EPA
United States
Environmental Protection
Agency

A High-Throughput Screening Assay to Detect Thyroperoxidase Inhibitors

Steve Simmons

The views expressed in this presentation are those of the author[s] and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency.

Office of Research and Development
National Center for Computational Toxicology

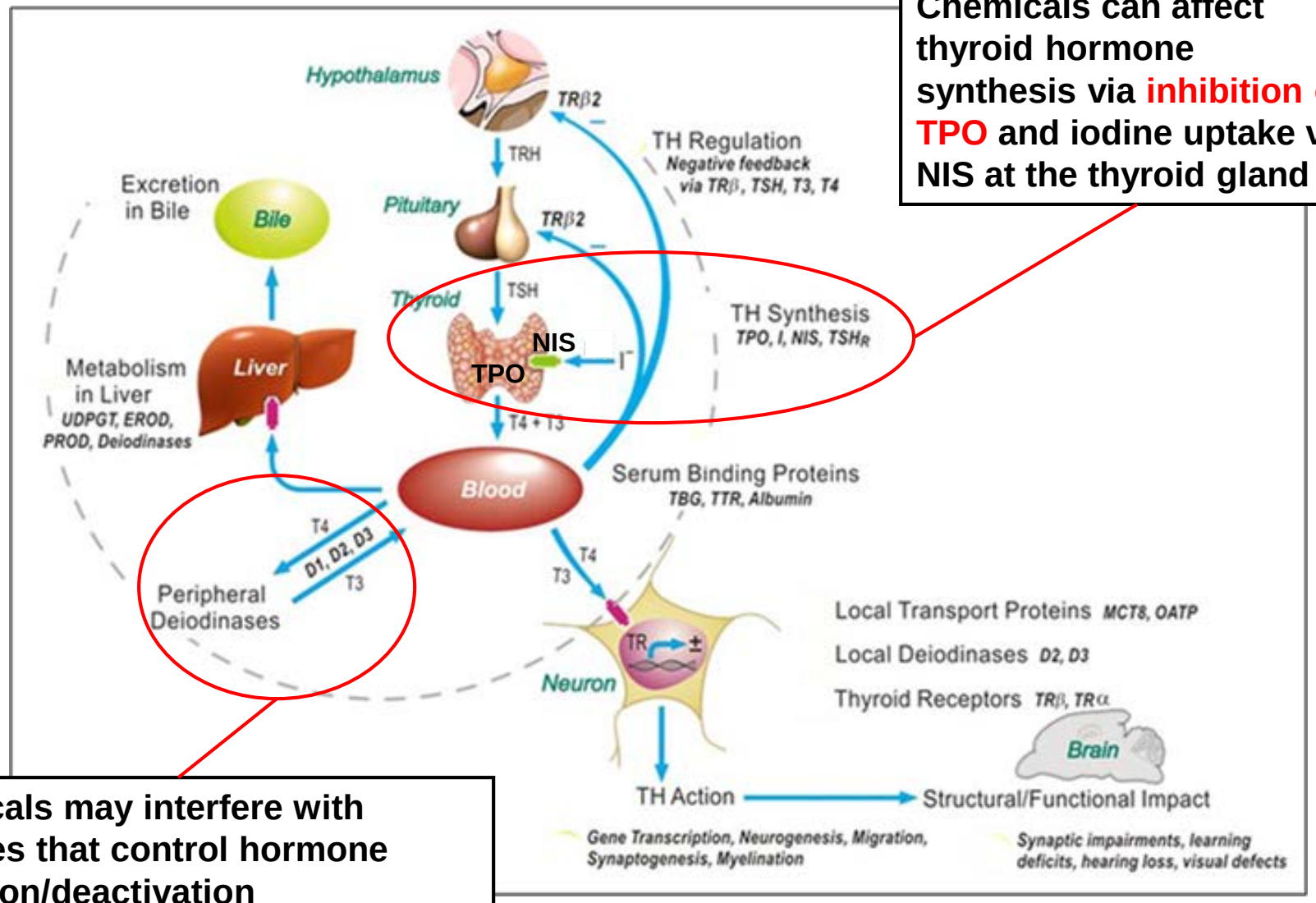
June 28, 2016

Disclosure

THE AUTHORS OF THIS RESEARCH HAVE NO FINANCIAL OR OTHER INTERESTS WHICH POSE A CONFLICT OF INTEREST

THIS RESEARCH WAS FUNDED BY THE U.S. ENVIRONMENTAL PROTECTION AGENCY

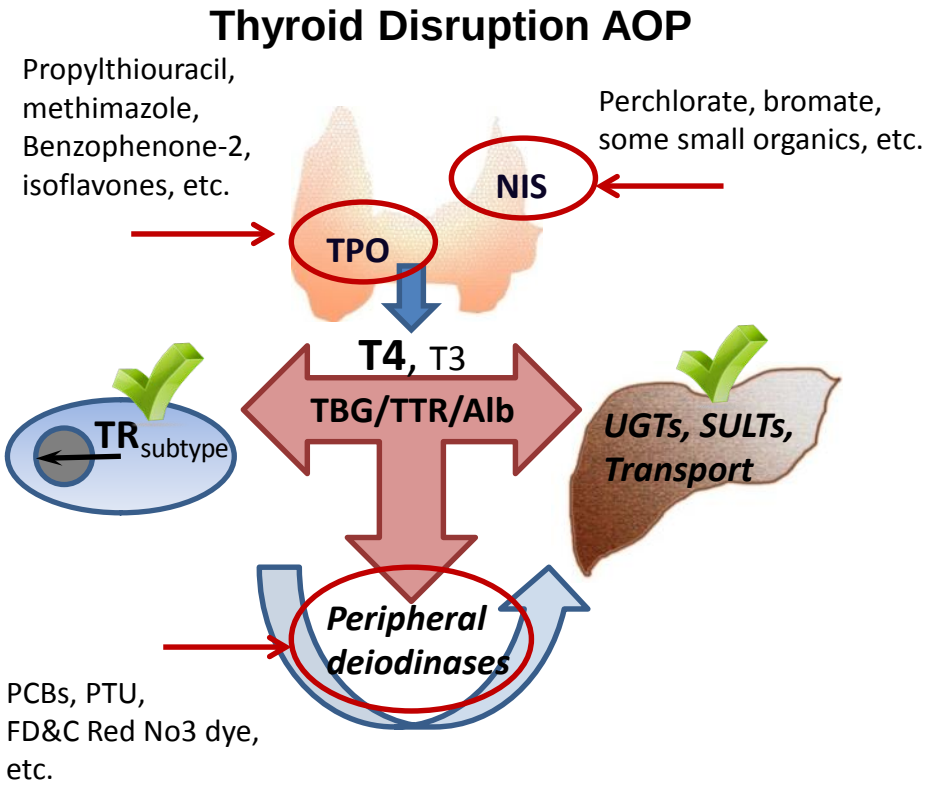
Critical HPT-Axis Endpoints Need to be Addressed



Chemicals can affect thyroid hormone synthesis via inhibition of TPO and iodine uptake via NIS at the thyroid gland

Chemicals may interfere with enzymes that control hormone activation/deactivation

ToxCast Thyroid Coverage



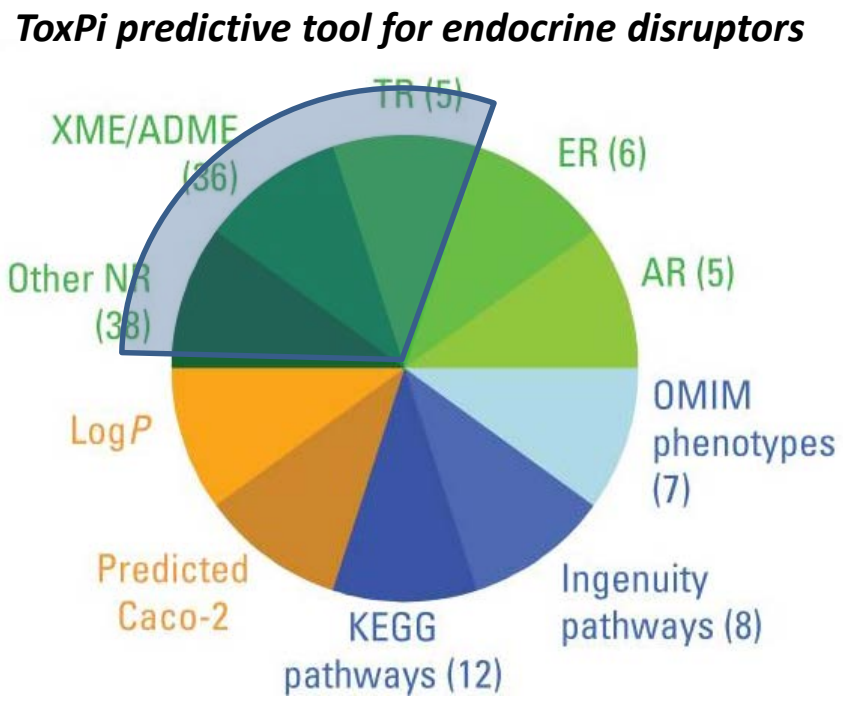


Figure reproduced from Reif et al. 2010 EHP

- Thyroid receptor is not a target for many environmental chemicals, nor for known environmental thyroid disruptors
- Disruption of TH synthesis (via TPO and/or NIS) and of T4→T3 hormone activation (via DIOs) are more common targets of environmental thyroid-disrupting chemicals
- No HTS-amenable solutions exist for TPO, NIS or DIOs

Courtesy of K. Paul-Friedman

ToxCast TPO Project

A. NHEERL/NCCT partnership to address critical assay gaps within the thyroid AOP

B. Development and deployment of new HTS assay for thyroperoxidase

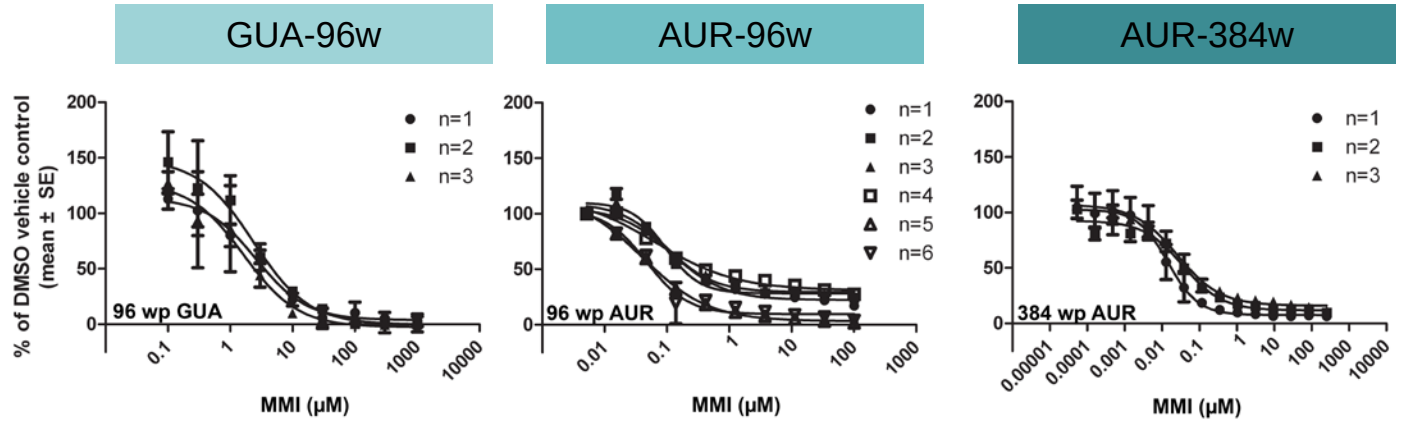
C. Screening strategy designed to prioritize TPO inhibitors

D. Retrospective comparison to legacy TPO inhibition assay (GUA)

E. Use of new HTS data to identify chemical features that drive TPO inhibition

TPO High-Throughput Assay Development

- TPO had been studied for decades using a number of oxidation assays
- The guaiacol assay (GUA) was the most widely used, but is wholly unsuited for HTS applications → only 114 unique chemicals tested in GUA as of 2015
- However, this method provided a roadmap for enzyme prep, H_2O_2 concentrations, buffer conditions, etc.
- Our experimental approach centered on finding a substitute for guaiacol among commercially-available peroxidase substrates that *was* HTS-amenable:
  - Stable, irreversible product of peroxidase activity (endpoint assay)
  - Luminescent, fluorescent, or colormetric (absorbance)
  - As sensitive/specific (or better) than guaiacol : discrimination and potency of known chemicals
  - Specific for TPO and not other peroxidases (using thyroid preps from animals)
  - Did not increase our animal needs compared to GUA
  - **Amplex Ultra Red (AUR) was the only tested substrate that satisfied all criteria**



* Substrate screening only required a single reference chemical
* Rat thyroid microsomal tissue used throughout this study

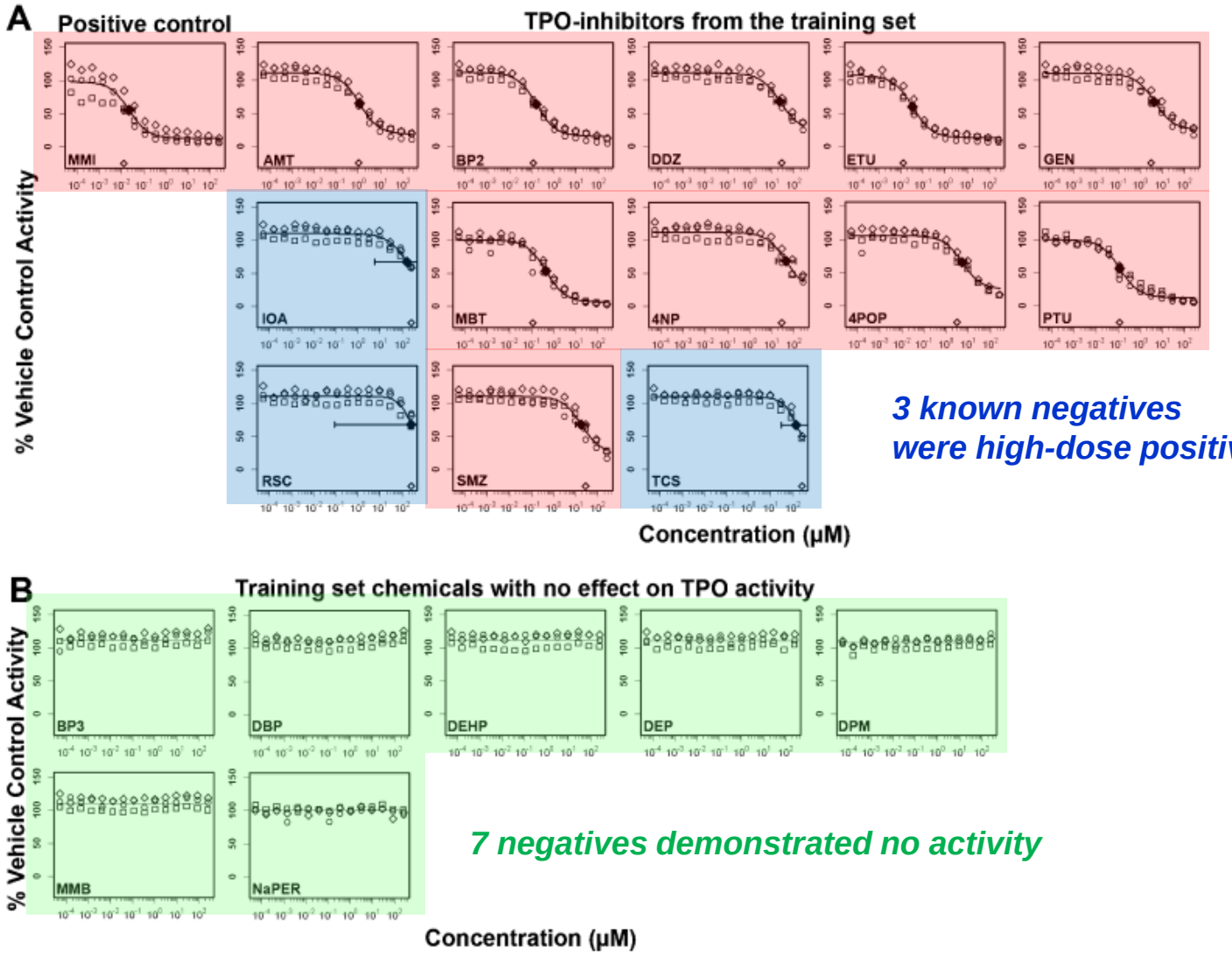
Paul et al., 2014, *Chem Res Tox*

Reference Chemicals

- A full evaluation of AUR-TPO required a robust training set of reference chemicals
- A good training set of reference chemicals should be balanced with known positive and known negatives
  - Potency across known positives
  - Selectivity among known positives and negatives
- Known negatives are often the hardest to find in the literature *
- We were the beneficiaries of decades of TPO research and had nearly 80 candidate chemicals in the literature from which to choose *
- We decided on a 21-chemical training set using chemicals published by multiple, independent laboratories
  - “Hits” determined by differing activity thresholds and Null Hypothesis Significance Tests
- Comprised of 11 known positives and 10 known negatives:

Methimazole	Diethylphthalate	Resorcinol
6-propylthiouracil	Diethylhexylphthalate	Ethylene thiourea
Sodium-perchlorate	Dibutylphthalate	3-Amino-1,2,4-Triazole (Amitrole)
2-mercaptobenzothiazole	Methyl 2-methyl-benzoate	Iopanoic Acid
3,5-Dimethylpyrazole-1-methanol	2,2',4,4'-Tetrahydroxy-benzophenone	Daidzein
4-propoxyphenol	4-nonylphenol	Genistein
Triclosan	2-hydroxy-4-methoxybenzophenone	Sulfmethazine

Validation of AUR-TPO Assay



11 known positives inhibited TPO

3 known negatives were high-dose positives

7 negatives demonstrated no activity

- Rank potency order nearly identical for those tested in GUA

Paul et al., 2014, *Chem Res Tox*

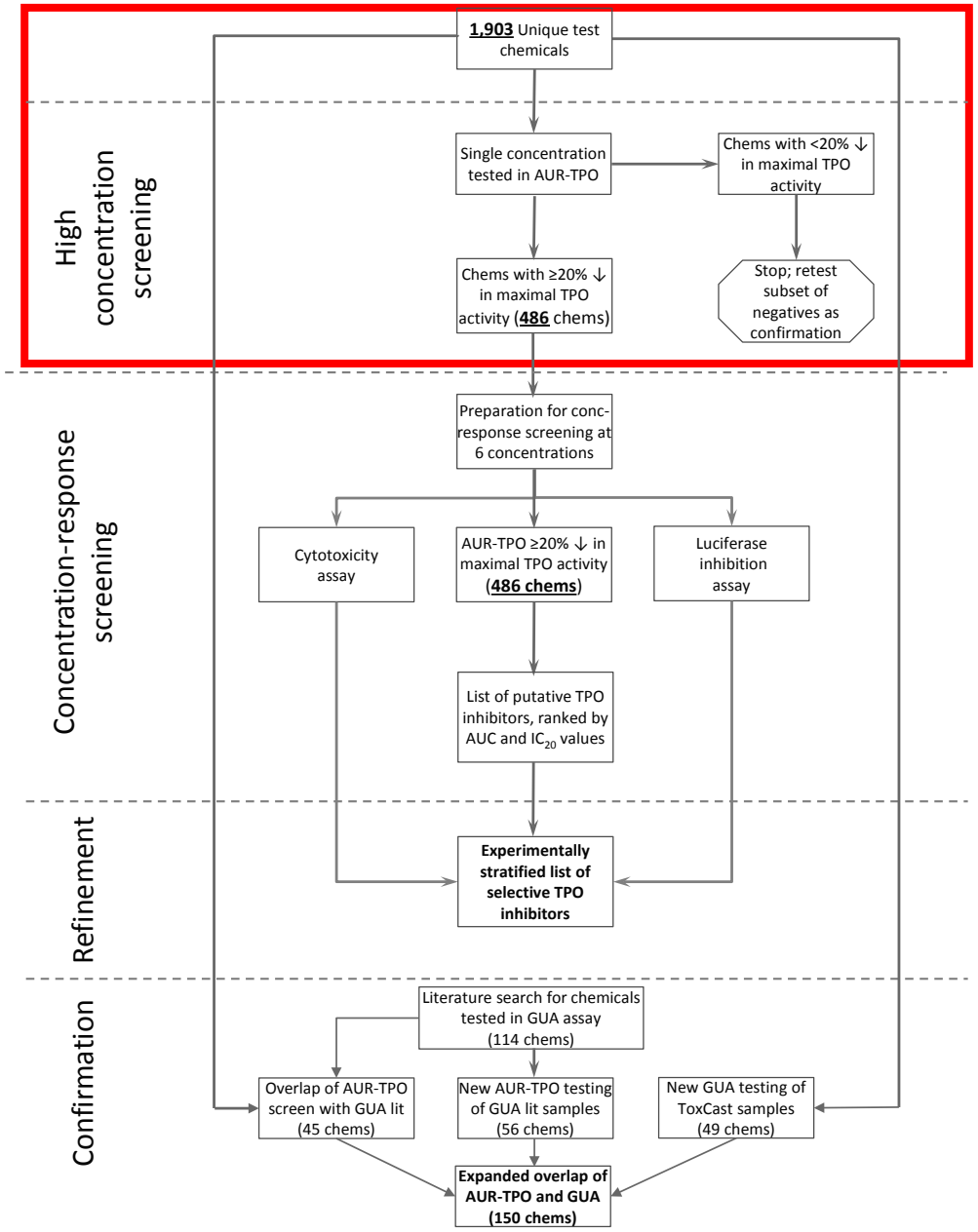
ToxCast Screen for TPO Inhibitors

- ToxCast chemicals are comprised of “Phases”
- Phase I (~300 chemicals) is comprised of mostly pesticide actives, some industrial
- Phase II (~800 chemicals) is comprised of pesticide inerts, TSCA and CCL
- E1k (~800 chemicals) is comprised of chemicals of interest to EDSP

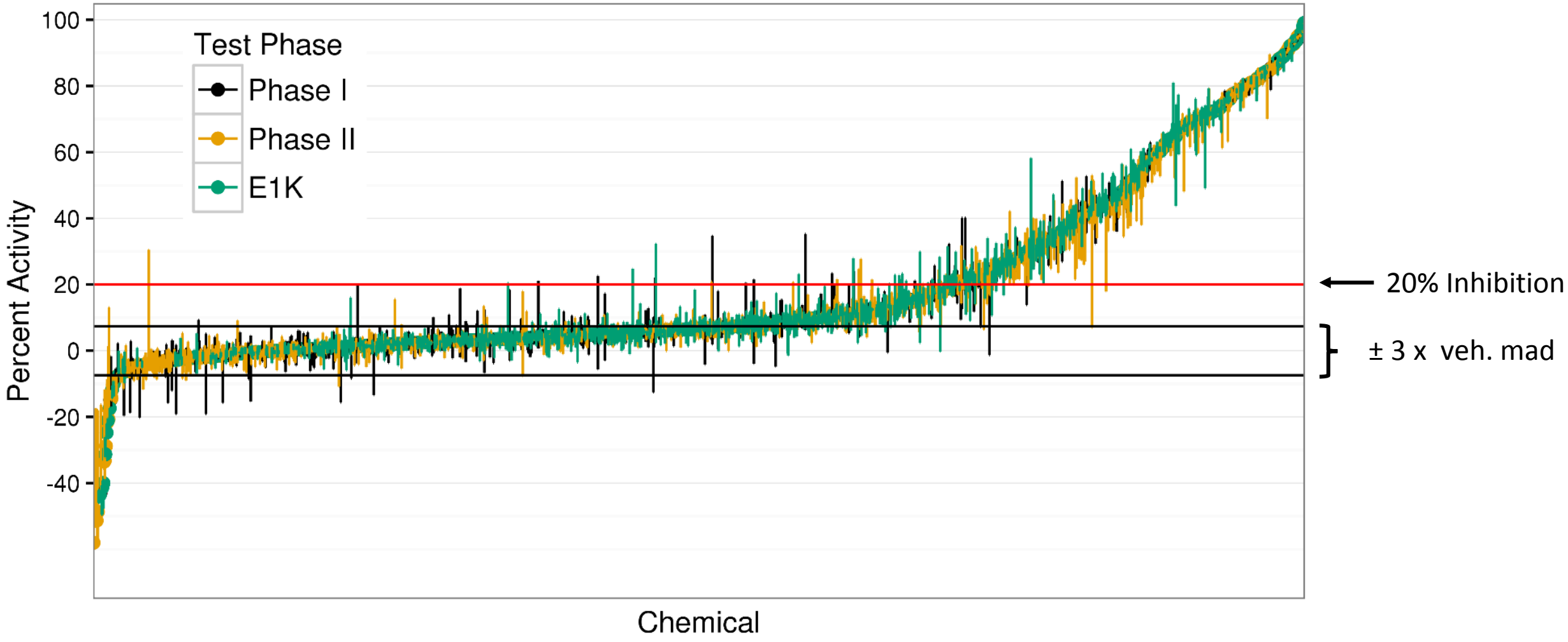
- With so many chemicals, we employed a tiered screening strategy that leveraged the loss of-signal aspect of the AUR-TPO assay
- Emphasis on “off-ramping” negatives
- Focuses resources (time, tissues) on characterizing positives (activity, potency)
- Uses parallel assays to discern “selectivity” to identify false positives and non-specific actors

- Follow-up with orthogonal legacy assay to confirm activity of putative TPO inhibitors

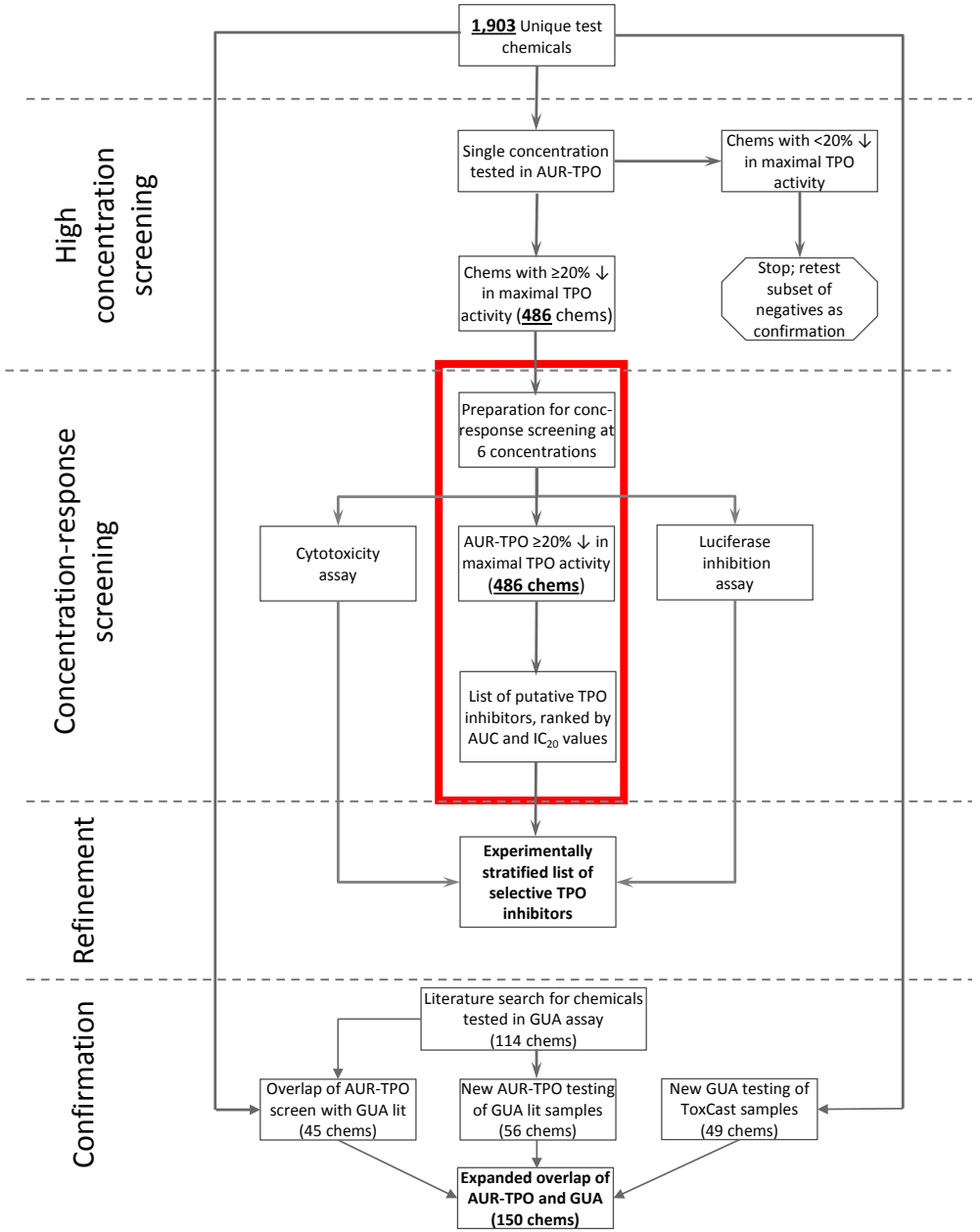
Tier 1: Single-concentration Screening



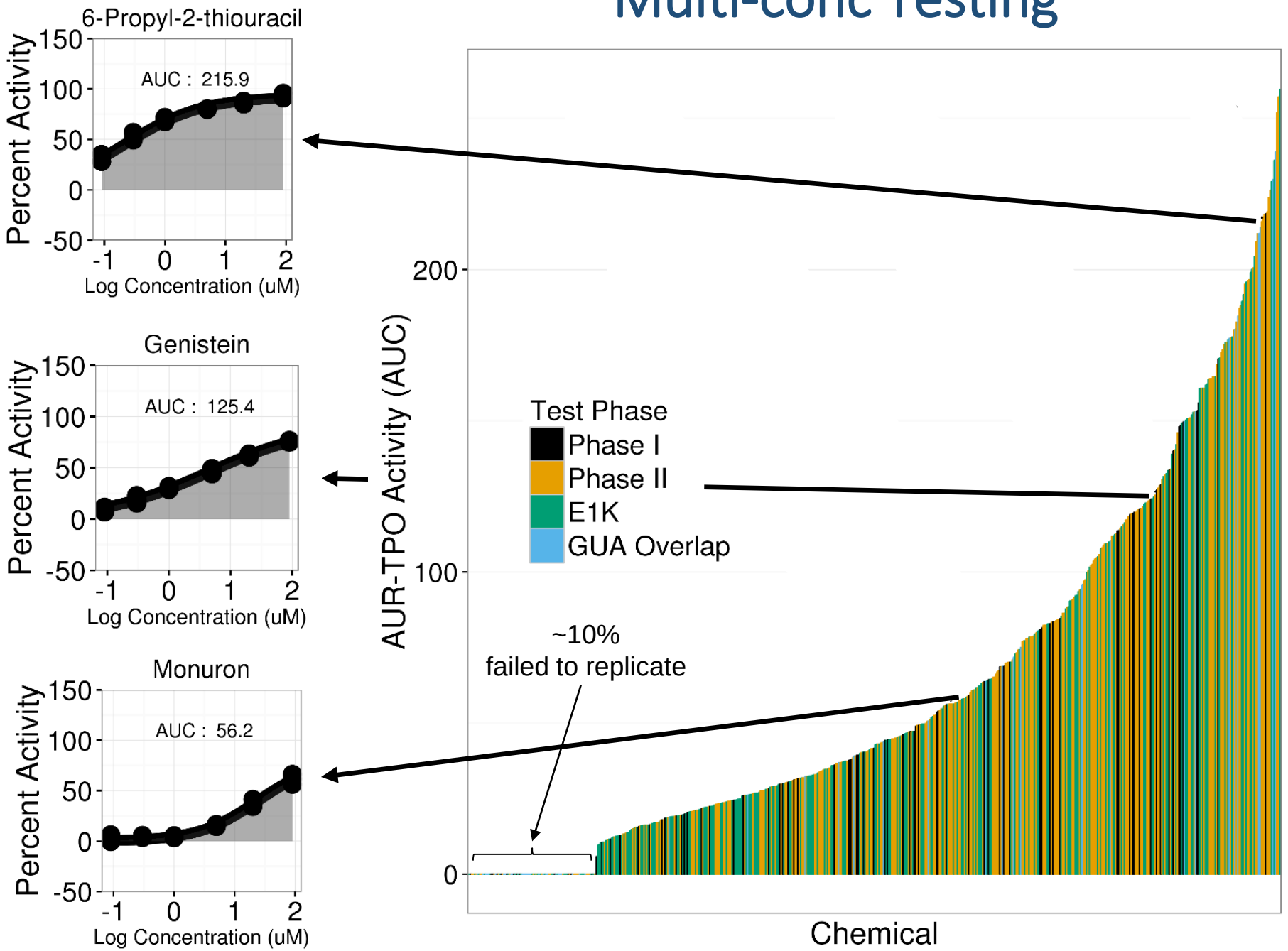
Single-conc Testing



Tier 2: Multi-concentration Screening

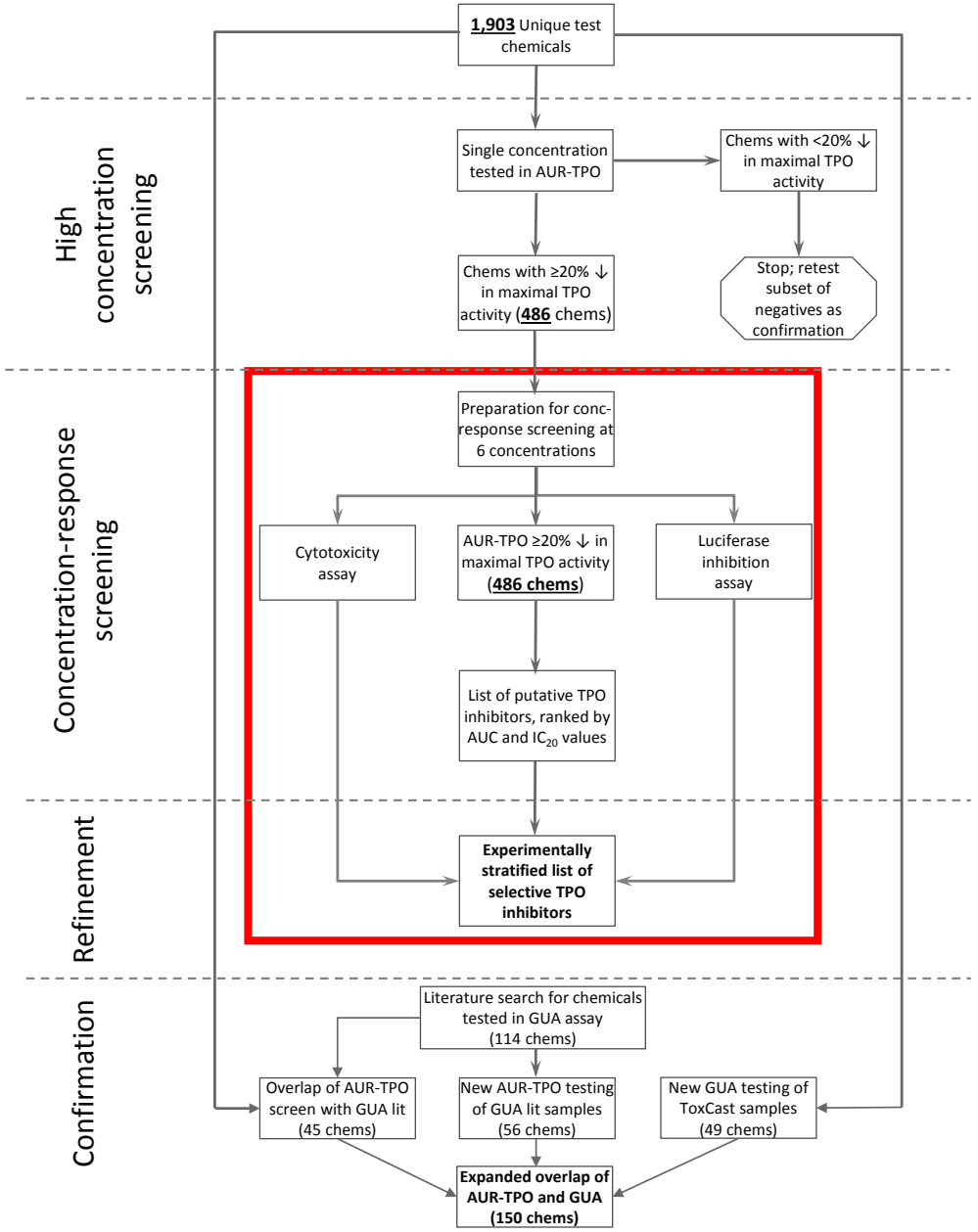


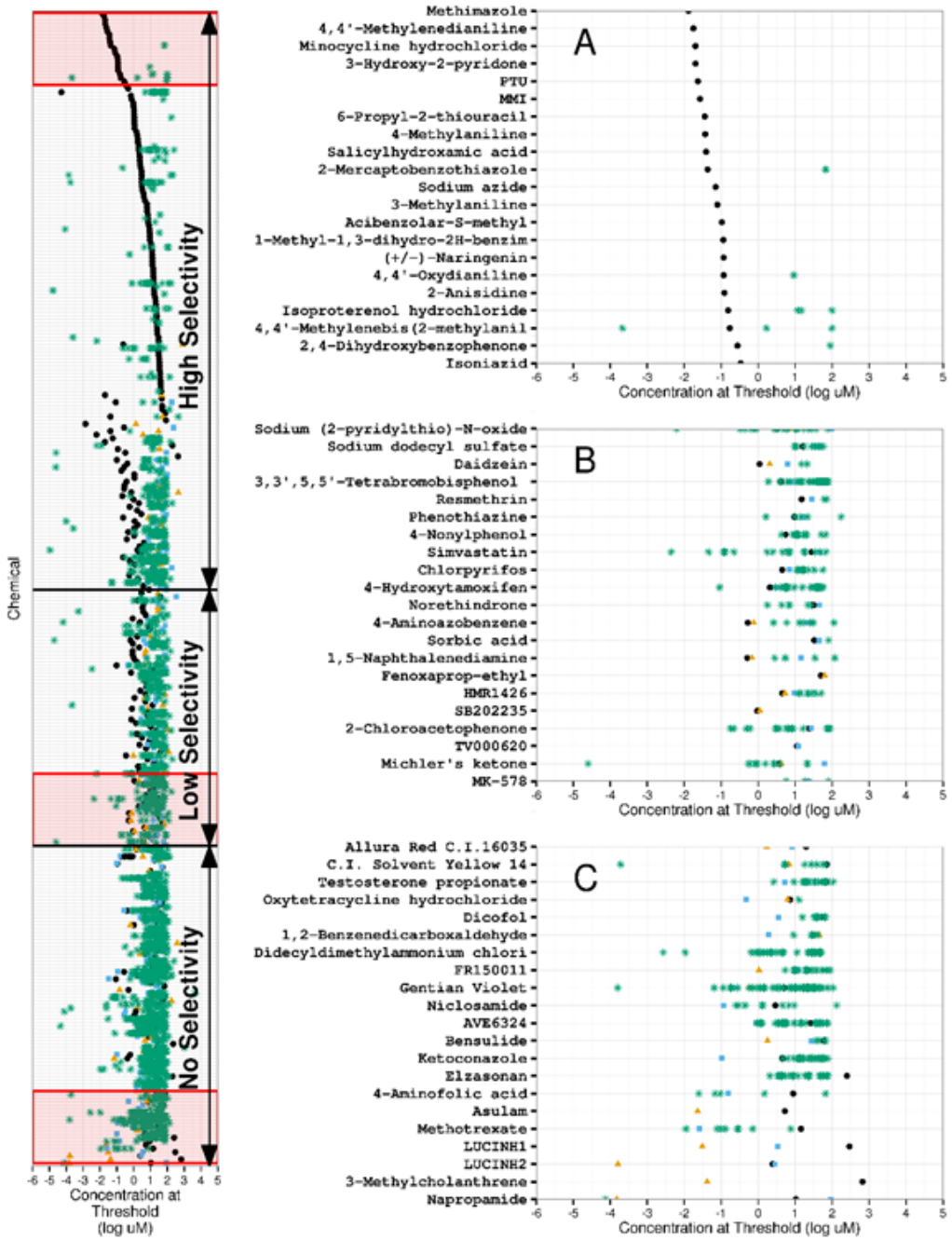
Multi-conc Testing



- Multi-conc data fit using Toxcast pipeline
- Area under fitted curve (AUC) calculated using integrate (base R) for hitc =1
- Inactive -> AUC = 0

Tier 2: Multi-concentration Screening (con't)





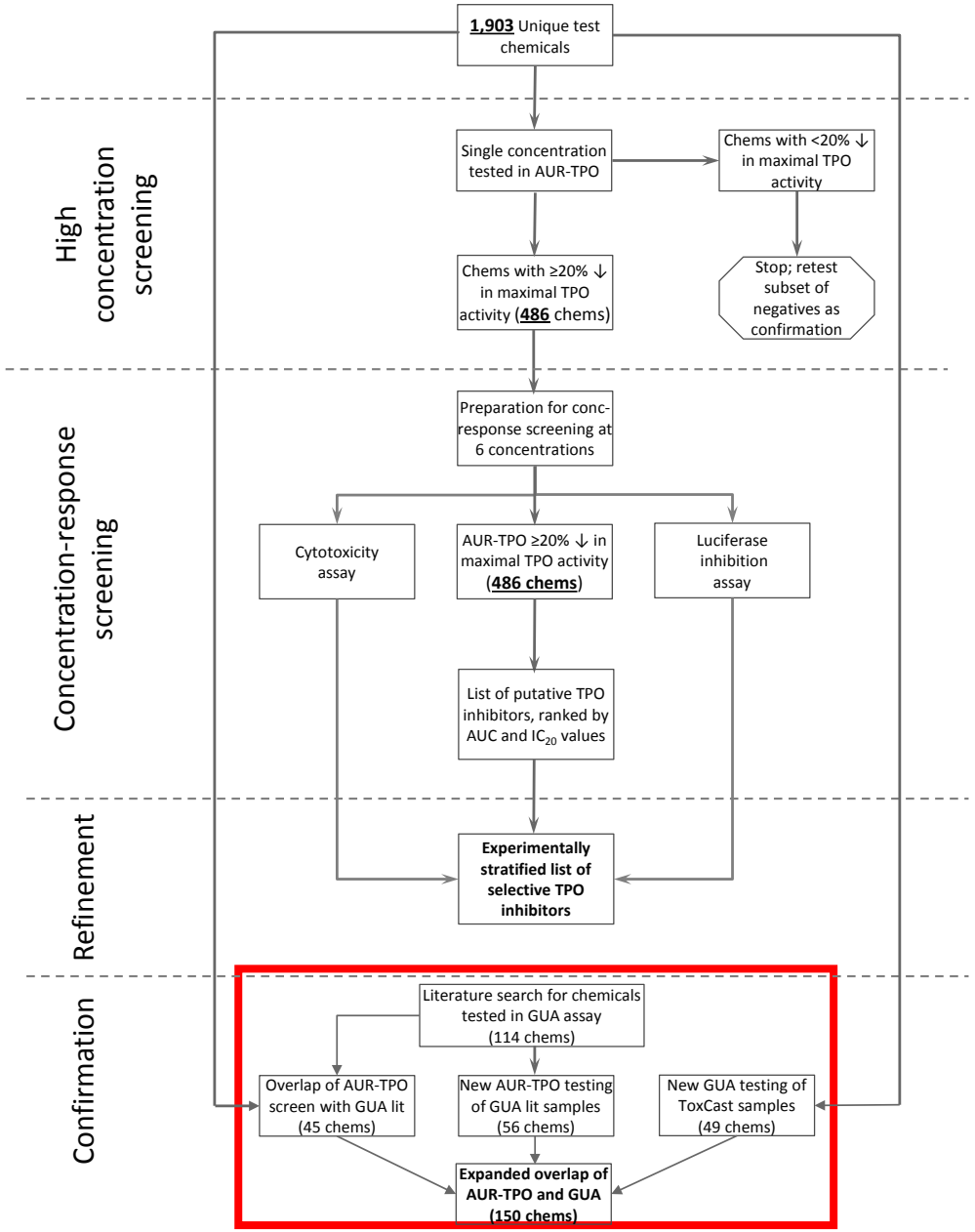
Prioritization by Selectivity

- Loss-of-signal assays like AUR-TPO often have high false positive rates owing to multiple non-specific mechanisms that deplete assay signal:
  - Detergents, acids, etc. that act a protein denaturants
  - Indiscriminately reactive chemicals
  - Fluorescent quenching chemicals
- Multiple, orthogonal HTS assays do not exist for thyroid MIEs like ER and AR → no assay read-across
- Two parallel assays deployed in multi-conc:
  - Luciferase inhibition
  - Cytotoxicty
- Selectivity defined as log M difference between AUR-TPO IC_{20} and IC_{20} of nearest parallel assay
- Over half of the putative TPO inhibitors identified in the HTS assay has low/no selectivity for TPO
- Those TPO inhibitors with the highest selectivity have structural similarity *

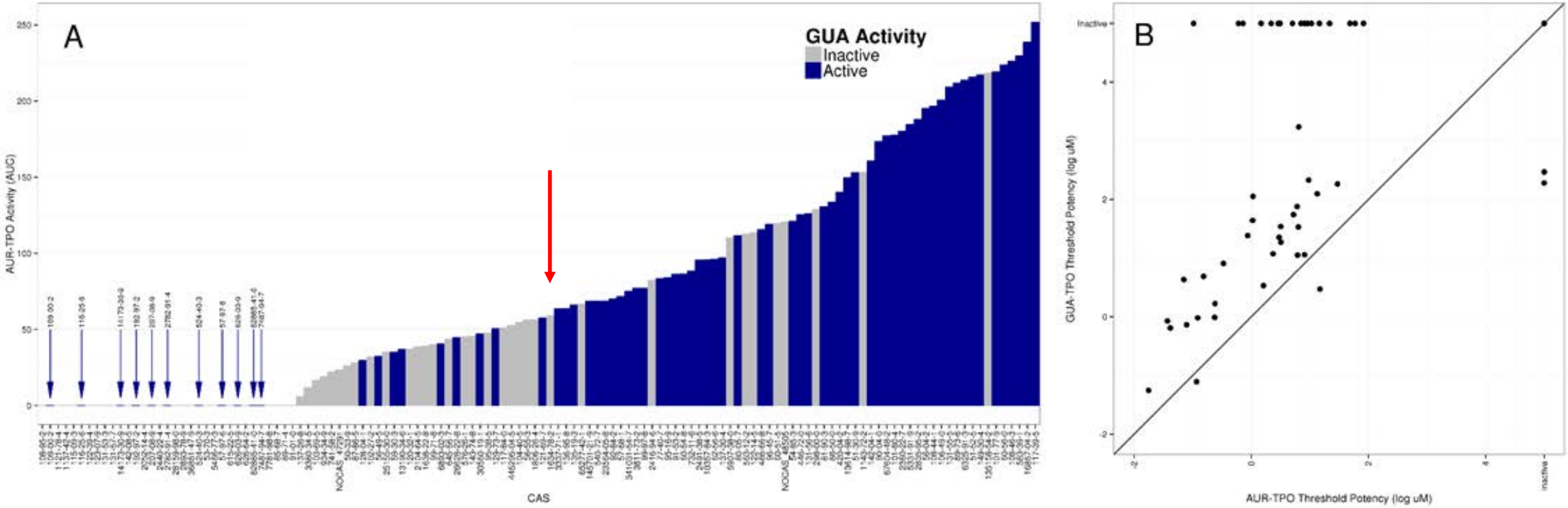
● AUR-TPO
■ Cytotoxicity
▲ Luciferase Inhibition
★ ToxCast Cytotoxicities

Paul Friedman and Watt et al., 2016, *Tox Sci*

Tier 3: Orthogonal Confirmation



Closing the Loop: Conformational Testing

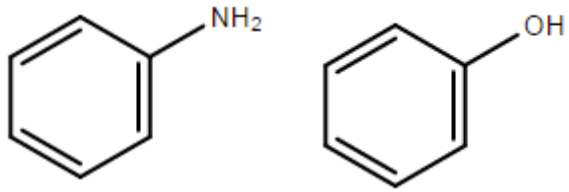


- We expanded the ~80 chemicals previously tested in GUA to 150 for comparison
- GUA activity scored as active/inactive due to heterologous study design/data analysis in literature
  - Enzyme sources, test chemical concentrations (ranges), solvents, hit-calling criteria
- AUR-TPO left-shifting was consistent with only a few exceptions
- Balanced accuracy was ~70% *

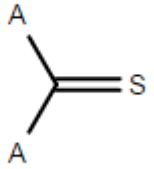
Paul Friedman and Watt et al., 2016, *Tox Sci*

Future Research

- AUR-TPO assay and deployment is a necessary step to addressing the knowledge gap for thyroid disrupting chemicals in the environment
- An assessment of how much AUR-TPO improves *in vivo* predictivity may be premature
- <u>HOWEVER</u>:
  - AUR-TPO likely overpredicts thyroid disruption *in vivo*: forced interactions vs. ADME
  - AUR-TPO will not likely identify thyroid disruptors that work through other MIEs
- Establishes the 'possible': ADME favorable + TPO most sensitive target = ↑ risk of thyroid disruption
- HTS assays for MIEs beyond TPO are currently be developed: NIS and DIOs
- AUR-TPO assay currently being used to develop QSAR model to predict putative TPO inhibitors:

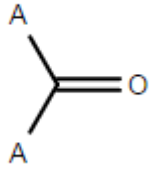


P - - S



C ------ SH

C ------ X



C --- O --- C

Acknowledgements

EPA-NCCT

Katie Paul-Friedman
Eric Watt
Kevin Crofton
Danielle Suarez
Richard Judson
Keith Houck
Ann Richard
Chris Grulke

EPA-NHEERL

Mike Hornung
Joan Hedge
Mary Gilbert