

A High-Throughput Screening Assay to Detect Thyroperoxidase Inhibitors

Steve Simmons



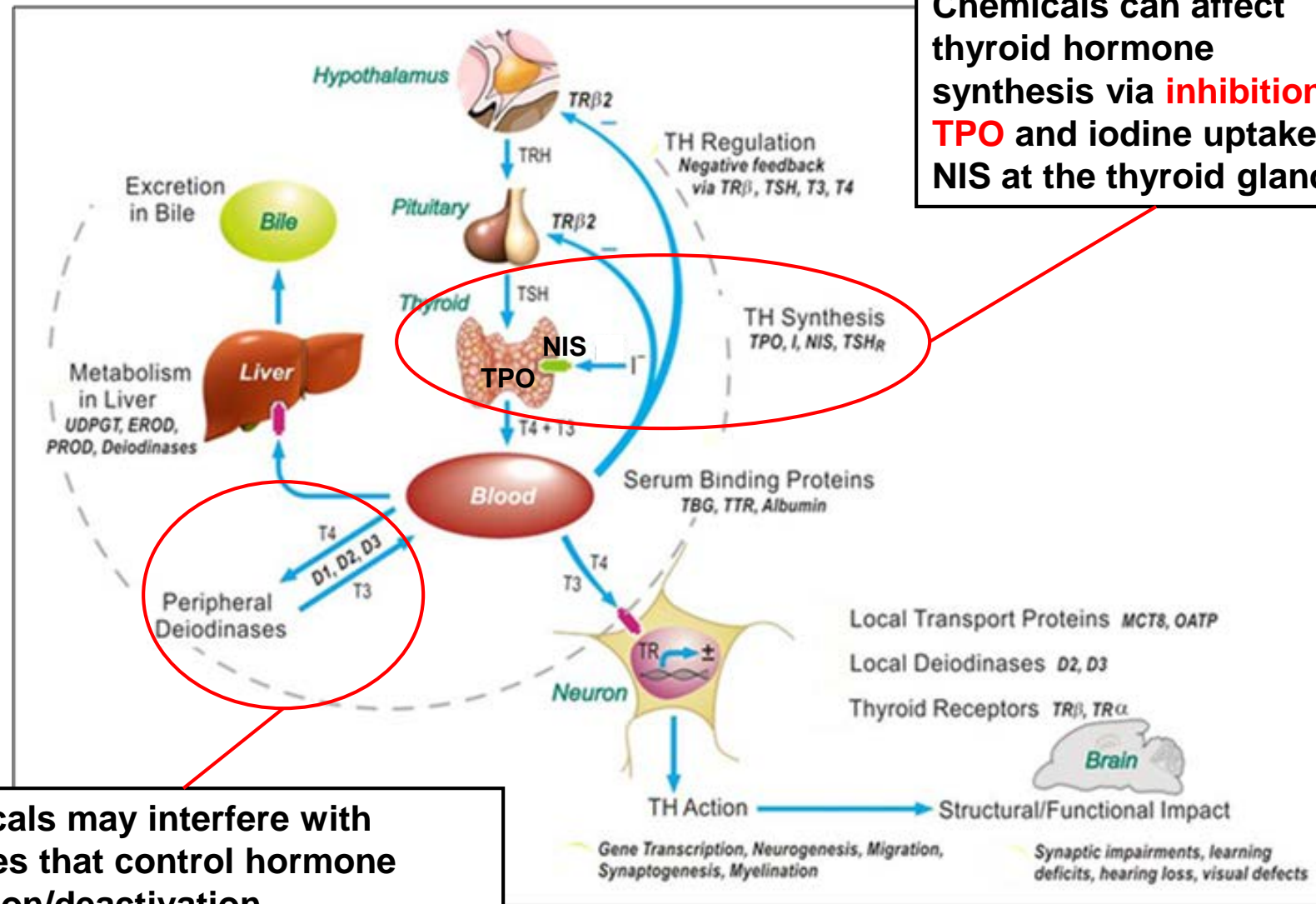
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Disclosure

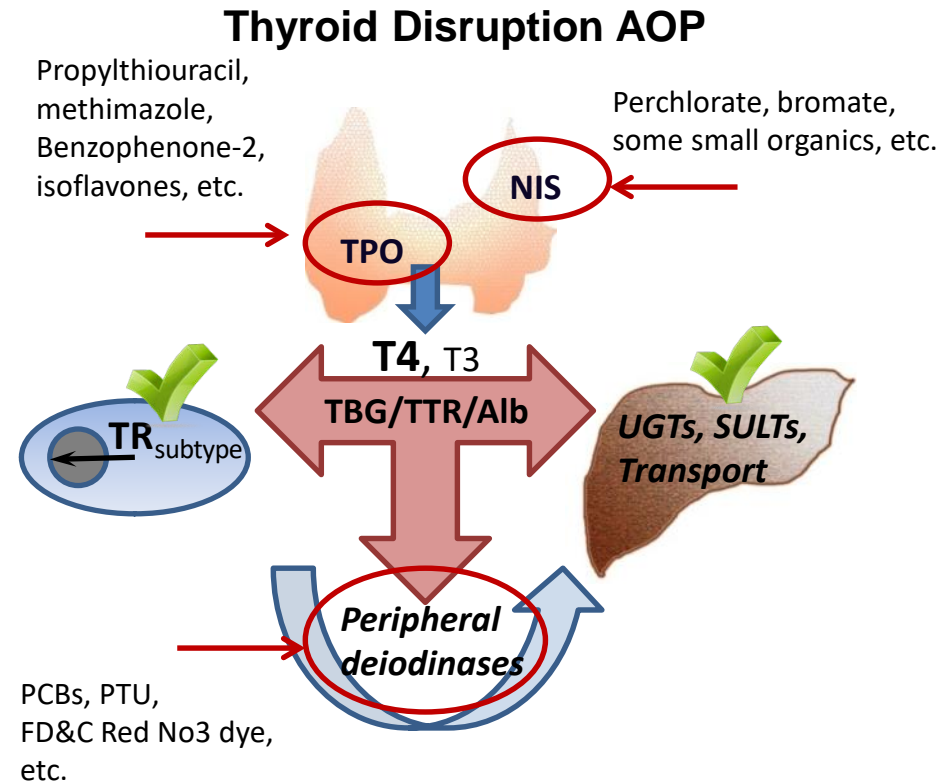
THE AUTHORS OF THIS RESEARCH HAVE NO FINANCIAL OR OTHER INTERESTS WHICH POSE
A CONFLICT OF INTEREST

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

Critical HPT-Axis Endpoints Need to be Addressed



ToxCast Thyroid Coverage



ToxPi predictive tool for endocrine disruptors

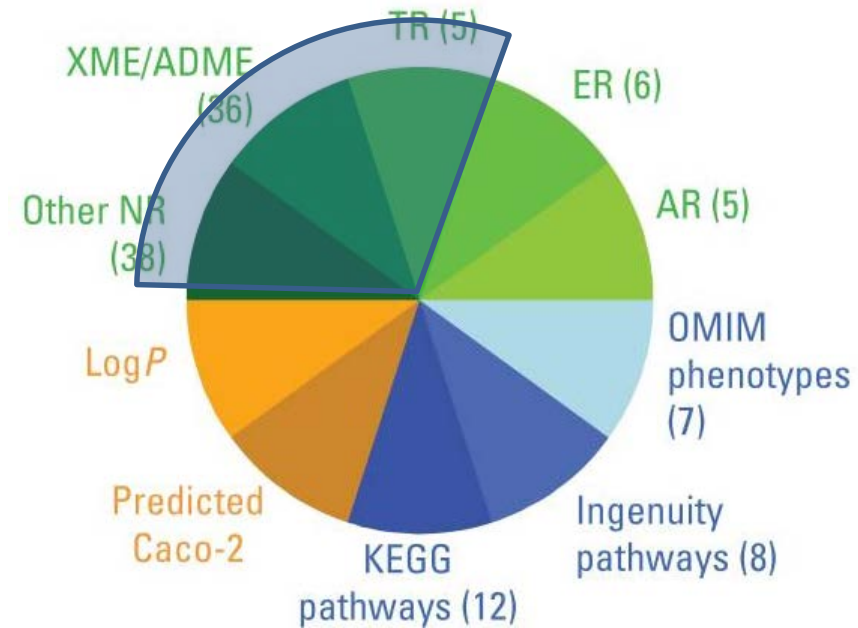


Figure reproduced from Reif et al. 2010 EHP

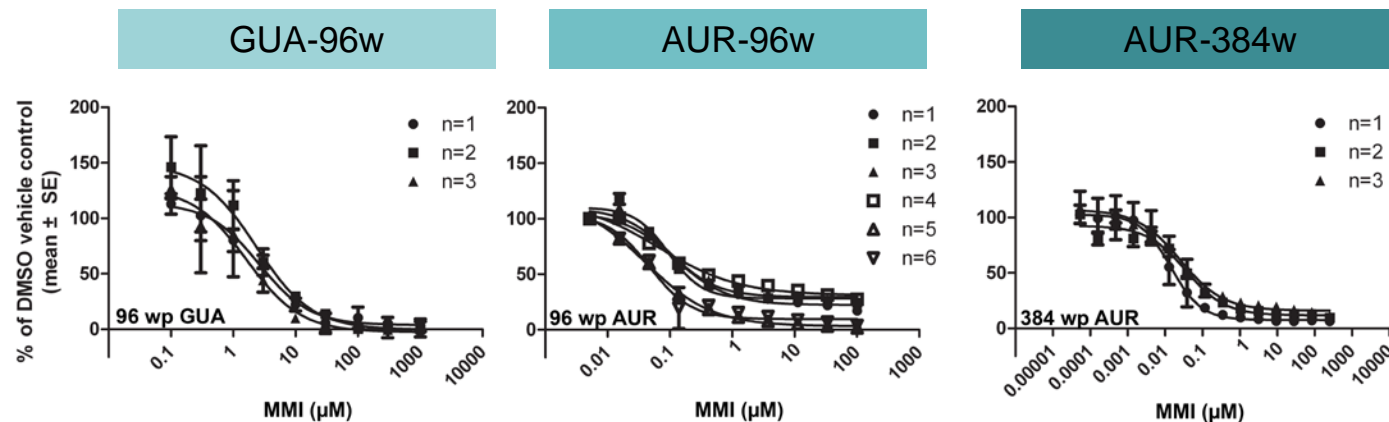
- Thyroid receptor is not a target for many environmental chemicals, nor for known environmental thyroid disruptors
- Disruption of TH synthesis (via TPO and/or NIS) and of T4→T3 hormone activation (via DIOs) are more common targets of environmental thyroid-disrupting chemicals
- No HTS-amenable solutions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

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

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

6-propylthiouracil

Sodium-perchlorate

2-mercaptobenzothiazole

3,5-Dimethylpyrazole-1-methanol

4-propoxyphenol

Triclosan

Diethylphthalate

Diethylhexylphthalate

Dibutylphthalate

Methyl 2-methyl-benzoate

2,2',4,4'-Tetrahydroxy-benzophenone

4-nonylphenol

2-hydroxy-4-methoxybenzophenone

Resorcinol

Ethylene thiourea

3-Amino-1,2,4-Triazole (Amitrole)

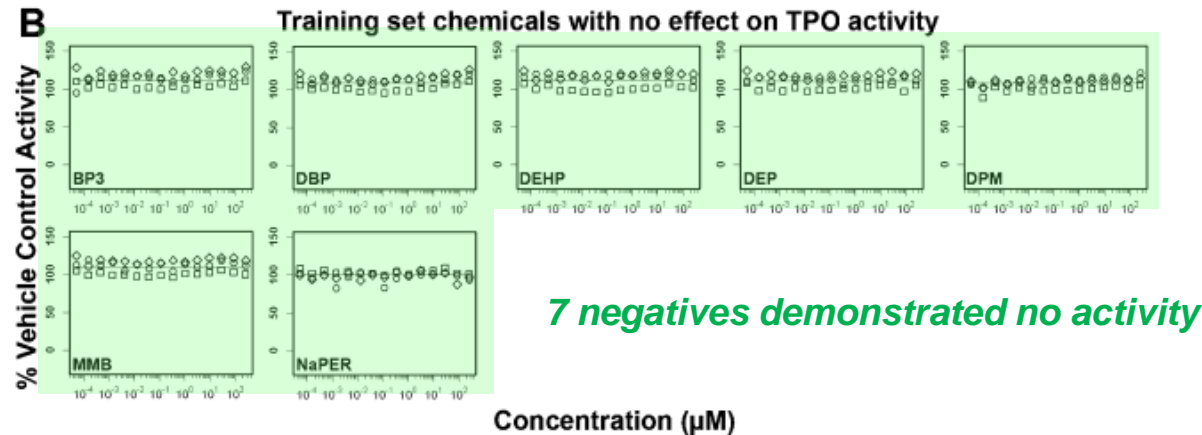
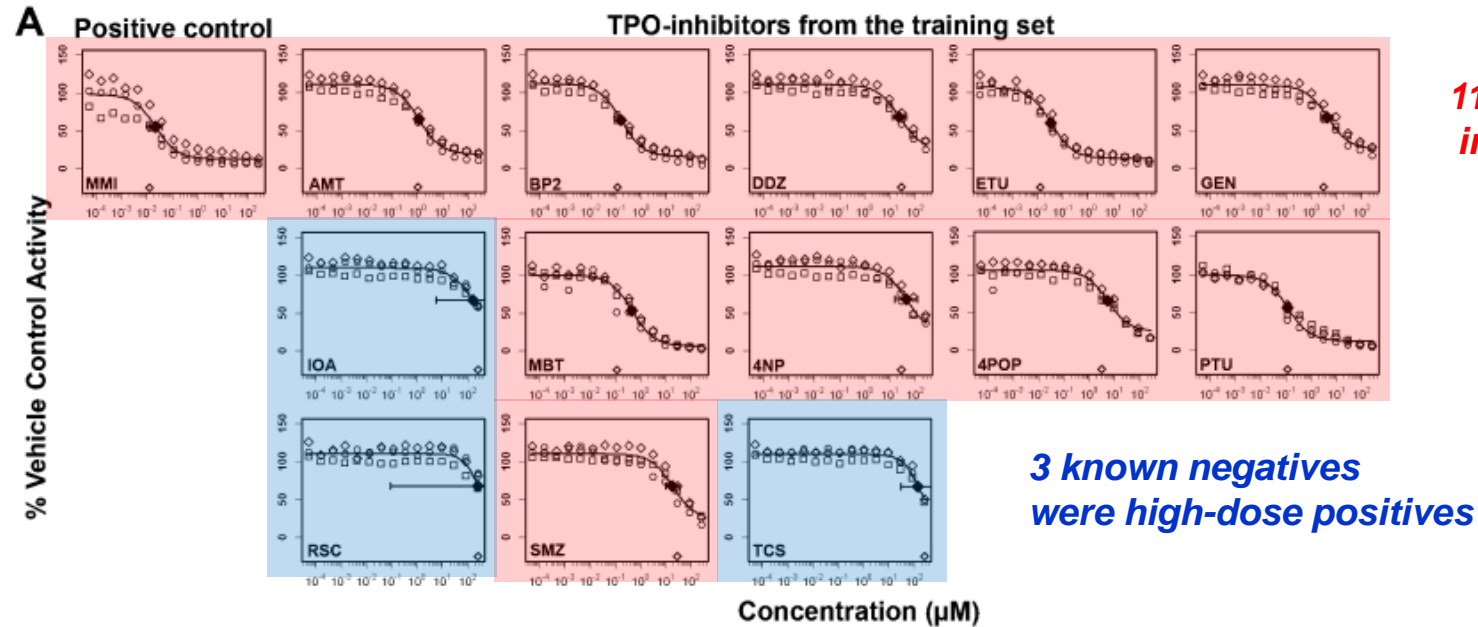
Iopanoic Acid

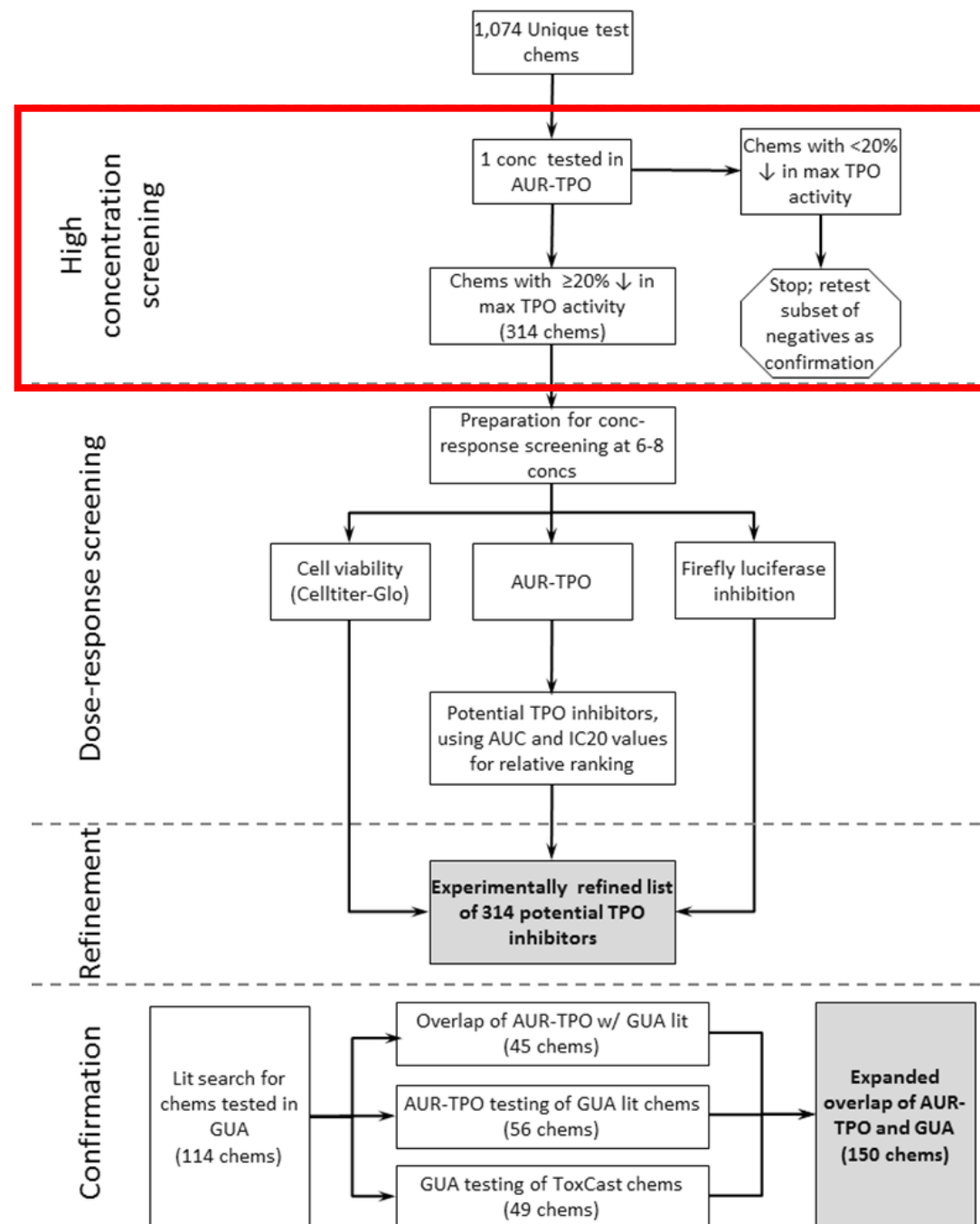
Daidzein

Genistein

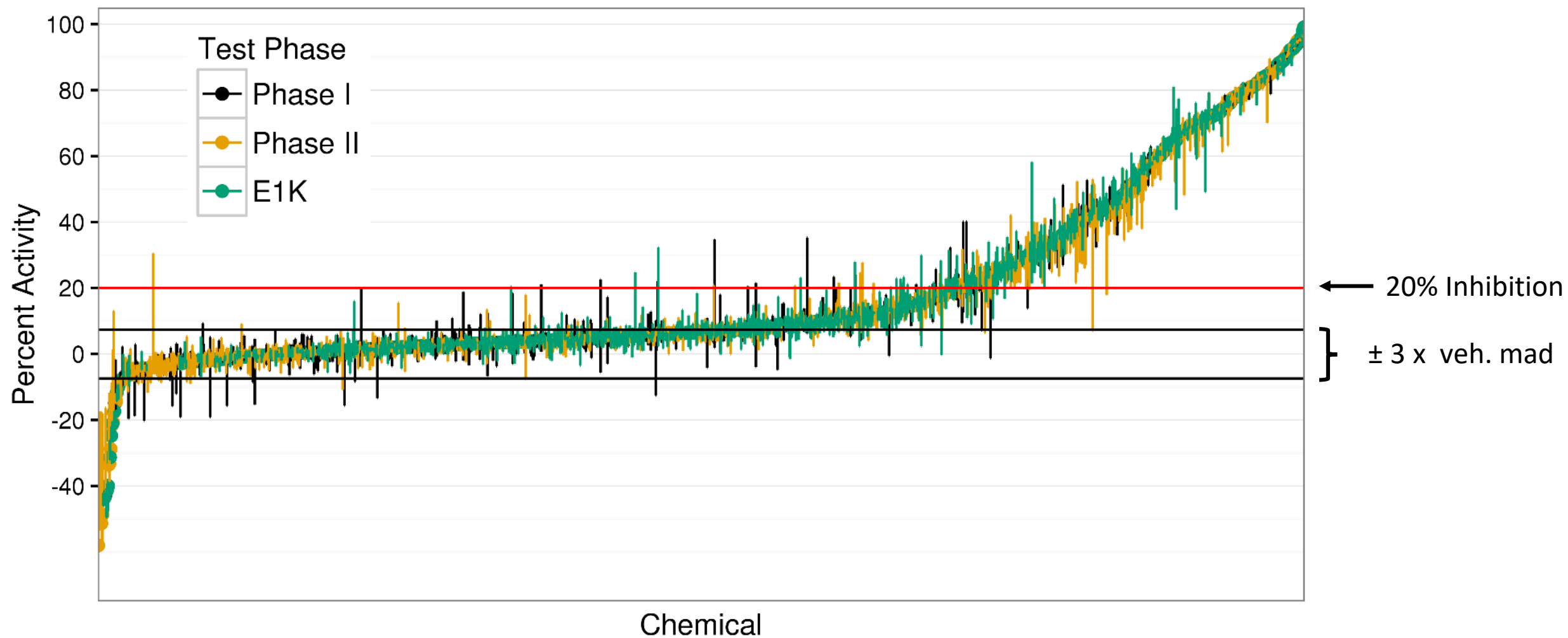
Sulfmethazine

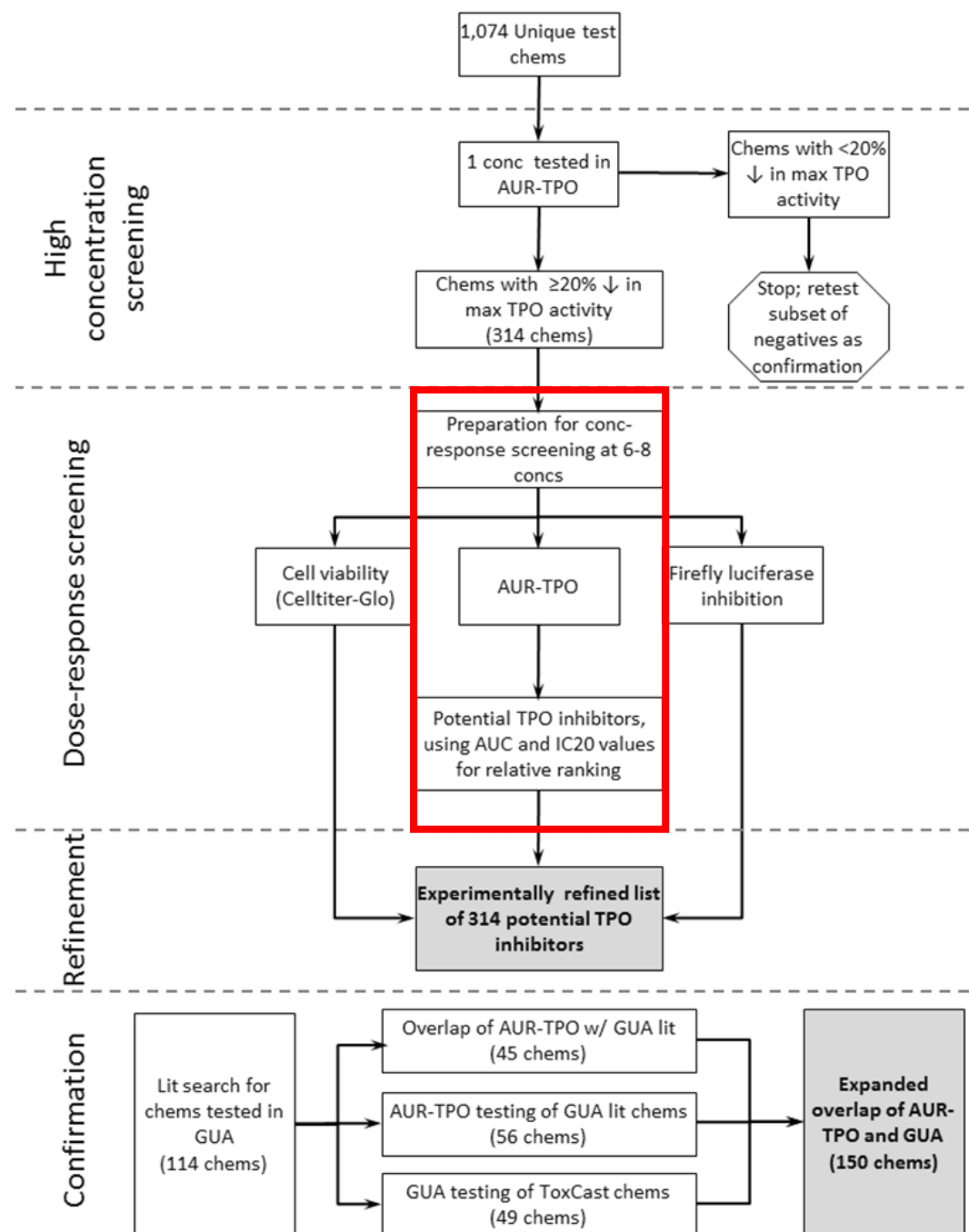
Validation of AUR-TPO Assay



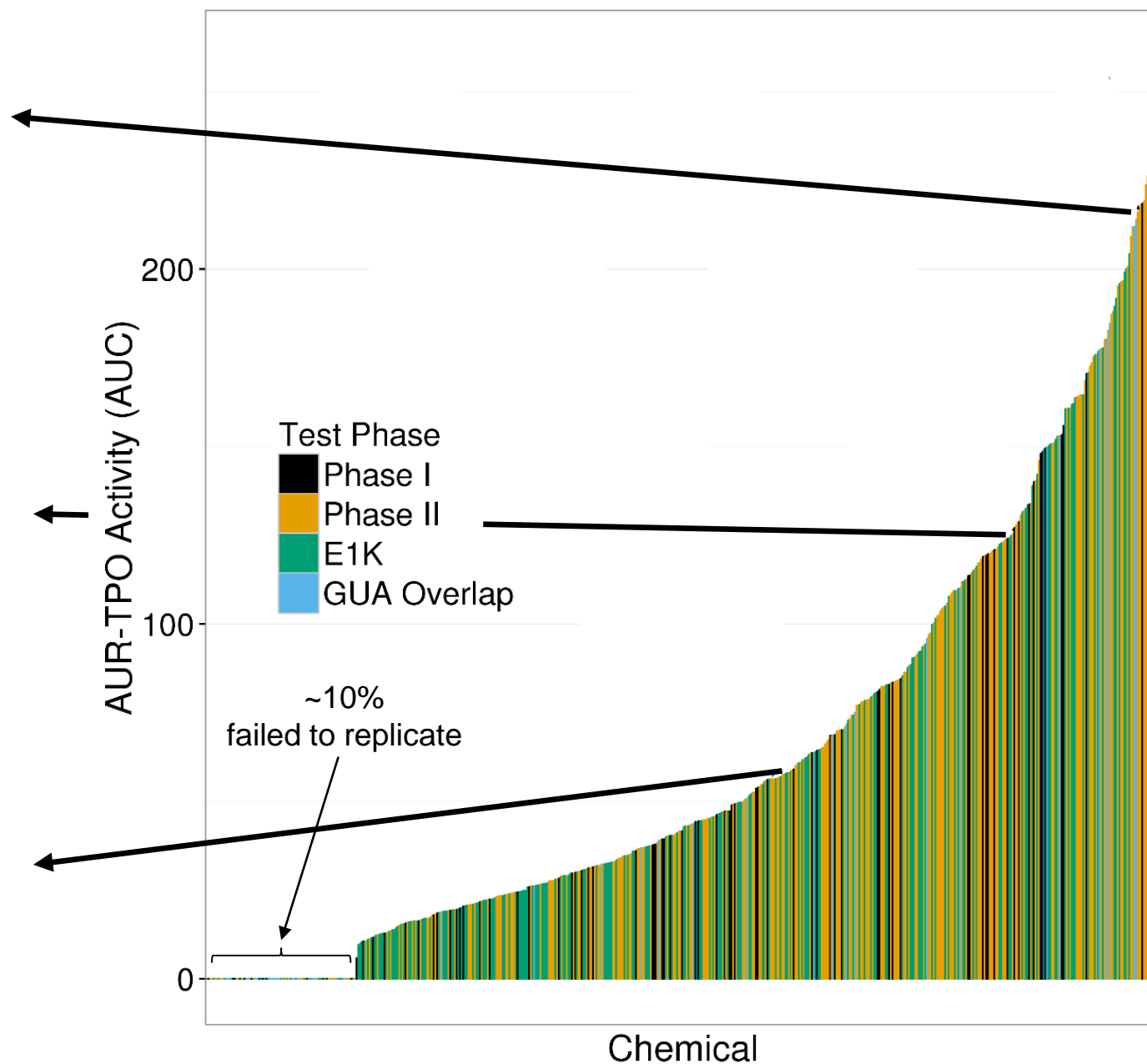


Single-conc Testing

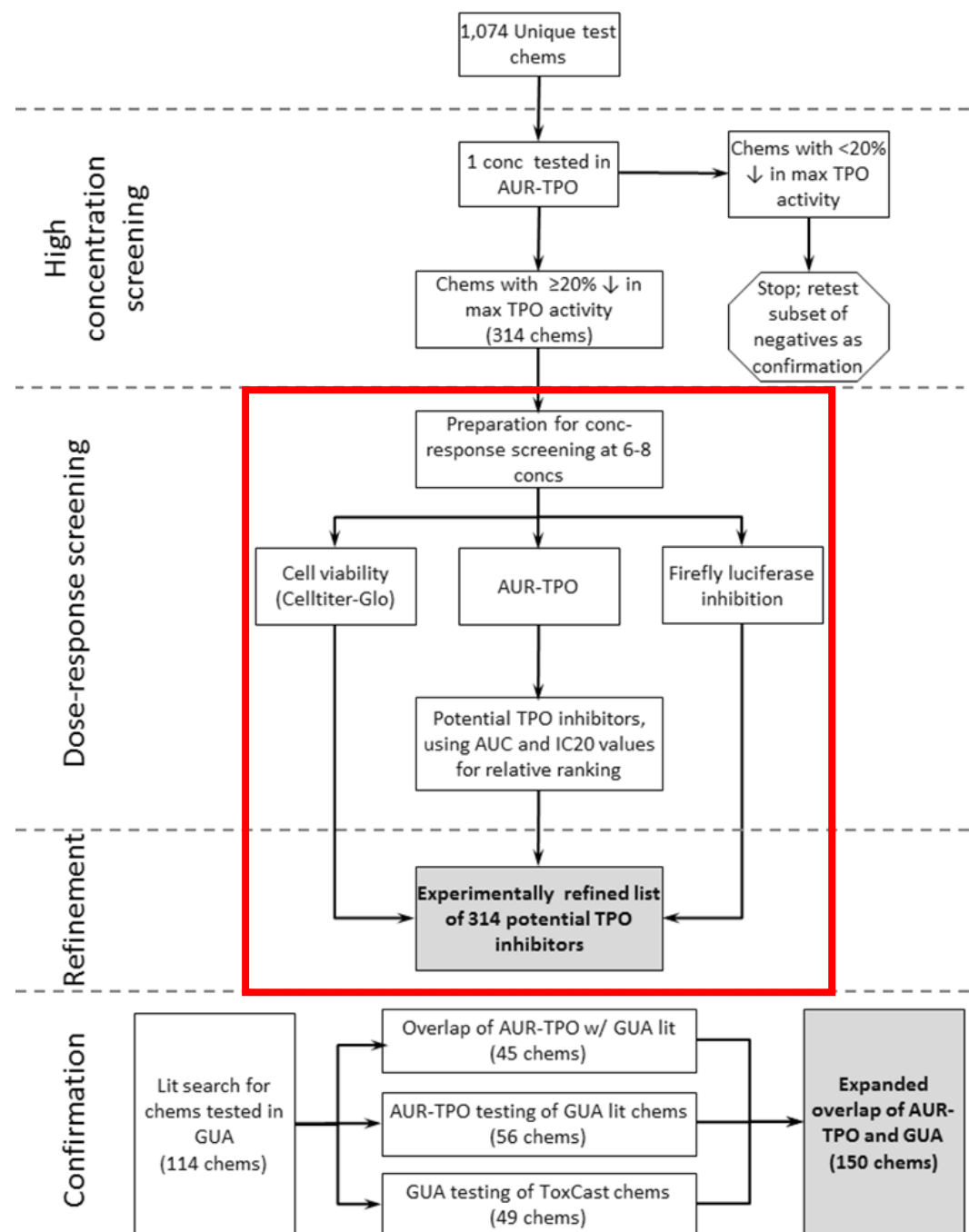




Multi-conc Testing



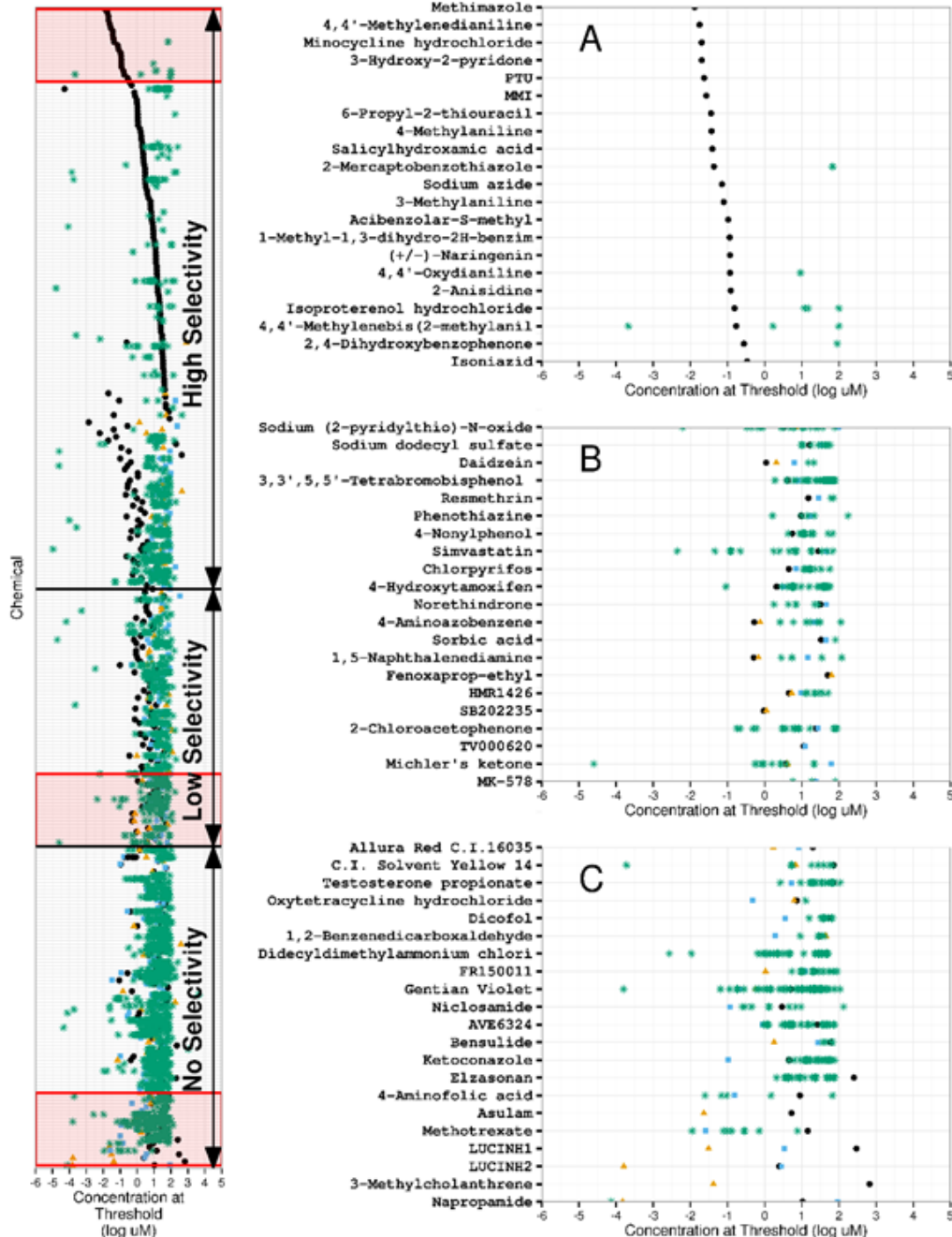
- Multi-conc data fit using Toxcast pipeline
- Area under fitted curve (AUC) calculated using integrate (base R) for hitc =1
- Inactive -> AUC = 0

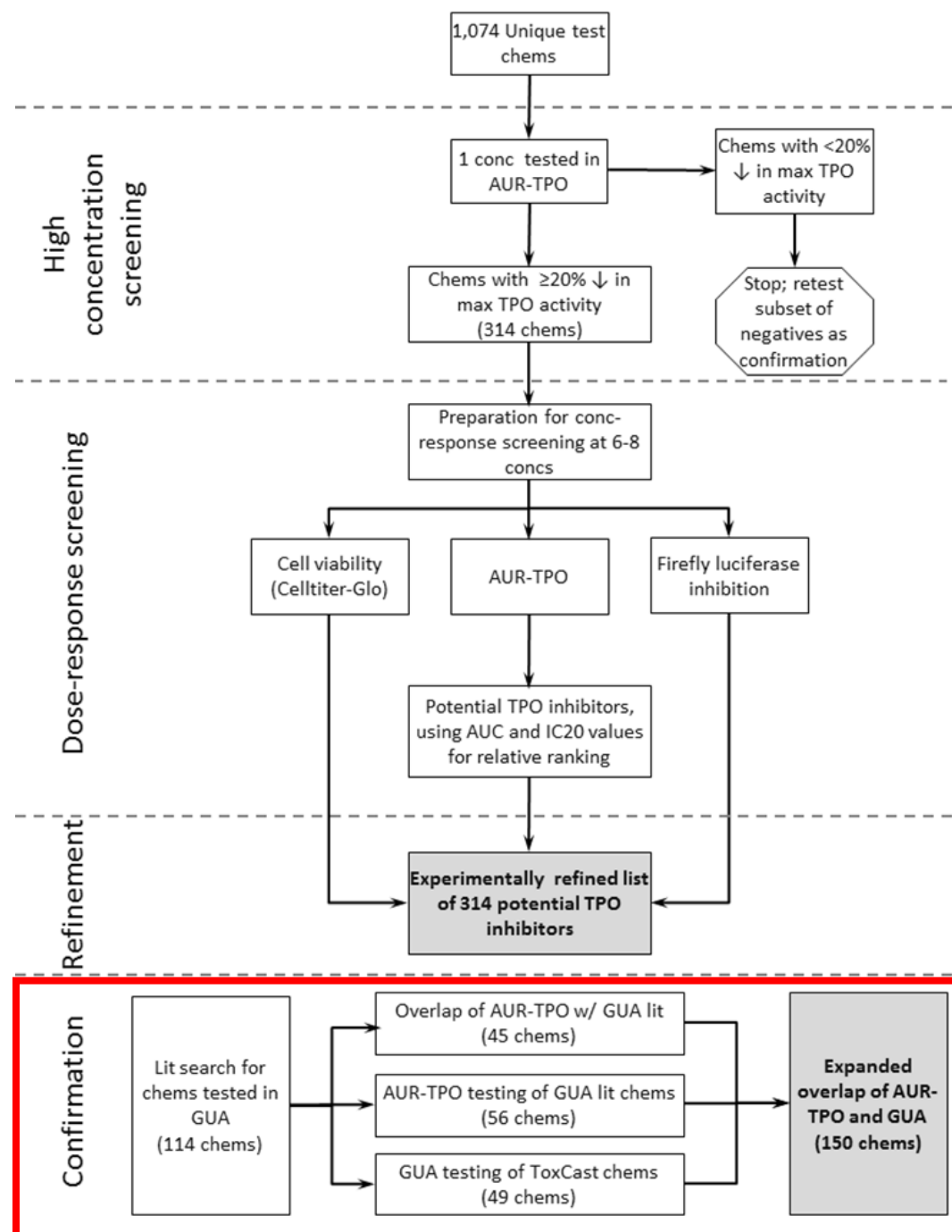


Prioritization by Selectivity

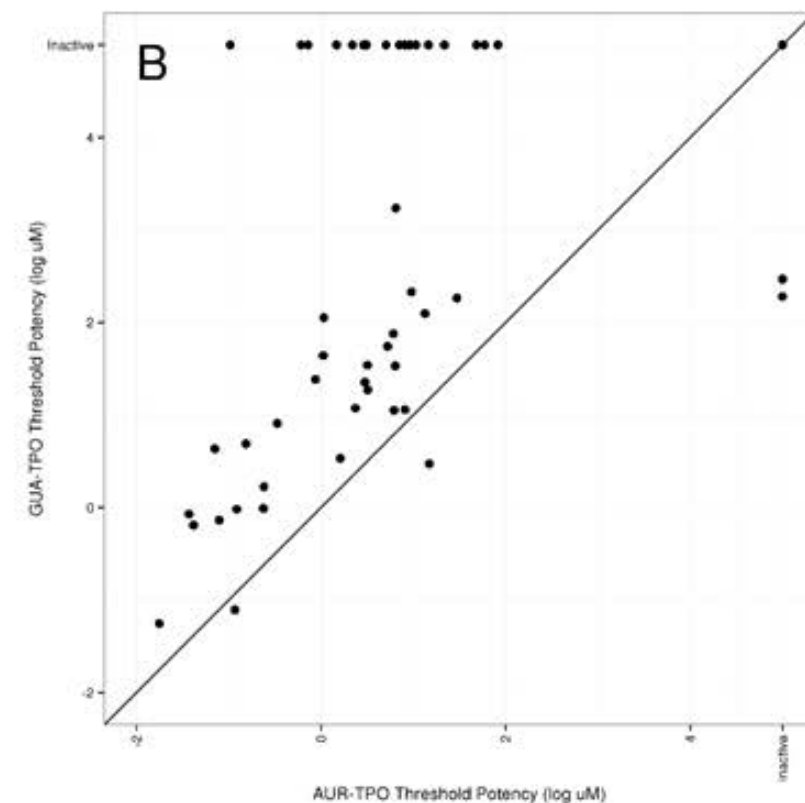
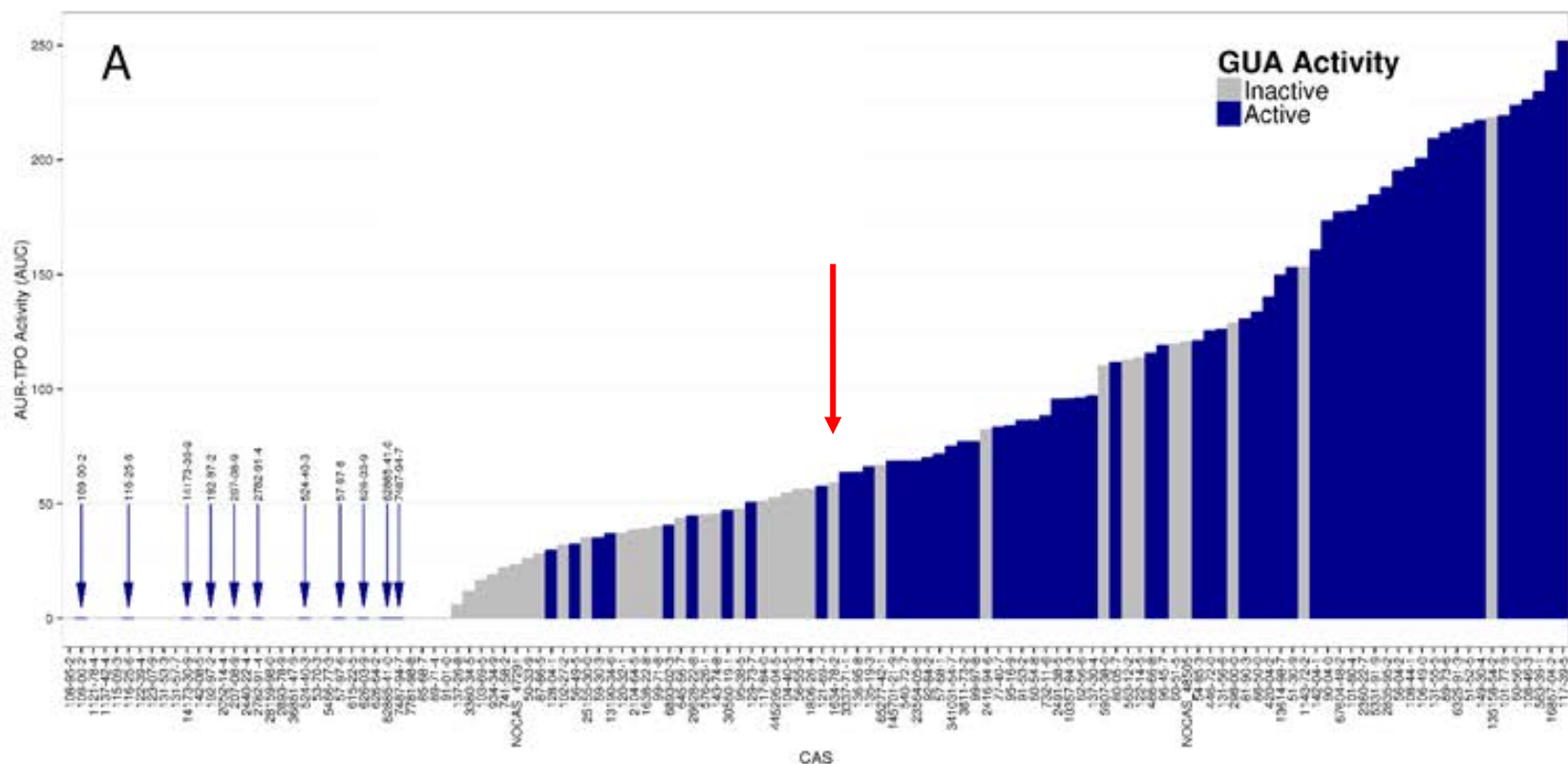
- Loss-of-signal assays like AUR-TPO often have high false positive rates owing to multiple non-specific mechanisms that deplete assay signal:
 - Detergents, acids, etc. that act as protein denaturants
 - Indiscriminately reactive chemicals
 - Fluorescent quenching chemicals
- Multiple, orthogonal HTS assays do not exist for thyroid MIEs like ER and AR → no assay read-across
- Two parallel assays deployed in multi-conc:
 - Luciferase inhibition
 - Cytotoxicity
- 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





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% *

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
- HOWEVER:
 - 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

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