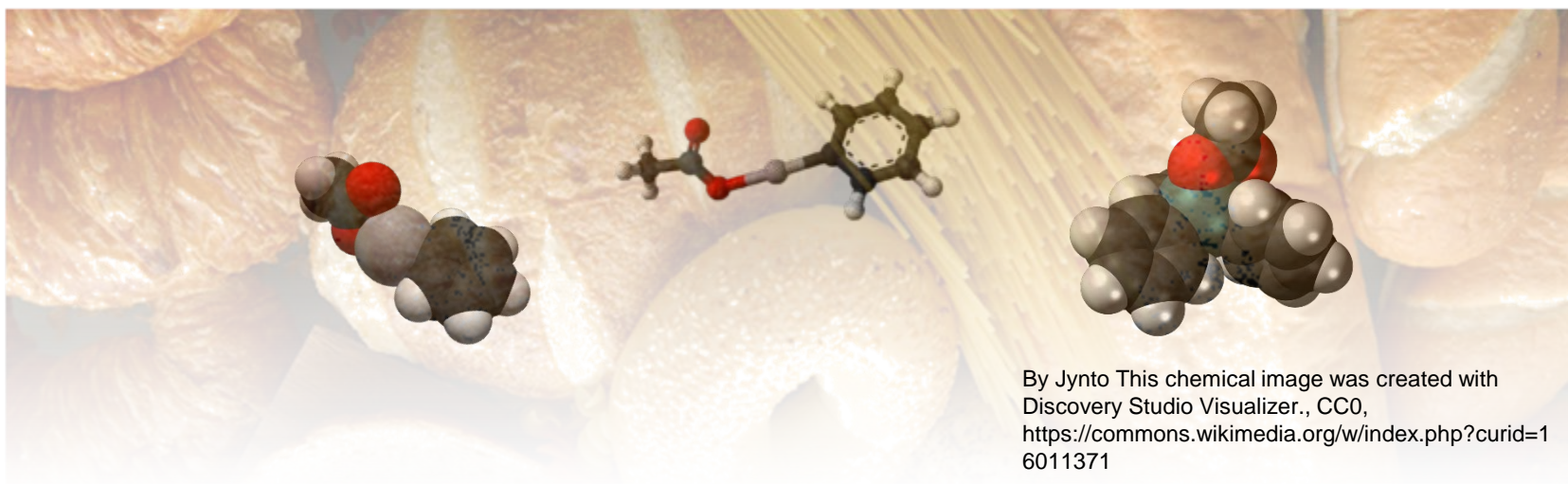
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The author has no conflict of interest disclosures.

Society of Toxicology 55th Annual Meeting
15 March 2016
NOLA

Outline

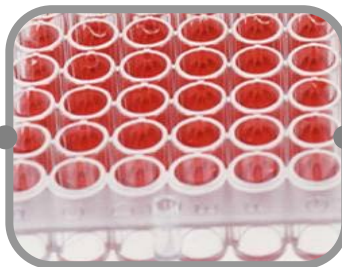
- Introduction to HTT
- Metals in Tox21/ToxCast
- Bioactivity screening
 - Transcription factor effects
 - Stress response
 - Nuclear receptor
 - Biochemical assays
 - Nuclear receptors
 - GPCRs
 - Tox21 assays
 - Nuclear receptors
 - Stress response
- Summary



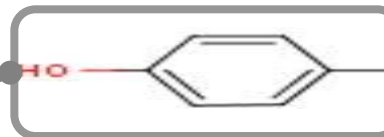
ToxCast/Tox21 :High-Throughput Screening (HTS): Hazard Predictions for Prioritization



Robots



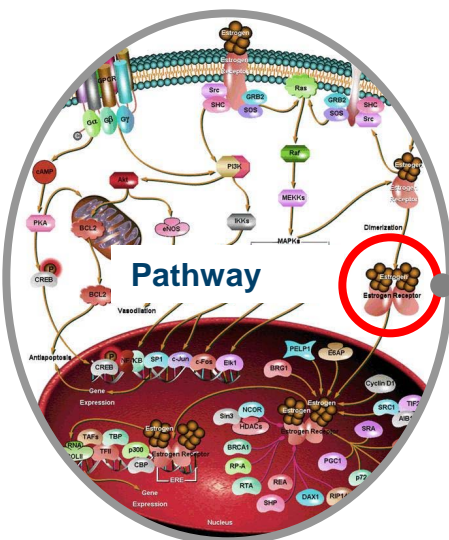
96-, 384-, 1536 Well Plates



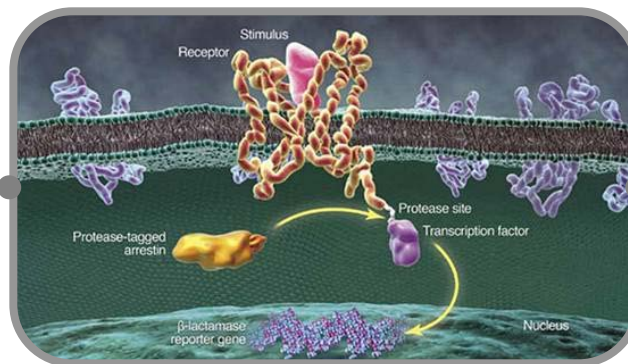
Chemical Exposure



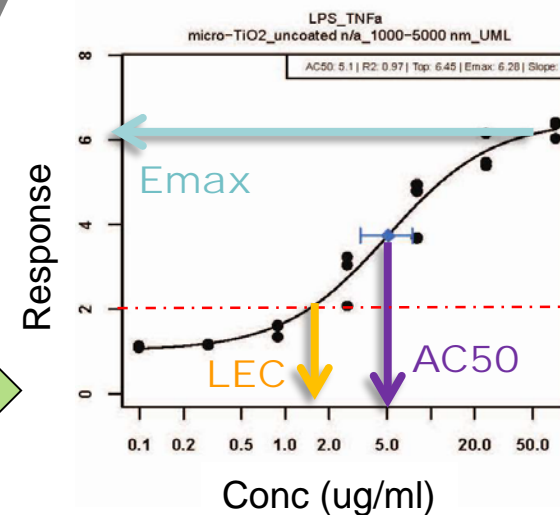
Cell Population



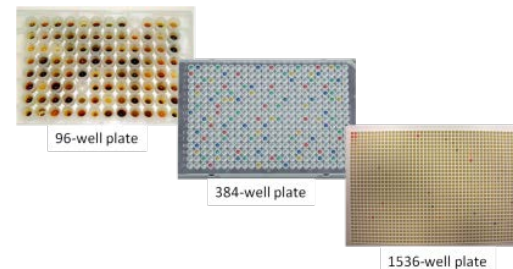
Pathway



Target Biology (e.g.,
Estrogen Receptor)



ToxCast Assays (>800 endpoints)



Assay Provider

ACEA
Apredica
Attagene
BioReliance
BioSeek
CeeTox
CellzDirect
Tox21/NCATS
NHEERL MESC
NHEERL Zebrafish
NovaScreen (Perkin Elmer)
Odyssey Thera
Vala Sciences

Biological Response

cell proliferation and death
cell differentiation
Enzymatic activity
mitochondrial depolarization
protein stabilization
oxidative phosphorylation
reporter gene activation
gene expression (qNPA)
receptor binding
receptor activity
steroidogenesis

Target Family

response Element
transporter
cytokines
kinases
nuclear receptor
CYP450 / ADME
cholinesterase
phosphatases
proteases
XME metabolism
GPCRs
ion channels

Assay Design

viability reporter
morphology reporter
conformation reporter
enzyme reporter
membrane potential reporter
binding reporter
inducible reporter

Readout Type

single
multiplexed
multiparametric

Cell Format

cell free
cell lines
primary cells
complex cultures
free embryos

Species

human
rat
mouse
zebrafish
sheep
boar
rabbit
cattle
guinea pig






Tissue Source

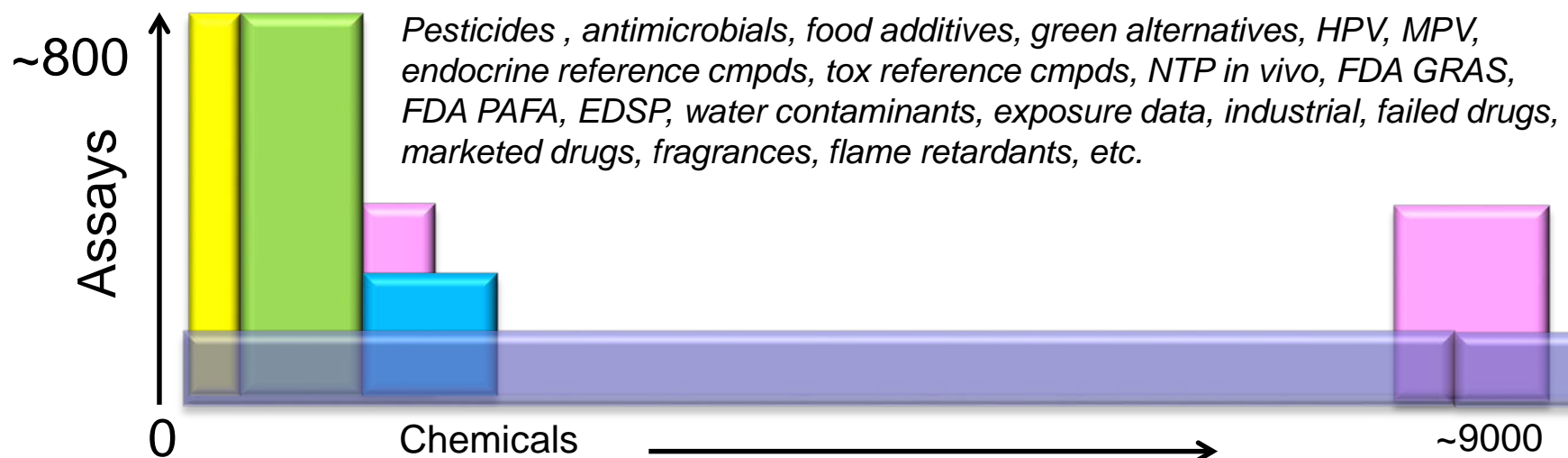
Lung	Breast
Liver	Vascular
Skin	Kidney
Cervix	Testis
Uterus	Brain
Intestinal	Spleen
Bladder	Ovary
Pancreas	Prostate
Inflammatory	Bone

Detection Technology

qNPA and ELISA
Fluorescence & Luminescence
Alamar Blue Reduction
Arrayscan / Microscopy
Reporter gene activation
Spectrophotometry
Radioactivity
HPLC and HPEC
TR-FRET

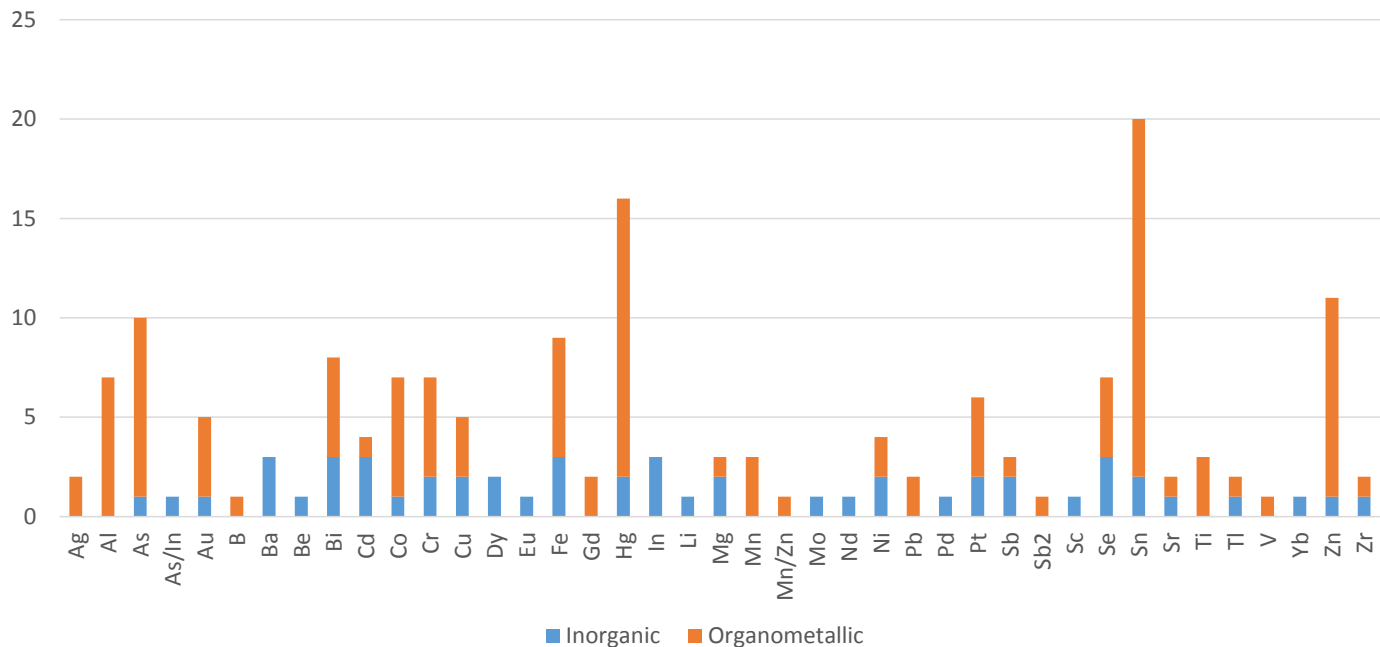
ToxCast & Tox21: Chemicals, Data and Release Timelines

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
ToxCast Phase III	 ~900	~300	~300	In progress	2016
Tox21	 ~9000	~80	~150	In progress	ongoing



Tox21 Metals Cross-Referenced with Food-Relevant Chemical Inventories

Tested Chemical Classes



Source Inventory

Alan Wood: Pesticide

FDA Everything Added to Food in the United States

FDA GRAS

FDA Inventory of Effective Food Contact Substance

FDA List of Indirect Additives Used in Food Contact Substances

Karmaus et al. (2015) Food & Chemical Toxicology, *in press*.

Office of Research and Development
National Center for Computational Toxicology

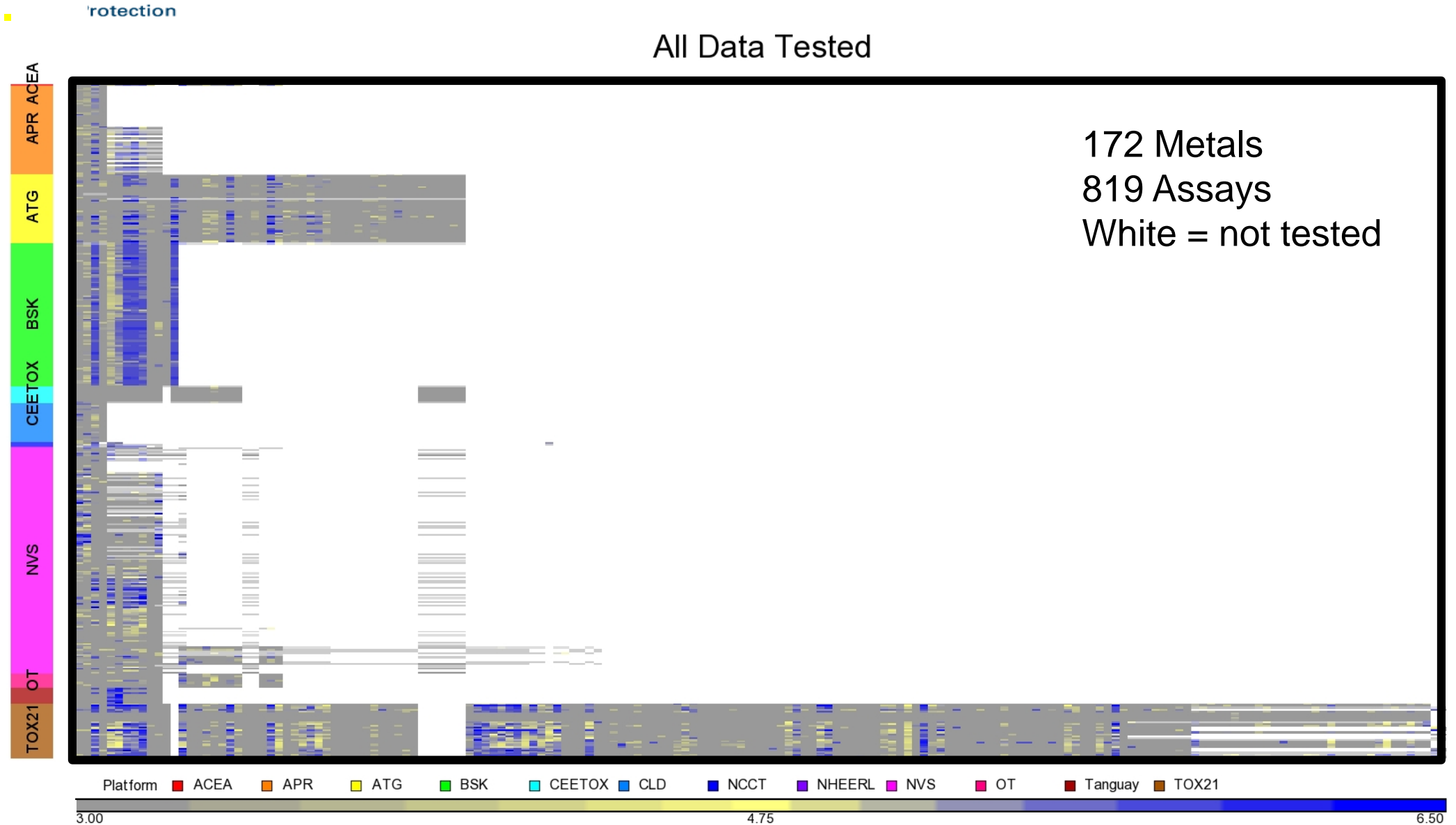
Chemical Name	Direct Food Additive	Food Contact Substance	Pesticide
Aluminum monostearate		1	
Aluminum tributoxide		1	
Arsenic trioxide			1
Bis(tributyltin)oxide		1	1
Cobalt(II) acetate		1	
Copper dimethyldithiocarbamate		1	
Copper(II) iodide		1	
Dibutyltin diacetate		1	
Dibutyltin dichloride		1	
Dibutyltin dilaurate		1	
Dichloro(dioctyl)stannane		1	
Dimethylarsinic acid			1
Ferbam			1
Ferric chloride		2	2
Iron(II) sulfate		2	1
Maneb			1
Manganese(II) acetate		1	
Mercuric chloride			1
Phenylmercuric acetate			1
Phenylmercuric chloride			1
Potassium dicromate		1	
Tetrachlorostannane		1	1
Thimerosal			1
Tin(II) chloride		2	
Tributyltin chloride		1	
Triphenyltin acetate			1
Triphenyltin chloride			1
Triphenyltin hydroxide			1
Vitamin B12		2	
Zinc acetate		2	
Zinc chloride		2	
Zinc dibutyldithiocarbamate		1	
Zinc diethyldithiocarbamate		1	
Zinc octanoate		1	
Zinc pyrrithione		1	
Zinc salicylate		1	
Ziram		1	1
Grand Total	14	19	14

- Broad definition for “metals”
- 172 in ToxCast and/or Tox21 chemical libraries
- 37 found on “food” inventories

Matrix of All Testing Results

All Data Tested

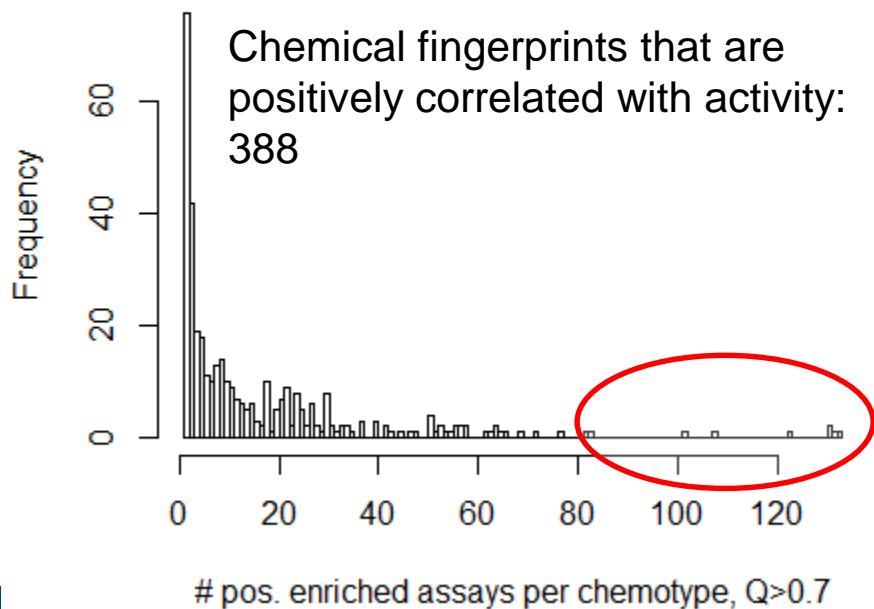
Assays



Chemicals

ToxPrint Chemotype Analysis

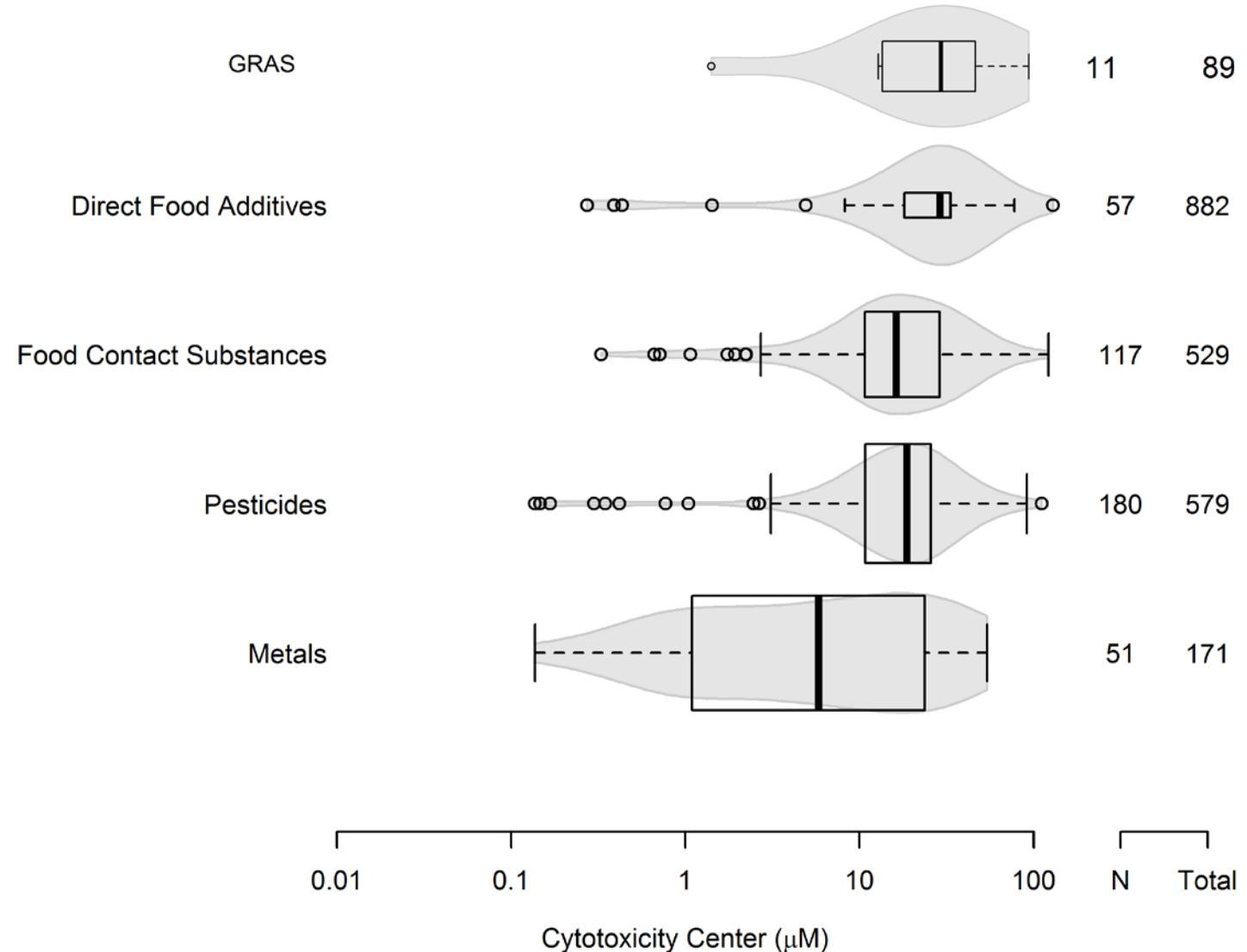
- Fingerprinting of chemical structures against chemotypes
 - Developed by Altamira for FDA
 - 729 chemotypes
- A single chemical may have multiple chemotype signatures
- Used odds ratio analysis to find chemotype enrichment across all ToxCast/Tox21 assays



ct_id	N
1:	atom.element_metal_poor_metal 131
2:	bond.metal_group_III_other_generic 133
3:	bond.metal_group_III_other_Sn_generic 131
4:	bond.metal_group_III_other_Sn_organo 132
5:	bond.C . O. N_carbamate_dithio 123
6:	atom.element_metal_transistion_metal 102
7:	chain.aromaticAlkane_Ph.C1.Ph 83
8:	bond.X.any_.C._halide_inorgani c 82
9:	bond.quatN_alkyl_acycli c 108

Cytotoxicity & Potency

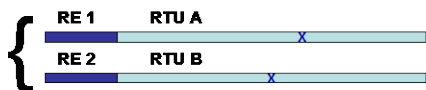
- Cytotoxicity measured through a variety of technologies in a variety of cell lines and primary cells
- Metals show high cytotoxicity, analogous to pesticides, but with even greater potency
- Question: is promiscuous and potent activity assay interference or are relevant biological/toxicological responses included?



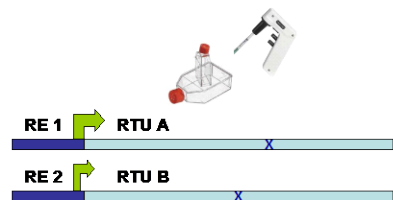
Attagene Technology: Multiplexed Reporter Gene Assays

Multiplexed Reporter Gene Assay

Library of RTUs



Cell Transfection



Transcription

RNA Isolation

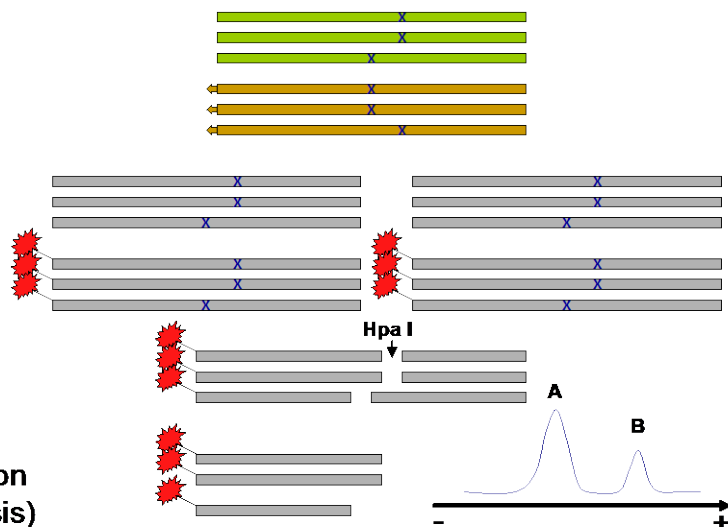
Reverse transcription

PCR amplification

Labeling

Processing (Hpa I)

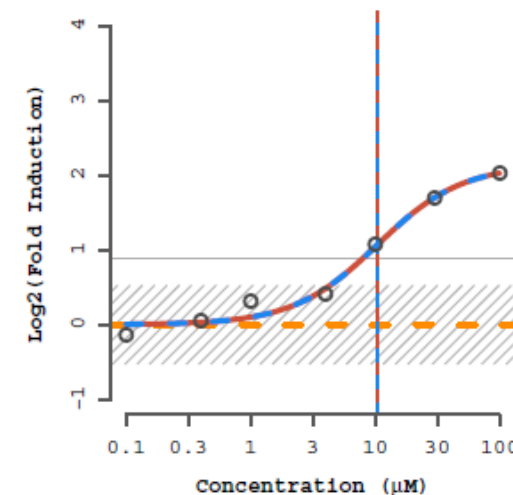
Separation and detection
(capillary electrophoresis)



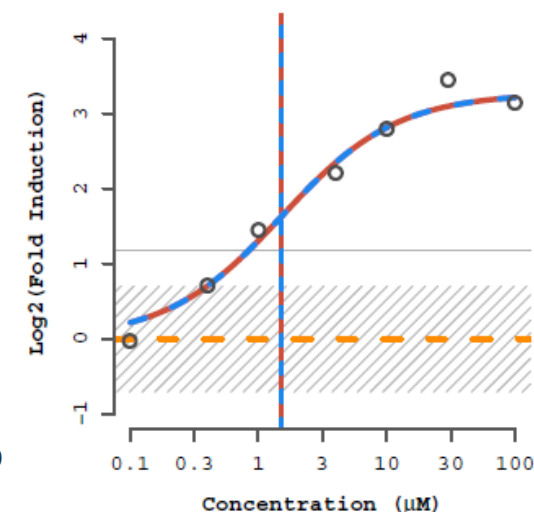
- HepG2 cells with enhanced CYP P450 activity
- Unique reporter genes controlled by transcription factor response elements
- Cis and trans formats
 - cis: endogenous TFs (52)
 - trans: Gal4-LBD NRs (30)

ASSAY: AEID102 (ATG_PPARE_CIS_up)

NAME: Lactofen
CHID: 24160 CASRN: 77501-63-4
SPID(S): TV000397
L4ID: 3591717



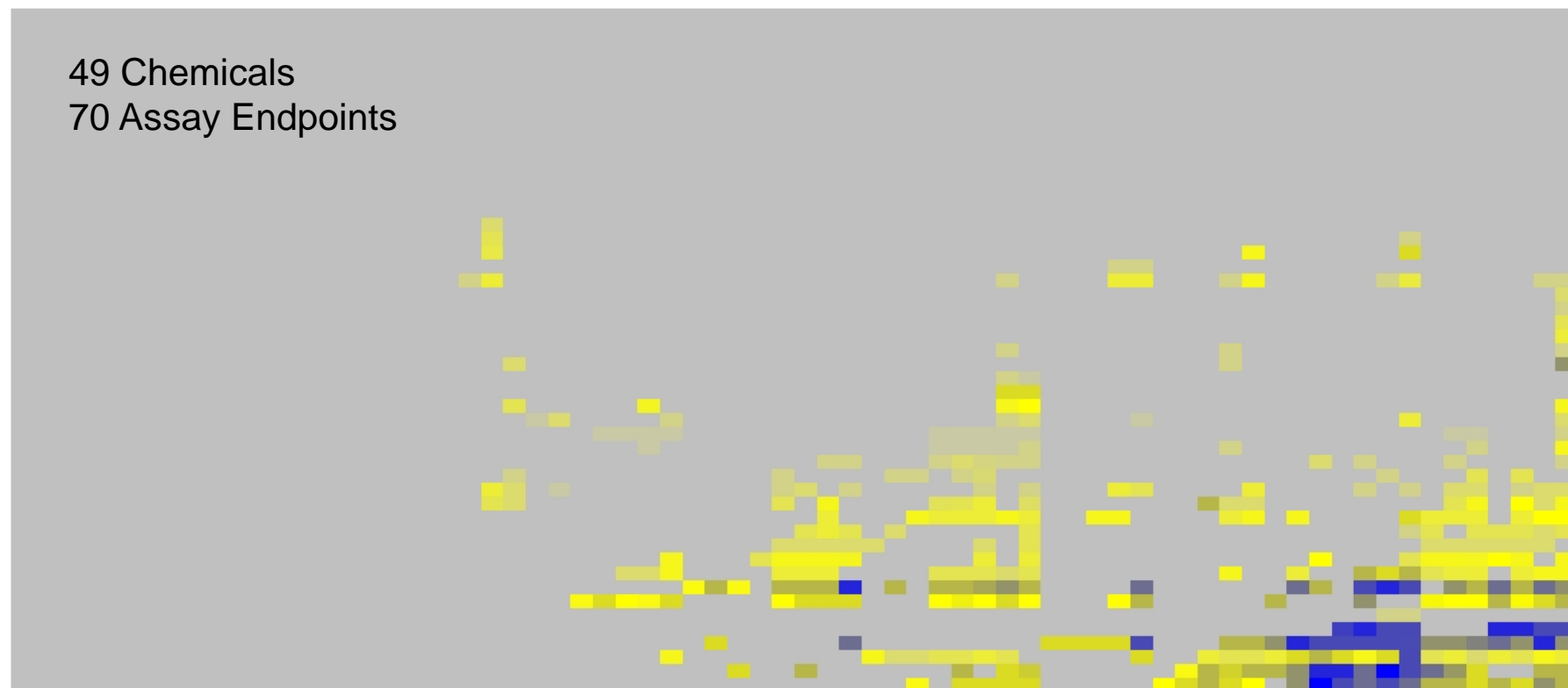
ASSAY: AEID132 (ATG_PPARE_TRANS_up)



ATG AC₅₀ Matrix

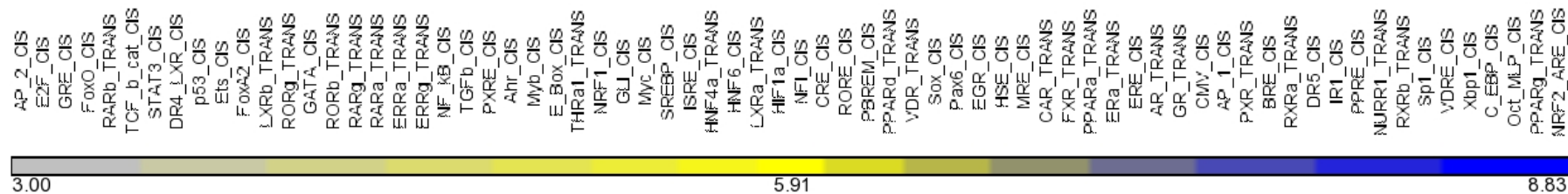


49 Chemicals
70 Assay Endpoints



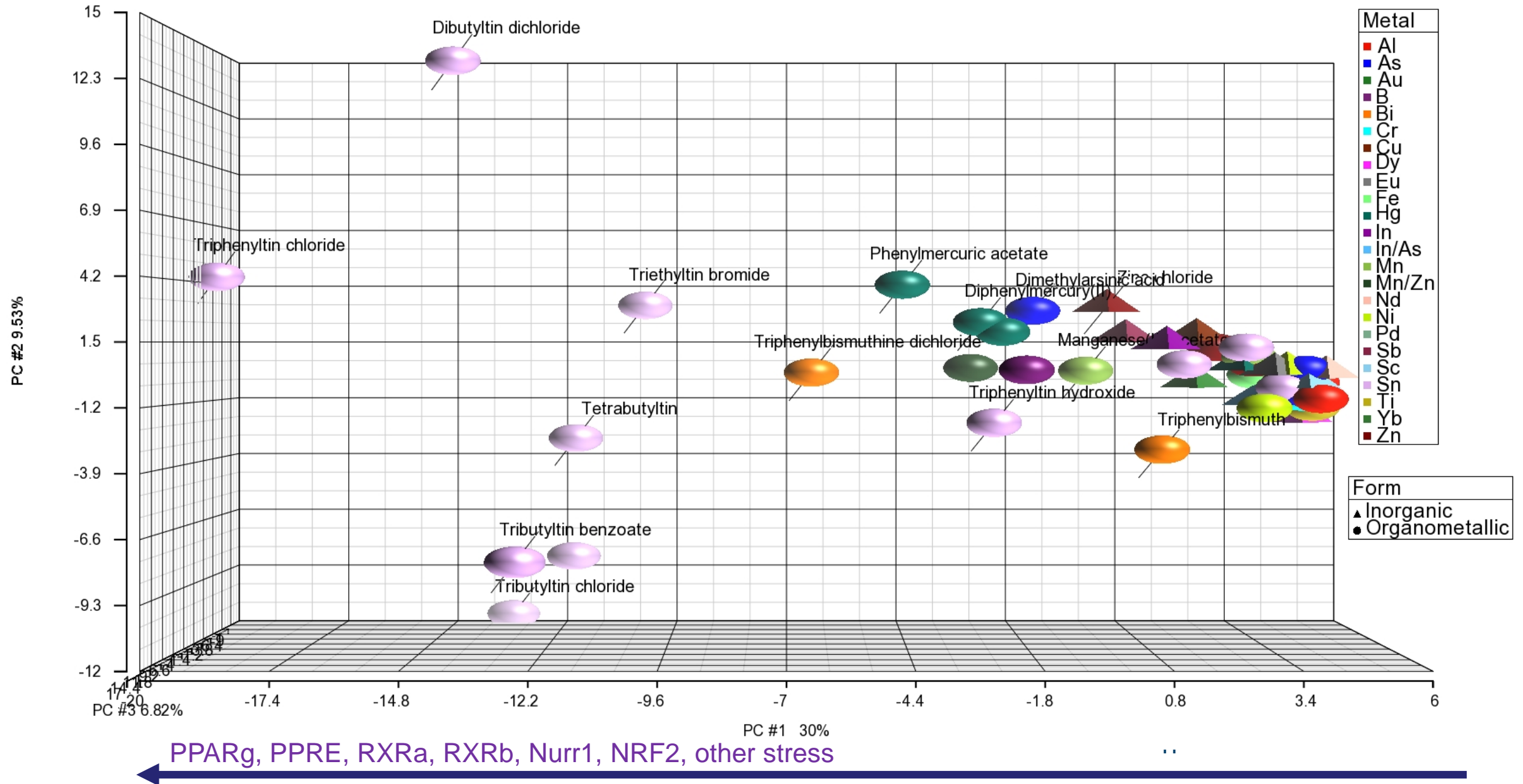
Metal
type

Organometal
vs inorganic



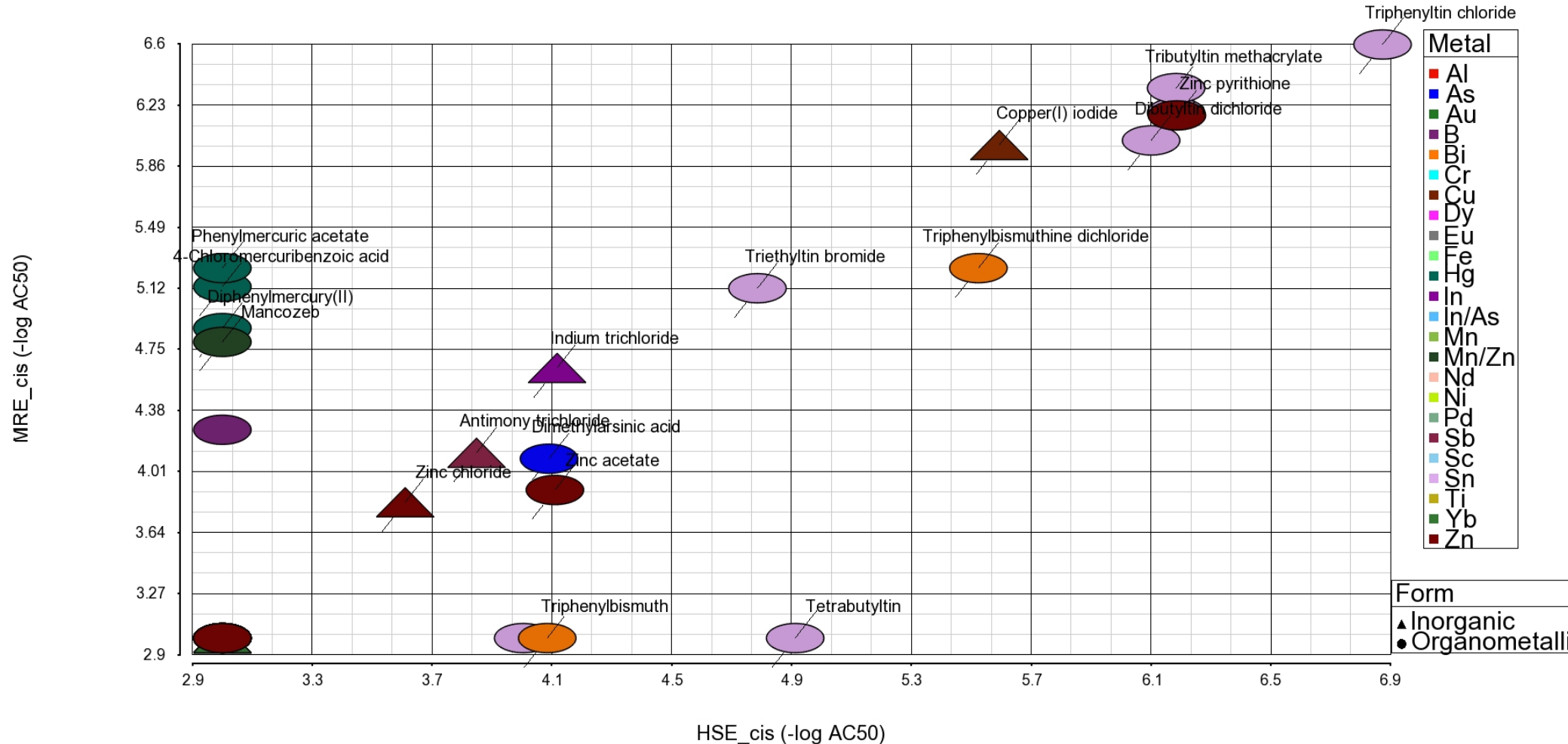
$-\log AC_{50}$

Principle Components Analysis of Transcription Factor Activation Assay



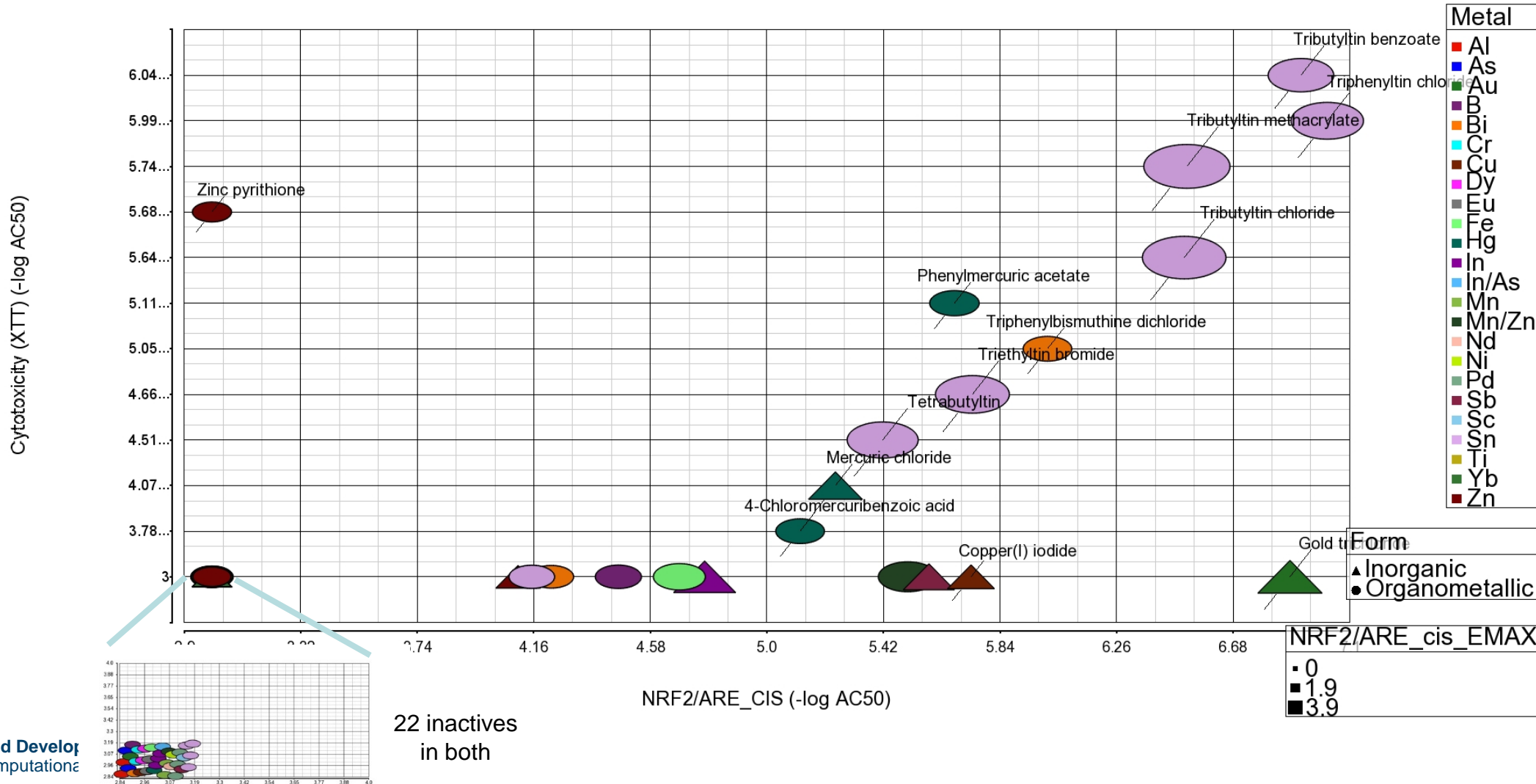
Expected Stress Pathways: Metal Response Element and Heat Shock Element

- Obvious effects on many stress pathways likely related to the cytotoxicity
- Many samples limited by cytotoxicity (no/poor RNA)
- Metal and heat shock are known stress pathways activated by metals
- 21 of 49 active in one or both



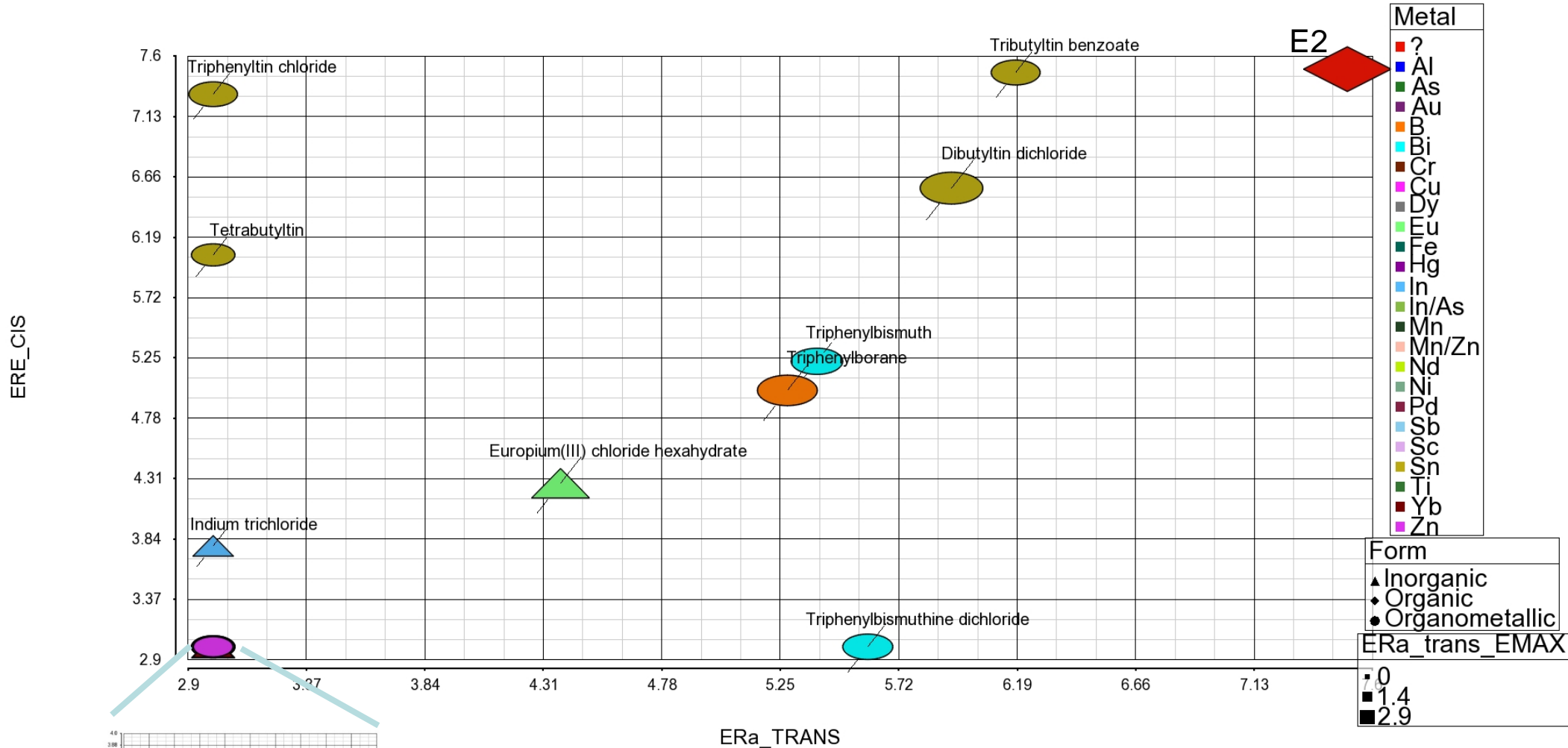
Oxidative Stress (NRF2) and Cell Death

- Many metals associated with oxidative stress
- 25 of 49 active in NRF2
- Good correlation for Sn, Hg between NRF2 and cytotox



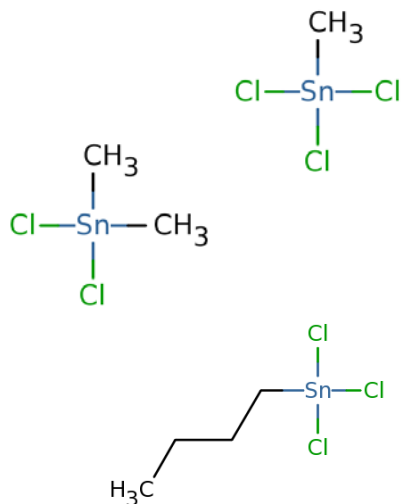
Estrogen Receptor Effects

- trans: ER α
(GAL4-LBD)
- cis: full-length
ER α
- Size by EMAX
(fold-induction)
- Sn, Bi, B active
- Some affect of
LBD vs full-
length receptor

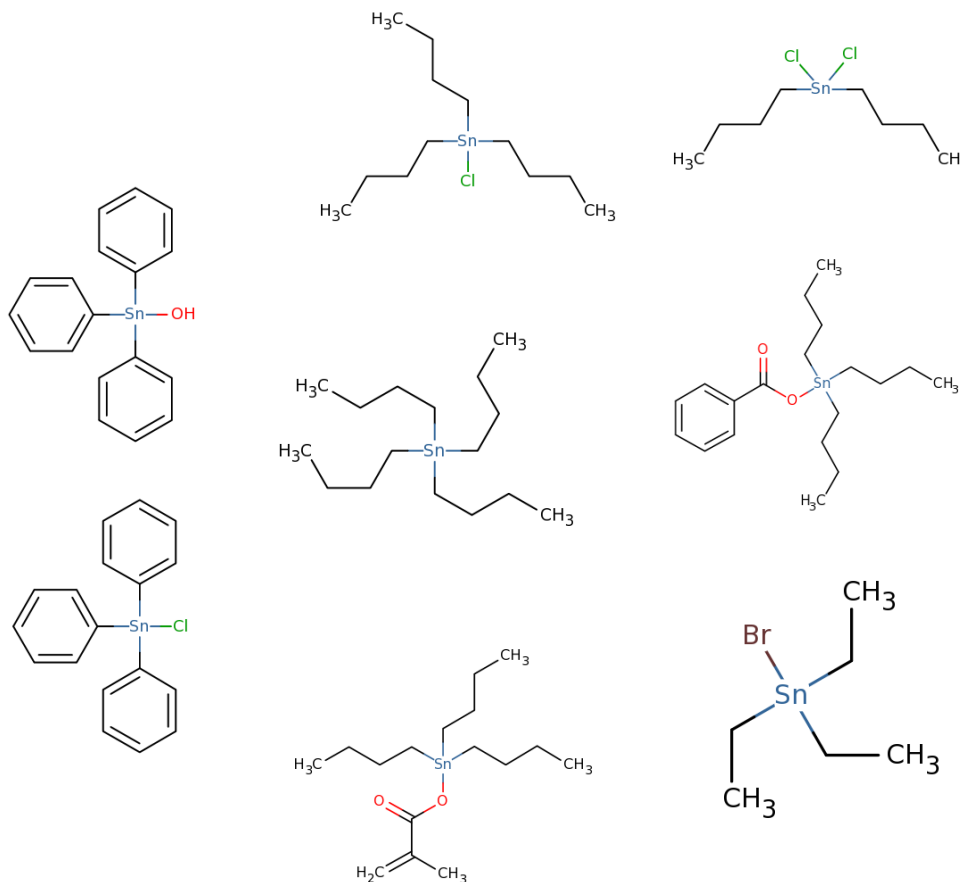


Structural Features of Agonists

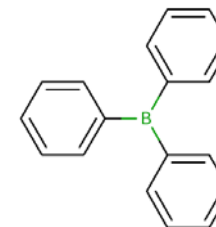
Inactive



Active



Weakly active

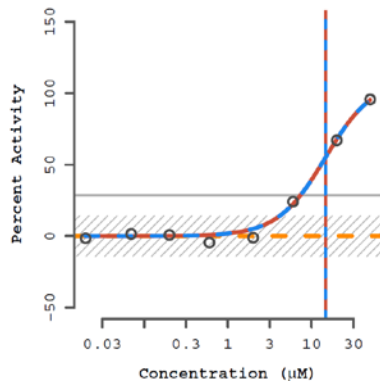


Biochemical Assays (NVS)

- **Protein super-families**

- GPCR
- Kinase
- Phosphatase
- Protease
- Ion channel
- Nuclear receptor
- Other enzyme
- CYP P450 inhibition
- Transporters

- 50 uM upper testing limit
- Many assay formats



172
Metals

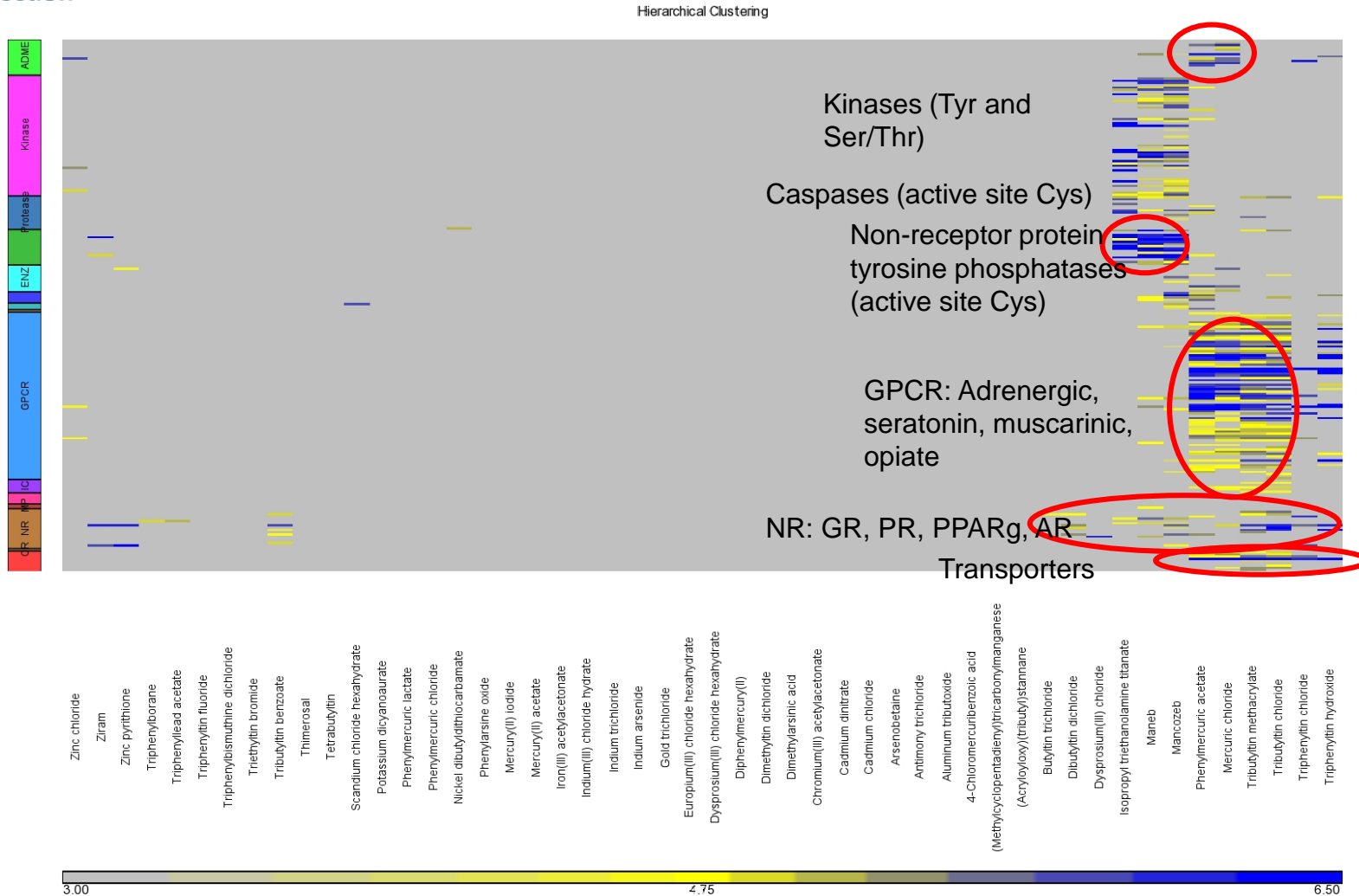
Tested Matrix: Biochemical Assays

50
65

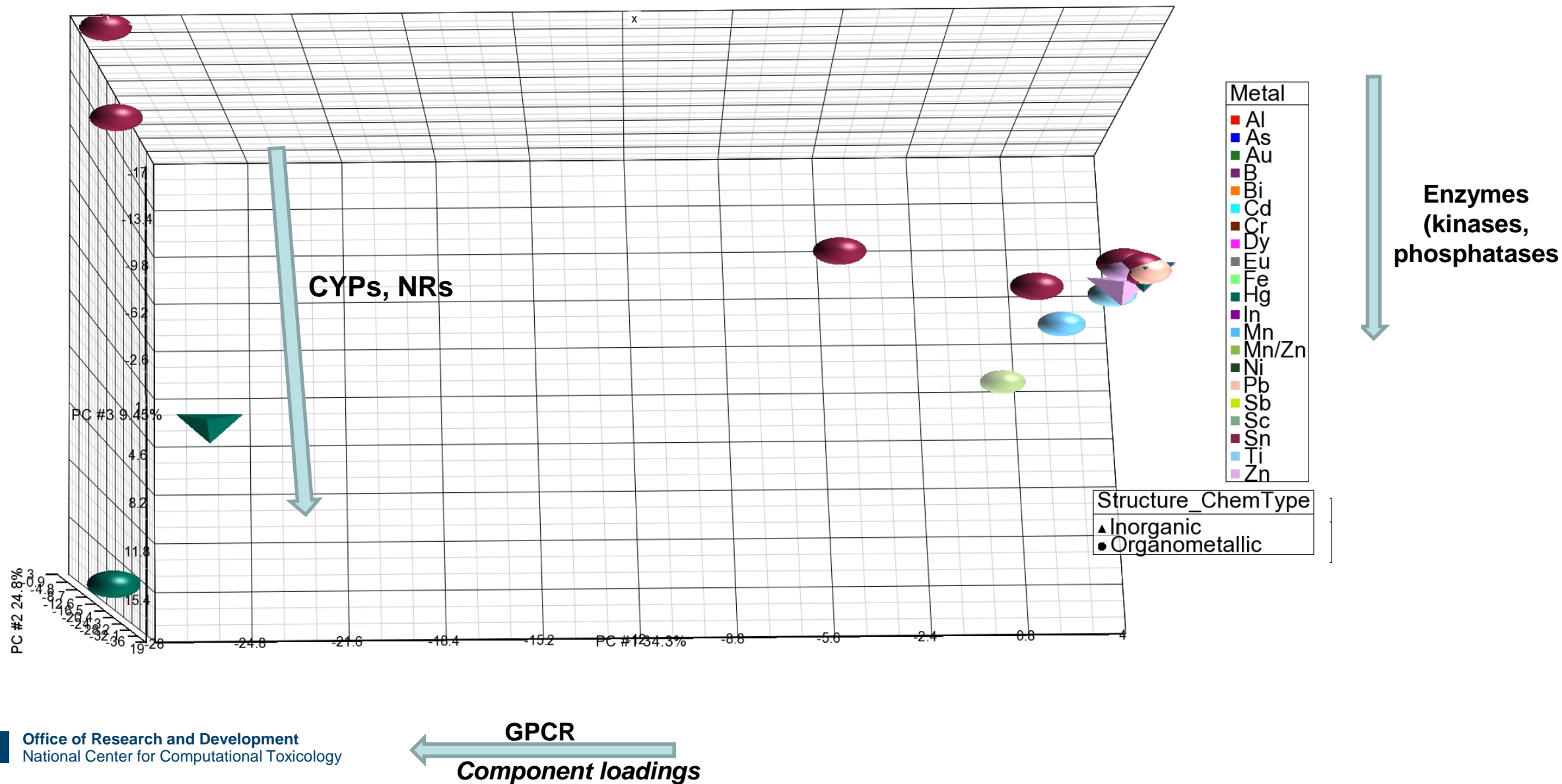
Blue = tested
Gray = not tested

277 Assays

Hierarchical Clustering of AC50's



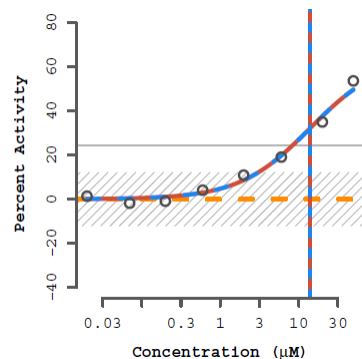
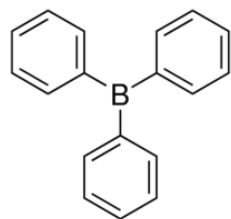
Principle Components Analysis of Biochemical Assay AC50's



Nuclear Receptor Radioligand Binding Assays

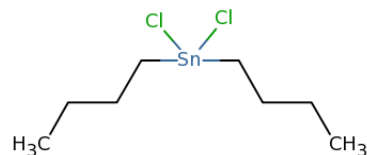
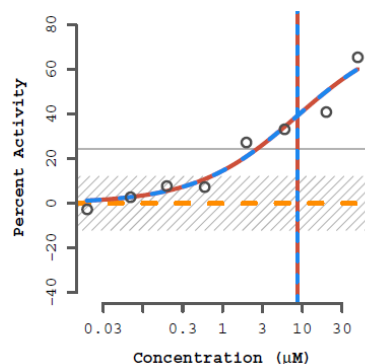
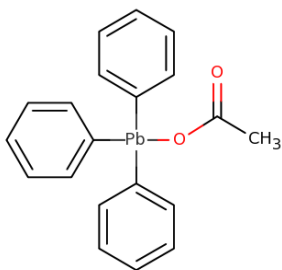
Triphenyl B/ Estrogen Receptor

NAME: Triphenylborane
CHID: 27345 CASRN: 960-71-4



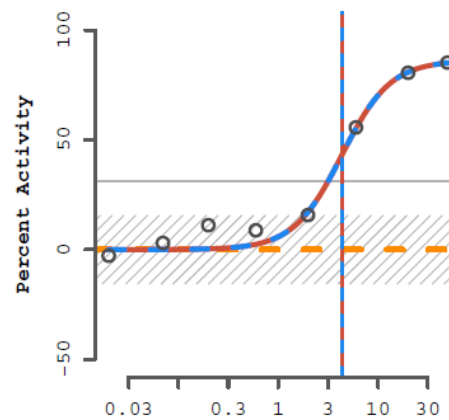
Triphenyl Pb/ Estrogen Receptor

NAME: Triphenyllead acetate
CHID: 47498 CASRN: 1162-06-7



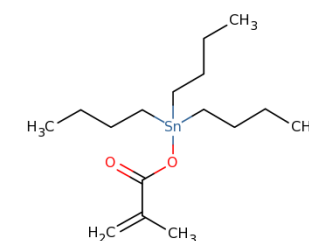
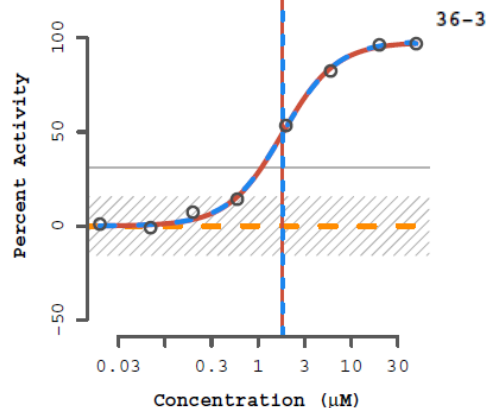
ASSAY: AEID717 (NVS_NR_hGR)

NAME: Dibutyltin dichloride
CHID: 27292 CASRN: 683-18-1



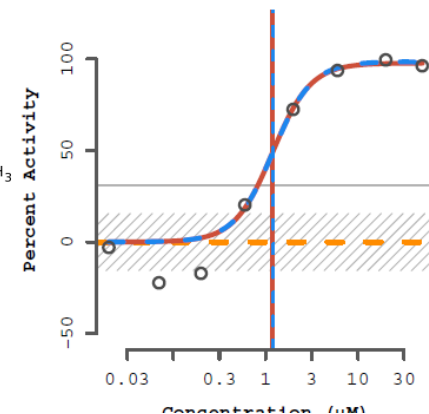
ASSAY: AEID717 (NVS_NR_hGR)

NAME: Tributyltin benzoate 36-3



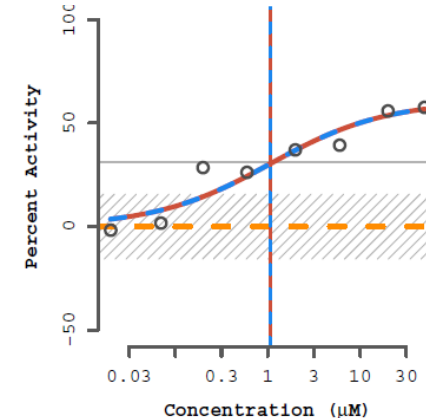
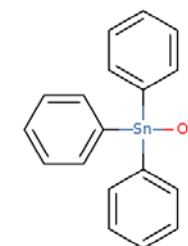
ASSAY: AEID717 (NVS_NR_hGR)

NAME: Tributyltin methacrylate
CHID: 35204 CASRN: 2155-70-6

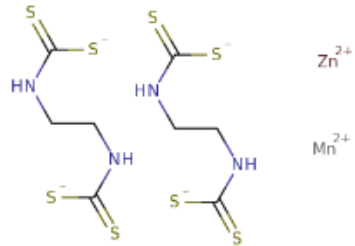


ASSAY: AEID717 (NVS_NR_hGR)

NAME: Triphenyltin hydroxide
CHID: 21409 CASRN: 76-87-9

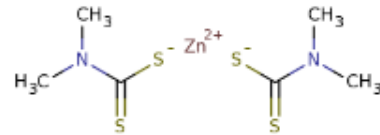
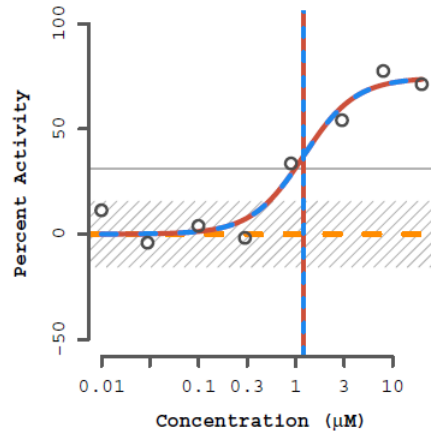


Additional GR Binders



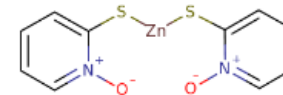
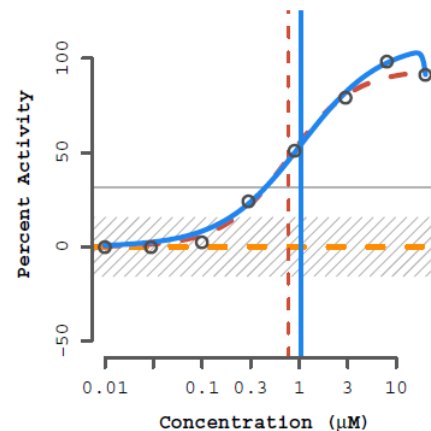
ASSAY: AEID717 (NVS_NR_hGR)

NAME: Mancozeb
CHID: 34695 CASRN: 8018-01-7



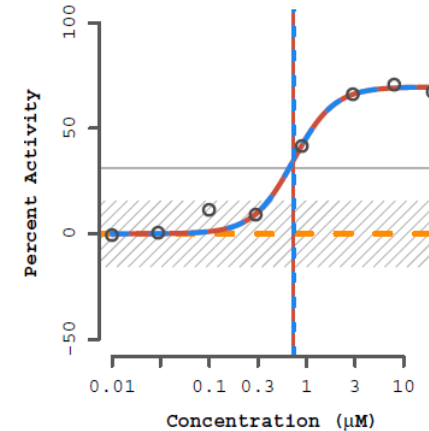
ASSAY: AEID717 (NVS_NR_hGR)

NAME: Ziram
CHID: 21464 CASRN: 137-30-4



ASSAY: AEID717 (NVS_NR_hGR)

NAME: Zinc pyrithione
CHID: 26314 CASRN: 13463-41-7

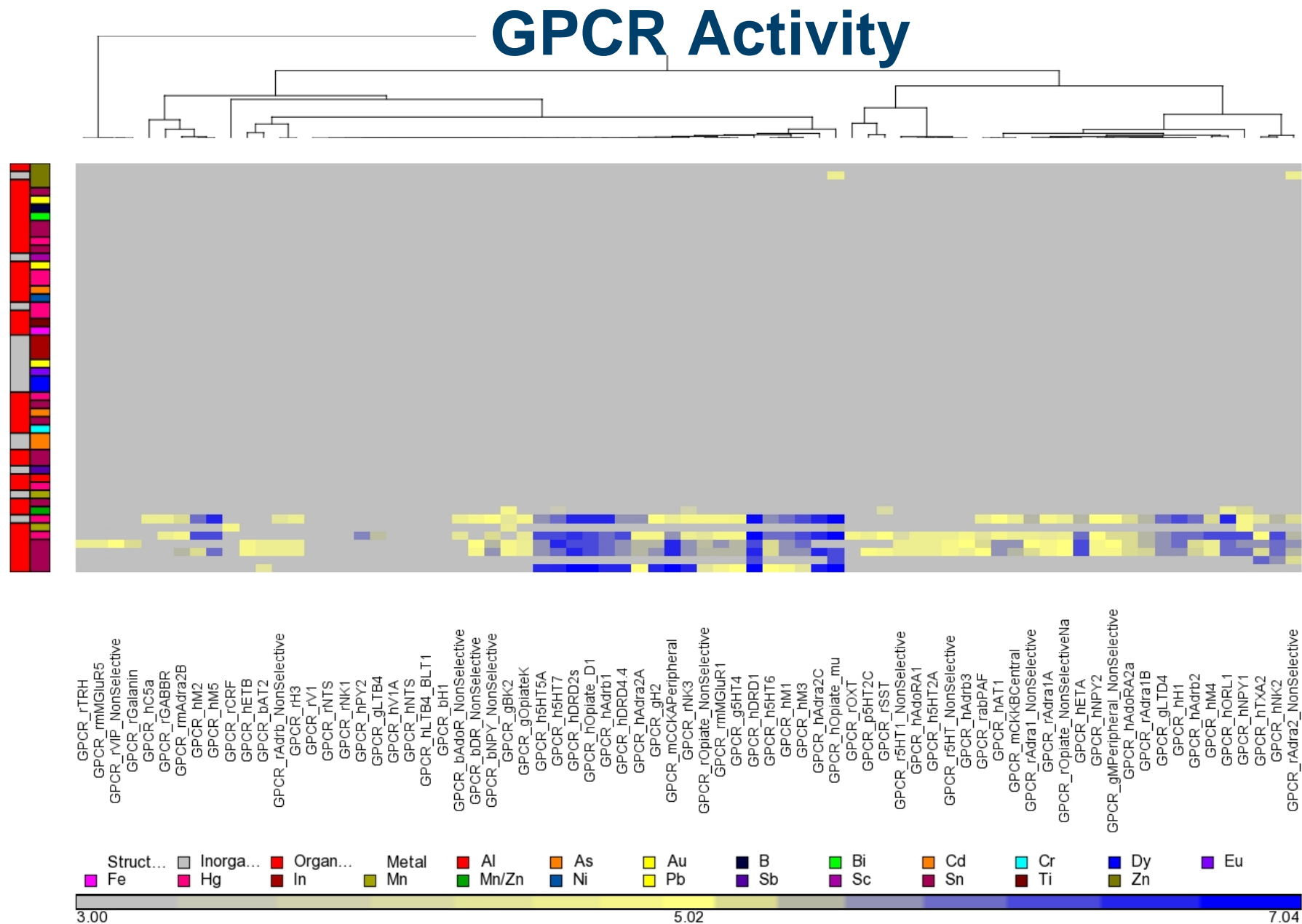


Mancozeb reported as an immunomodulator in humans: targets NFκB activation which is also the pathway of GR trans-repression.



- Binding assays
- Activity mainly in organo Sn and Hg
- Well known neurotoxic effects of these organometals

Tributyltin chloride
Tributyltin methacrylate
Phenylmercuric acetate
Triphenyltin chloride
Mercuric chloride
Triphenyltin hydroxide
Mancozeb



Tox21 qHTS Assays

- 1536-well format
- All cell-based
- 12-15 point concentration response
- Most 24 hr exposure



Nuclear Receptors		Stress Pathways		EDC Other		Other	
Completed	In Queue	Completed	In Queue	Completed	In Queue	Completed	In Queue
Androgen receptor, full	Constitutive androstane receptor, full	AP-1	Real time cytotoxicity and viability: HEK293	Aromatase	Thyroid stimulating hormone receptor (TSHR)	Auto fluorescence	Luciferase, biochemical
Androgen receptor, full	Constitutive androstane receptor, full	DNA repair, rad54/ku70 (-/-)	Real time cytotoxicity and viability: HepG2		Thyroid stimulating hormone receptor (TSHR)		Luciferase, cellular
Androgen receptor, LBD		DNA repair, Rev3 (-/-)	Real time cytotoxicity and viability: Stem cell				
Androgen receptor, LBD		DNA repair, wild type					
Aryl hydrocarbon receptor (AhR), full		ELG1					
Estrogen receptor alpha, full		EndoR (ESRE)					
Estrogen receptor alpha, full		HSE					
Estrogen receptor alpha, LBD		Hypoxia/Hif-1					
Estrogen receptor alpha, LBD		Mitochondrial membrane potential					
Farnesoid X receptor, LBD		NFkB					
Farnesoid X receptor, LBD		Nrf2/ARE					
Glucocorticoid receptor, full		P53					
Glucocorticoid receptor, full		pH2AX					
PPAR delta receptor, LBD							
PPAR delta receptor, LBD							
PPAR gamma, LBD							
PPAR gamma, LBD							
Retinoid X receptor alpha, LBD							
Retinol signaling pathway (RSP)							
Retinol signaling pathway (RSP)							
ROR gamma							
ROR gamma							
Thyroid receptor, full							
Thyroid receptor, full							
Vitamin D receptor, LBD							
Vitamin D receptor, LBD							

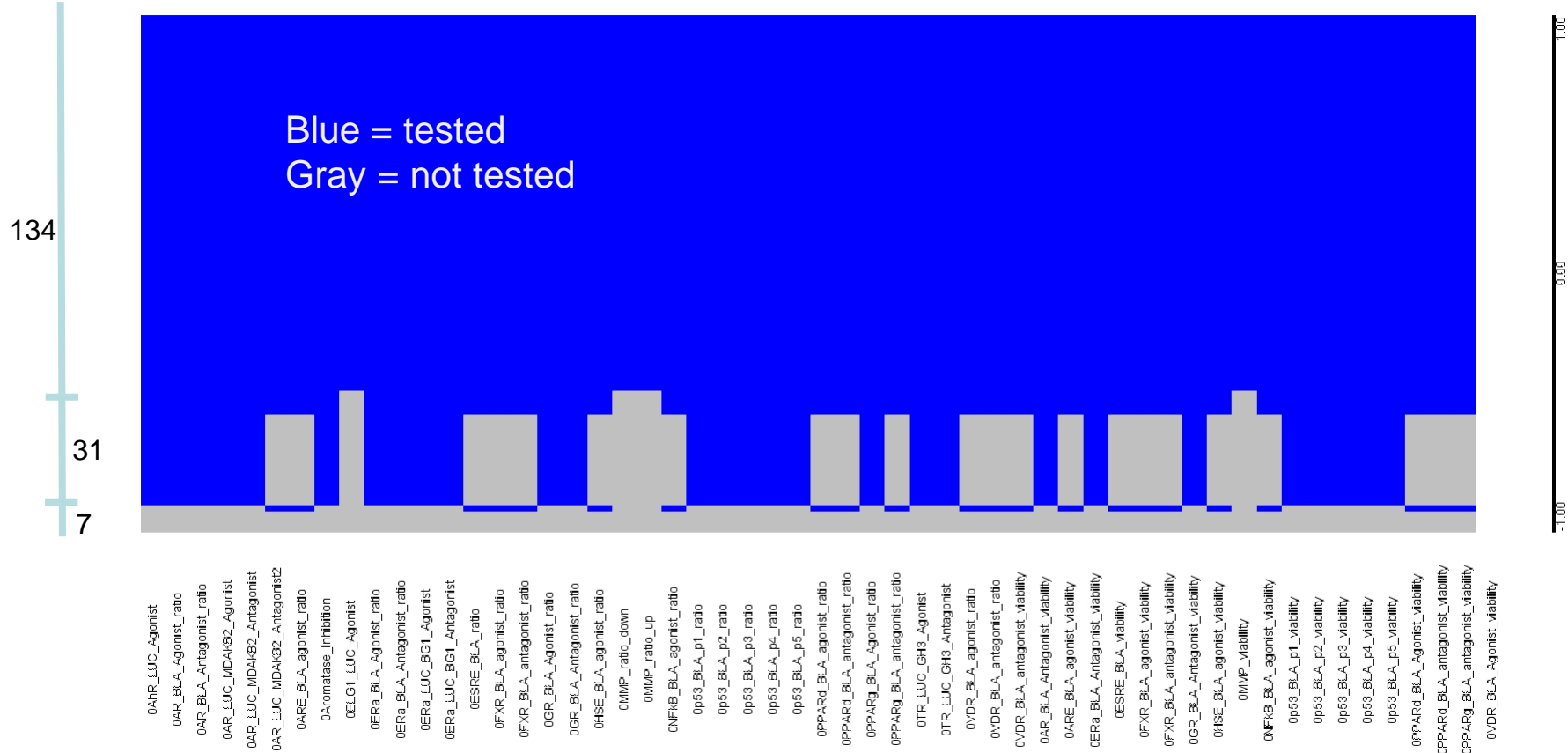
Agonist

Antagonist

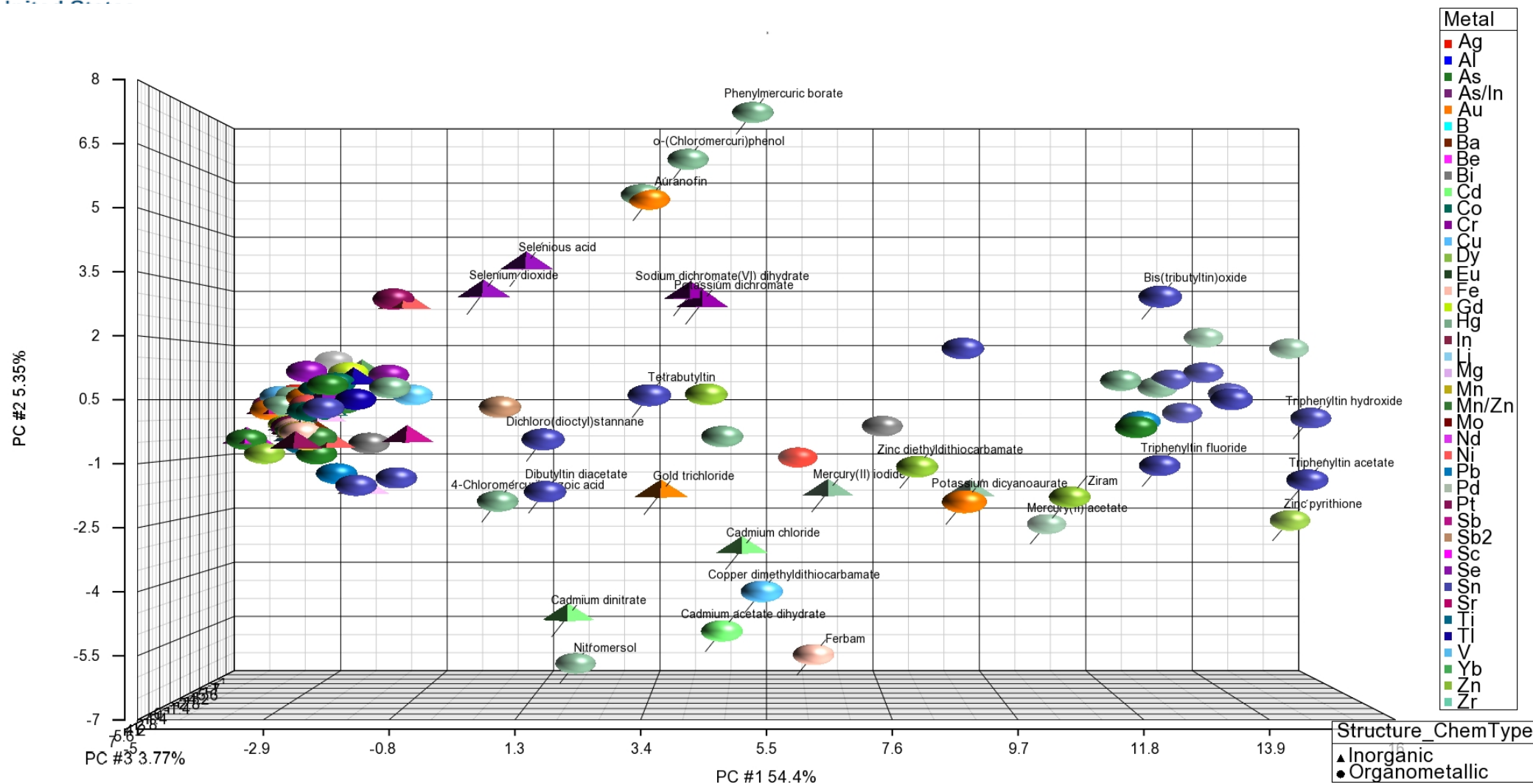
Metal Testing Matrix in Tox21

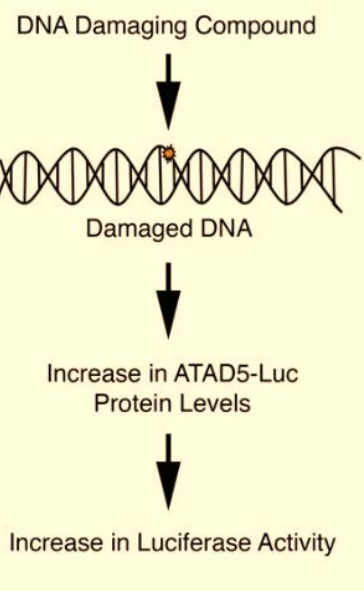
**172
Metals**

Tested Matrix: Tox21



Principal Components Analysis of All Tox21 Data





DNA Damage:

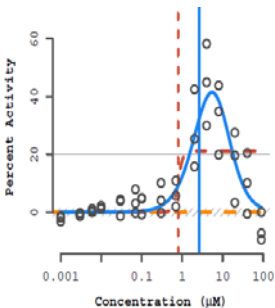
Induction of ATAD5/ELG1 by Cadmium

Jennifer T. Fox et al. PNAS
2012;109:5423-5428

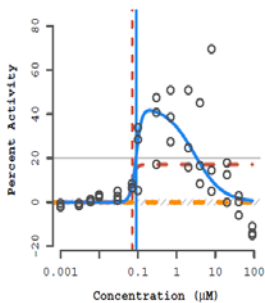
ELG1 Induction
(TOX21_ELG1_LUC_Agonist)

Top Actives
Triphenyltin acetate
Cadmium acetate dihydrate
Cadmium chloride
Zinc pyrithione
Copper dimethyldithiocarbamate
Cadmium dinitrate
Triethyltin bromide
Zinc diethyldithiocarbamate
Potassium dicyanoaurate
Ziram
Thimerosal
Zinc dibutyldithiocarbamate
Methylmercuric(II) chloride
Ferbam
Dibutyltin dichloride
Triphenyltin hydroxide
Triphenyllead acetate

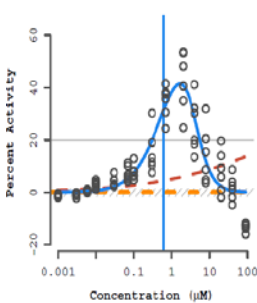
NAME: Cadmium dinitrate
CHID: 44504 CASRN: 10325-94-7



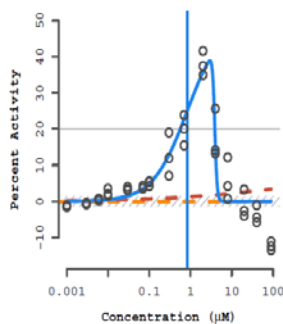
NAME: Cadmium acetate dihydrate
CHID: 33281 CASRN: 5743-04-4



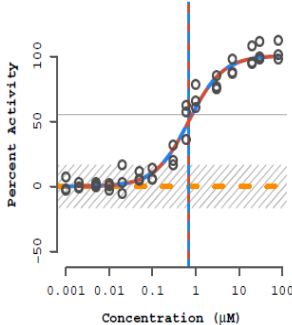
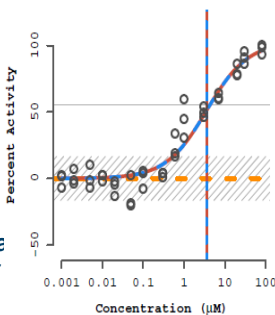
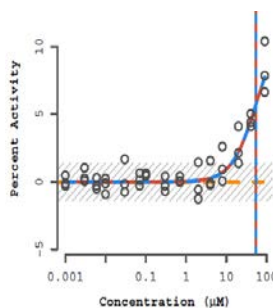
NAME: Cadmium chloride
CHID: 20226 CASRN: 10108-64-2



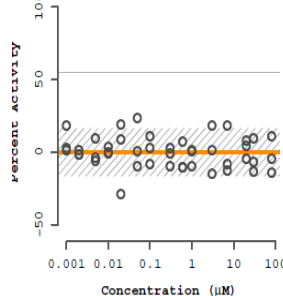
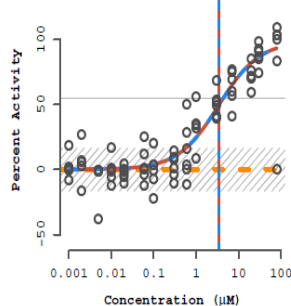
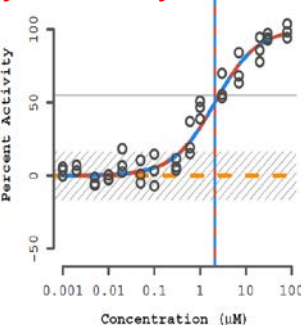
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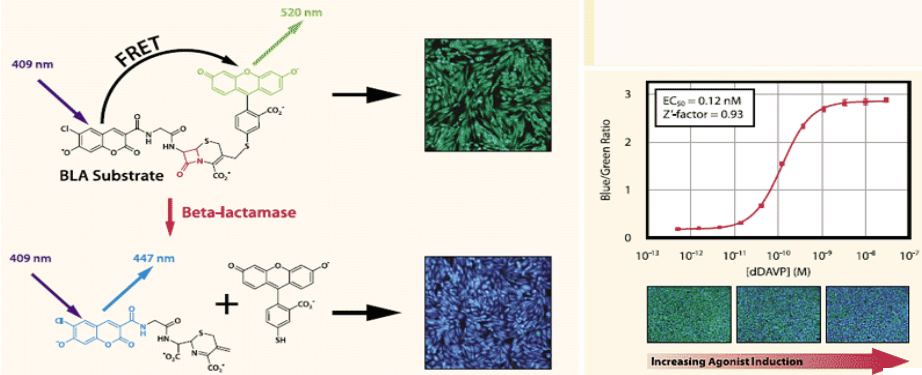


NAME: Cadmium oxide
CHID: 24715 CASRN: 1306-19-0



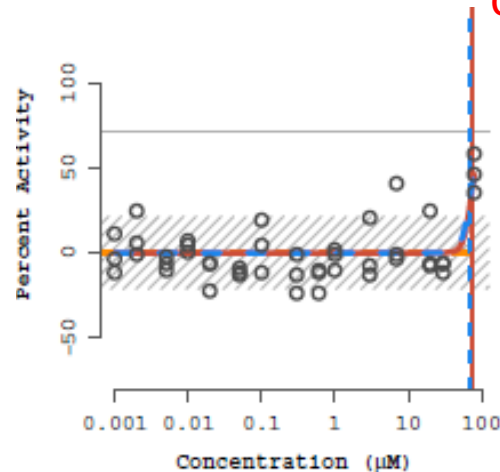
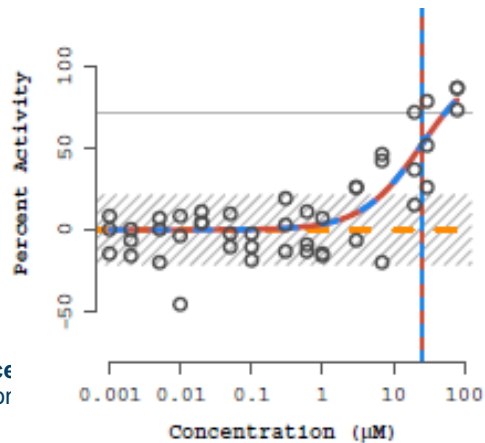
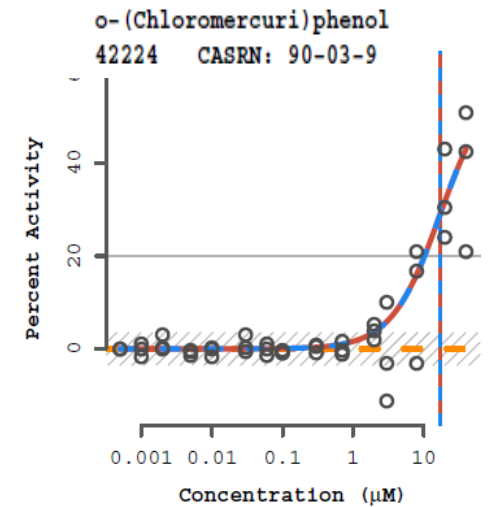
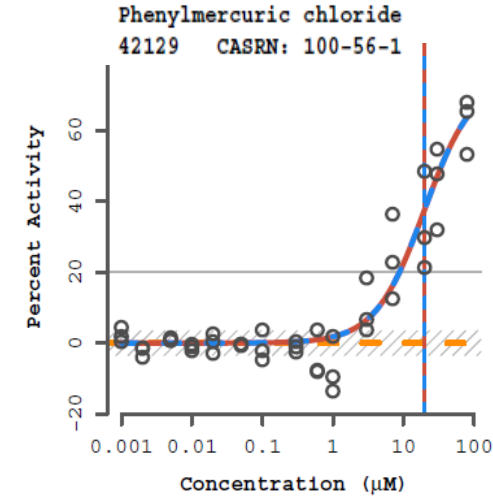
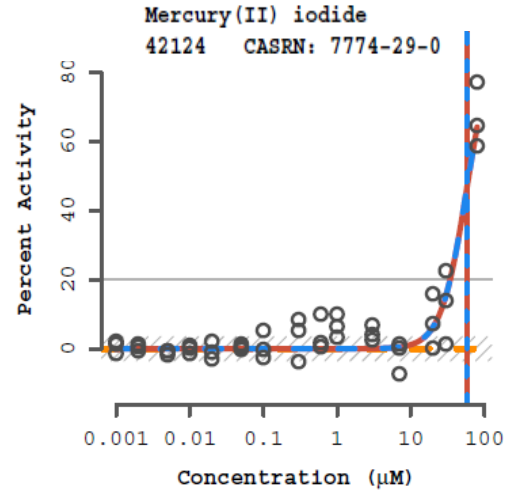
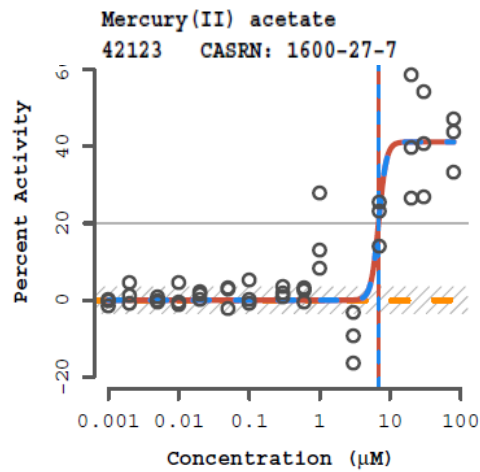
cytotoxicity



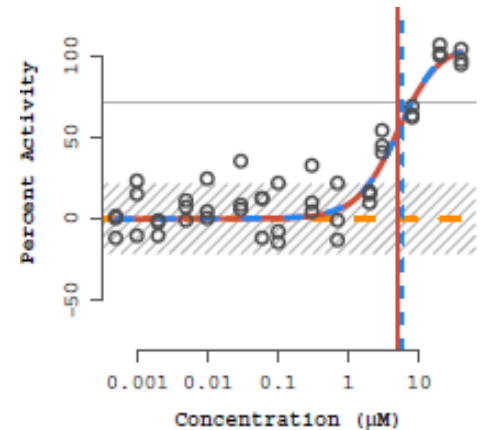
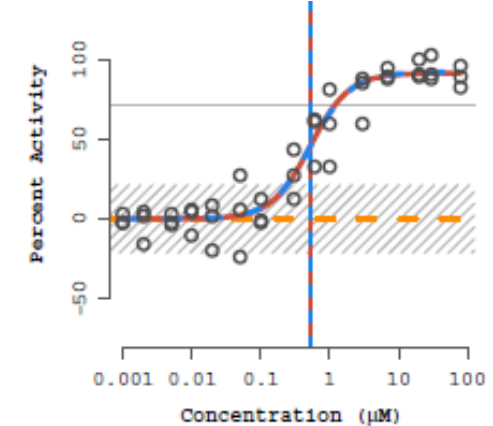


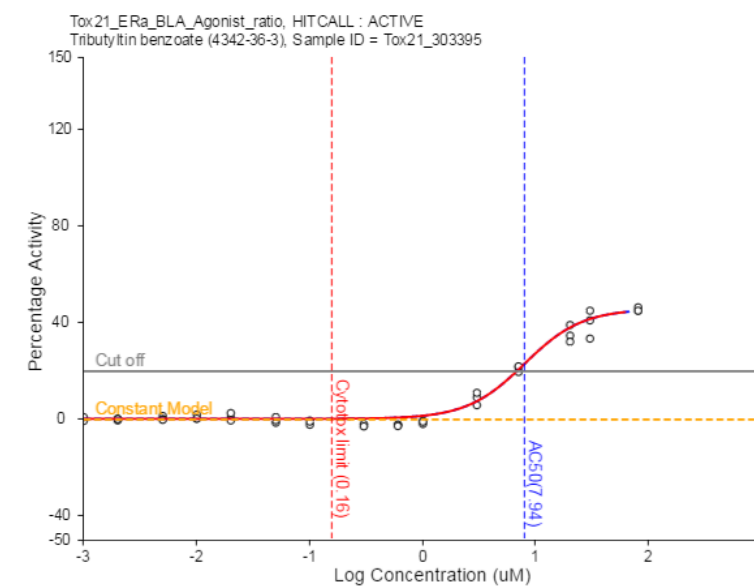
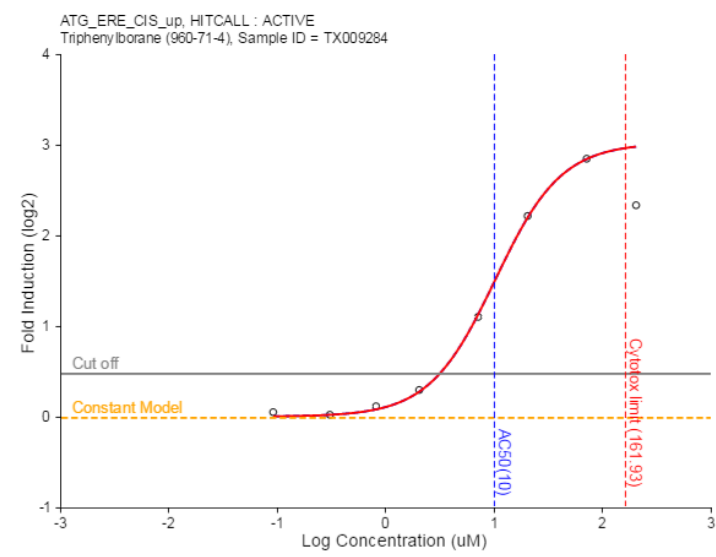
Cytotoxicity Effects: Hg Example with Glucocorticoid Receptor Activation

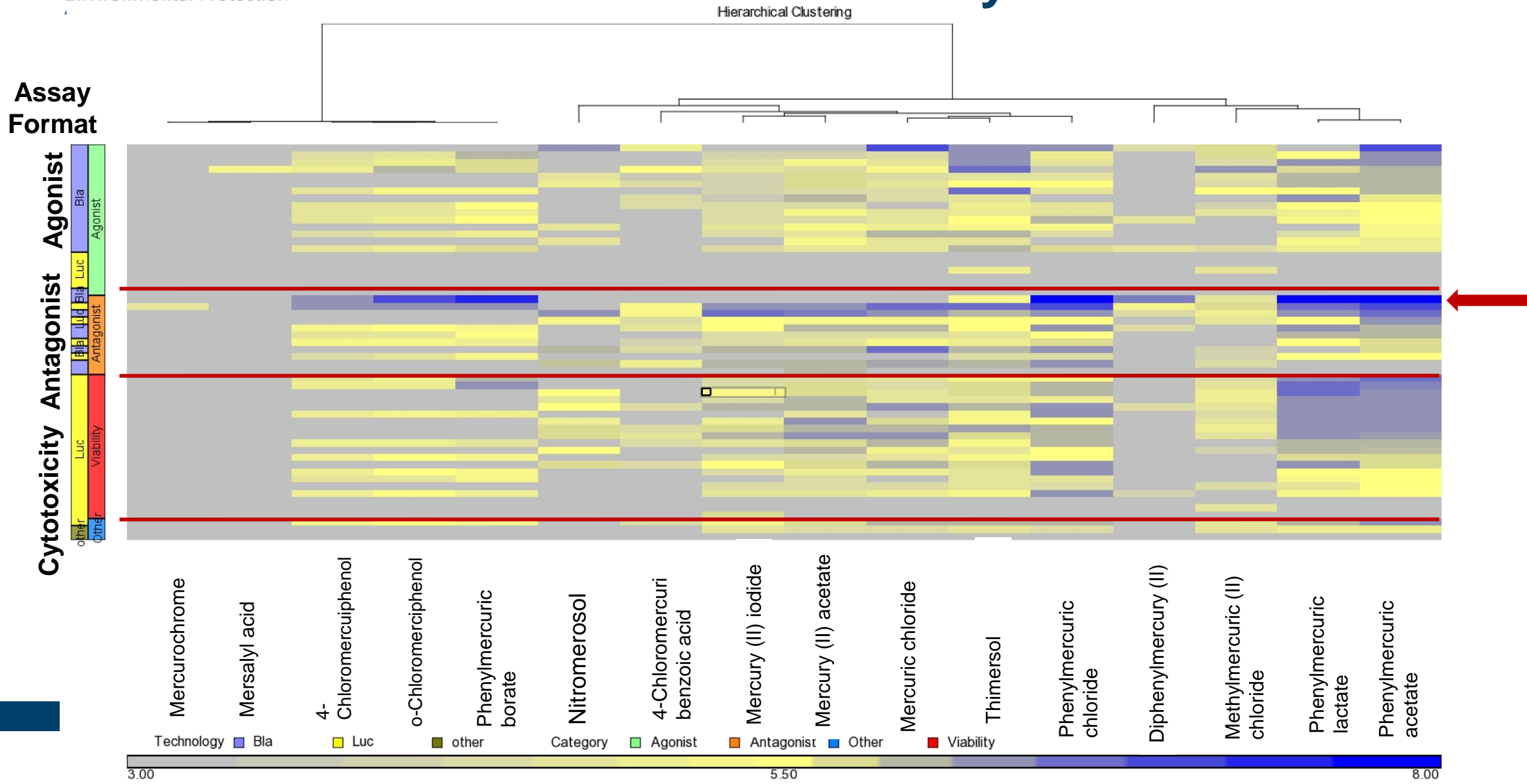
Glucocorticoid Receptor Activation



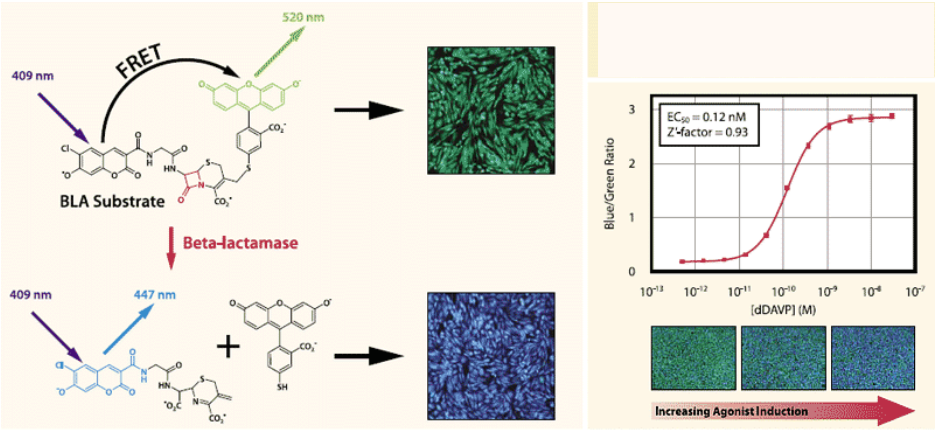
cytotoxicity



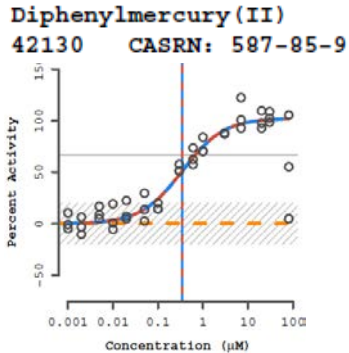
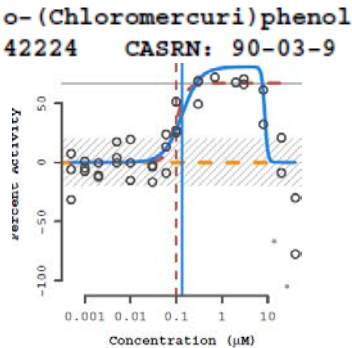
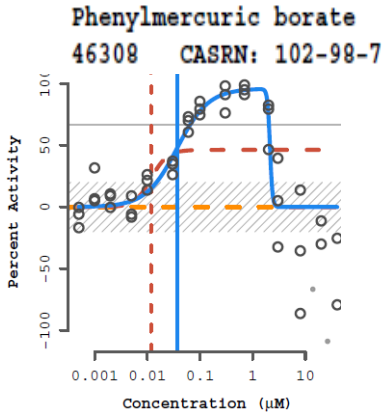
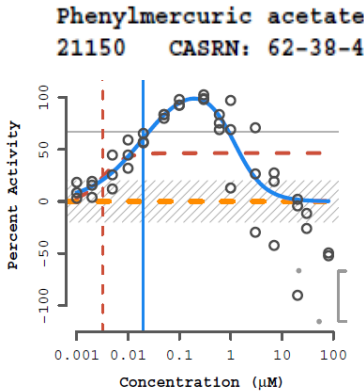
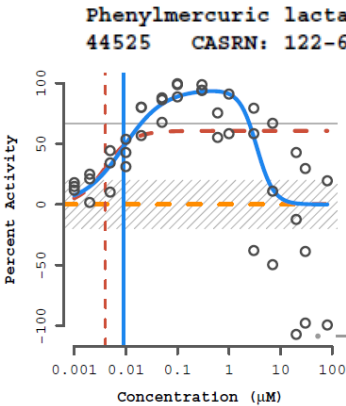
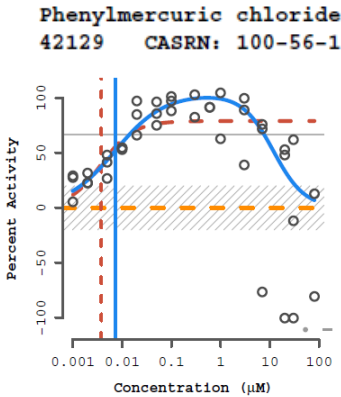




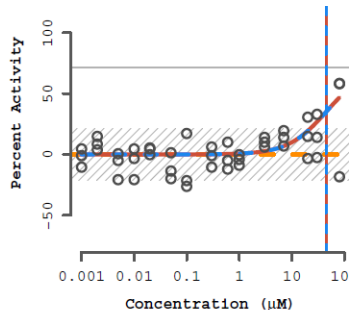
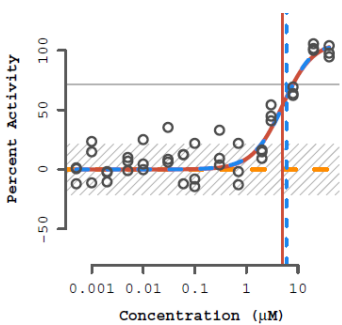
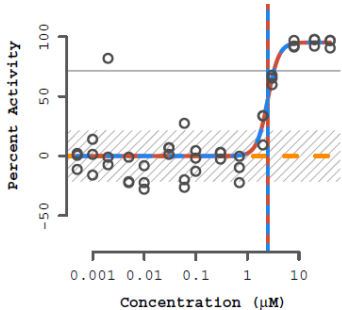
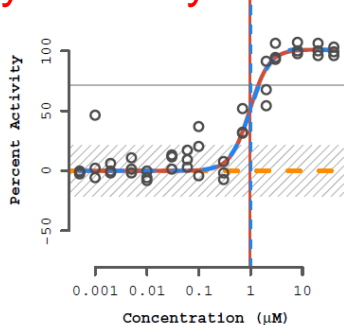
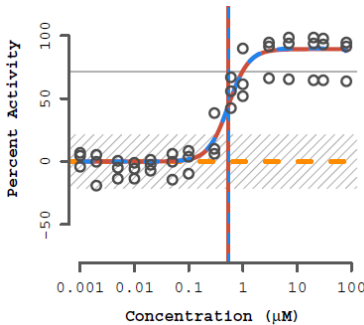
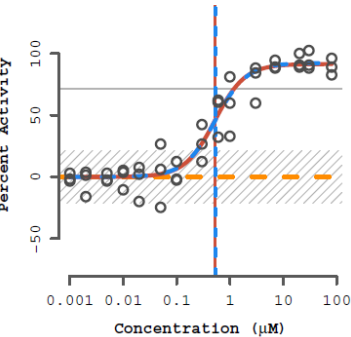
Glucocorticoid Receptor Antagonists



TOX21_GR_BLA_Antagonist



cytotoxicity



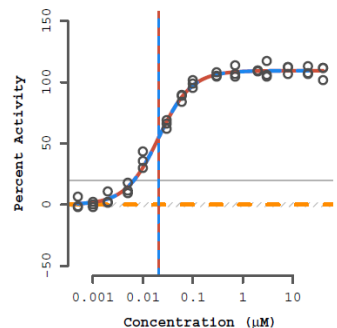
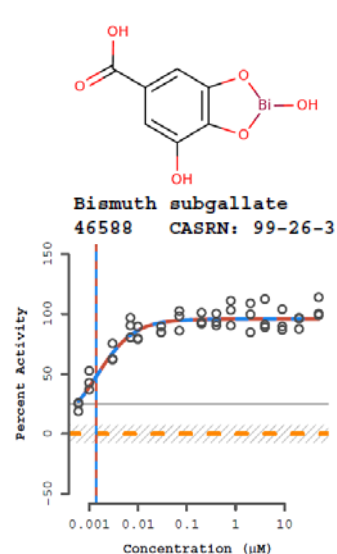
Chemical Name	-logAC50
Phenylmercuric chloride	8.138
Phenylmercuric lactate	8.041
Phenylmercuric acetate	7.760
Dibutyltin dichloride	7.658
Triphenyltin hydroxide	7.443
Triphenyltin acetate	7.296
Triphenyllead acetate	7.225
Phenylmercuric borate	7.223
o-(Chloromercuri)phenol	6.874
Triphenyltin fluoride	6.570
Diphenylmercury(II)	6.460
Triphenylbismuthine dichloride	6.454

Estrogen Receptor Activity

- ERa_Bla: GAL4-LBD with β -lactamase reporter gene
- BG1: endogenous ER with luciferase reporter
- Again, differences in LBD vs full-length
- Bismuth subgallate, ingredient in an OTC med, is very potent agonist

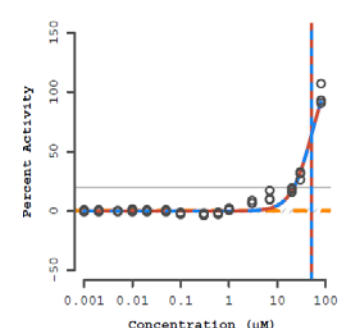
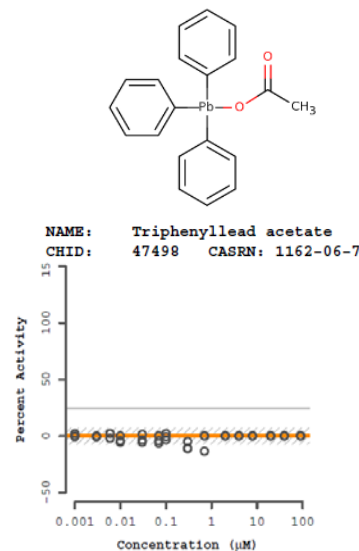
BG1

Bla

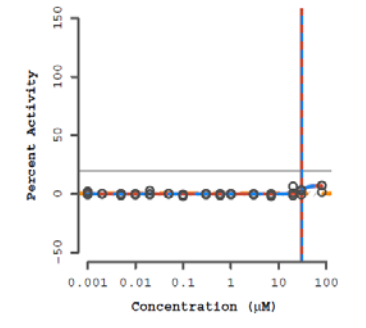
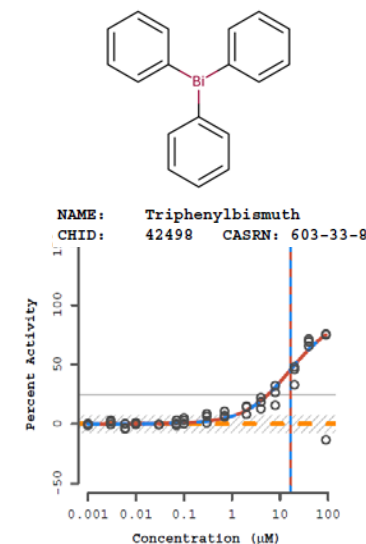


Bismuth subgallate

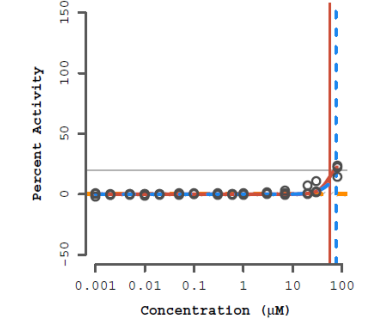
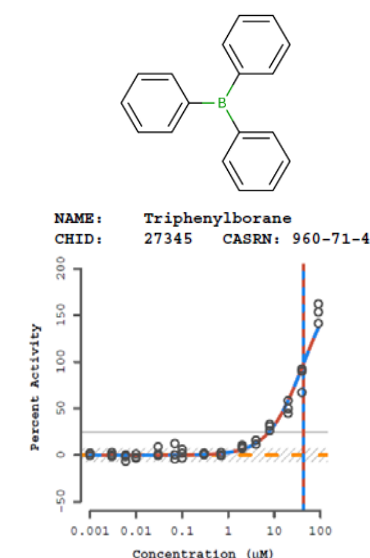
2-20 nM AC50



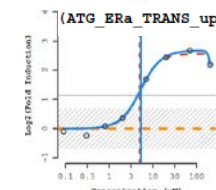
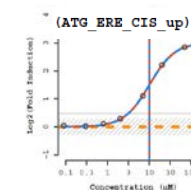
Triphenyl lead
acetate



Triphenyl bismuth



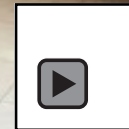
Triphenylborane
ER Agonist Model
Score 0.101



Summary

- Screening of metals in high-throughput assays if feasible
 - Some evidence of chemical-specific assay interferences
 - Cytotoxicity a frequent occurrence which can confound interpretation
- Analyzing data across many related structures increases confidence level
- Orthologous assay formats increase confidence in results
- Many known effects were captured
- Nuclear receptors and GPCR's particularly sensitive
- Triphenyl groups + metal = increased likelihood of NR activity
- Novel effects need to be examined using the bigger picture to fully appreciate biological significance

US EPA, Research Triangle Park, NC



NCCT 2014

- NCATS/NIH
- Menghang Xia
- Ruili Huang