Update on U.S. progress on *in vitro* thyroid-axis endpoint screening assays

17th Meeting of the Validation Management Group on Non-animal Testing

OECD, Advisory Group on Endocrine Disrupters Testing and Assessment (EDTA) of the Test Guidelines Programme.

Virtual Meeting/Teleconference

Thursday, November 7, 2019

Contributors

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Thyroperoxidase Inhibition

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Radioactive Iodide Uptake Inhibition

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TSHR / TRHR

Katie Paul-Friedman

Thyronine and Tyrosine Deiodinase Inhibition

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Thyroid Hormone Activation

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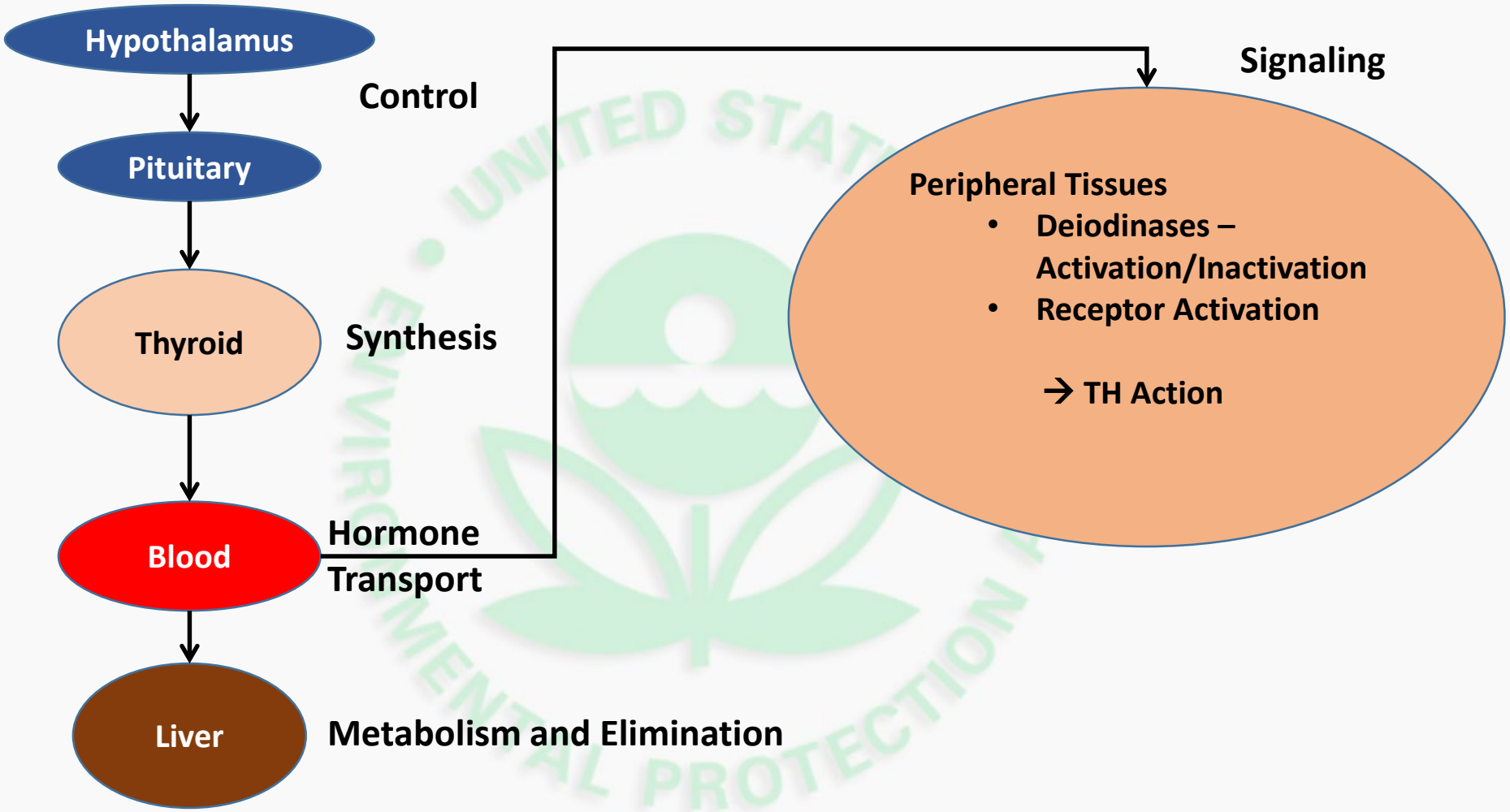
Thyroid Axis



Screening Assays

Control
- **TSHR → stimulation of hormone production**
- **TRHR**

HTP screening assays in Tox21, but more work needed to establish specificity.

Synthesis
- **TPO Inhibition → hormone iodination and coupling**
- **NIS Inhibition → iodide uptake**
- **IYD Inhibition → iodide recycling**

Signaling
- **DIO 1, DIO2, DIO3 Inhibition → hormone activation / inactivation control**
- **Thyroid hormone receptor transactivation assays → ultimate signaling**

Thyroid Hormone Synthesis

Synthesis MIEs

<u>Iodide Transport</u>

Sodium-Iodide Symporter (NIS)

Pendrin (PEN)

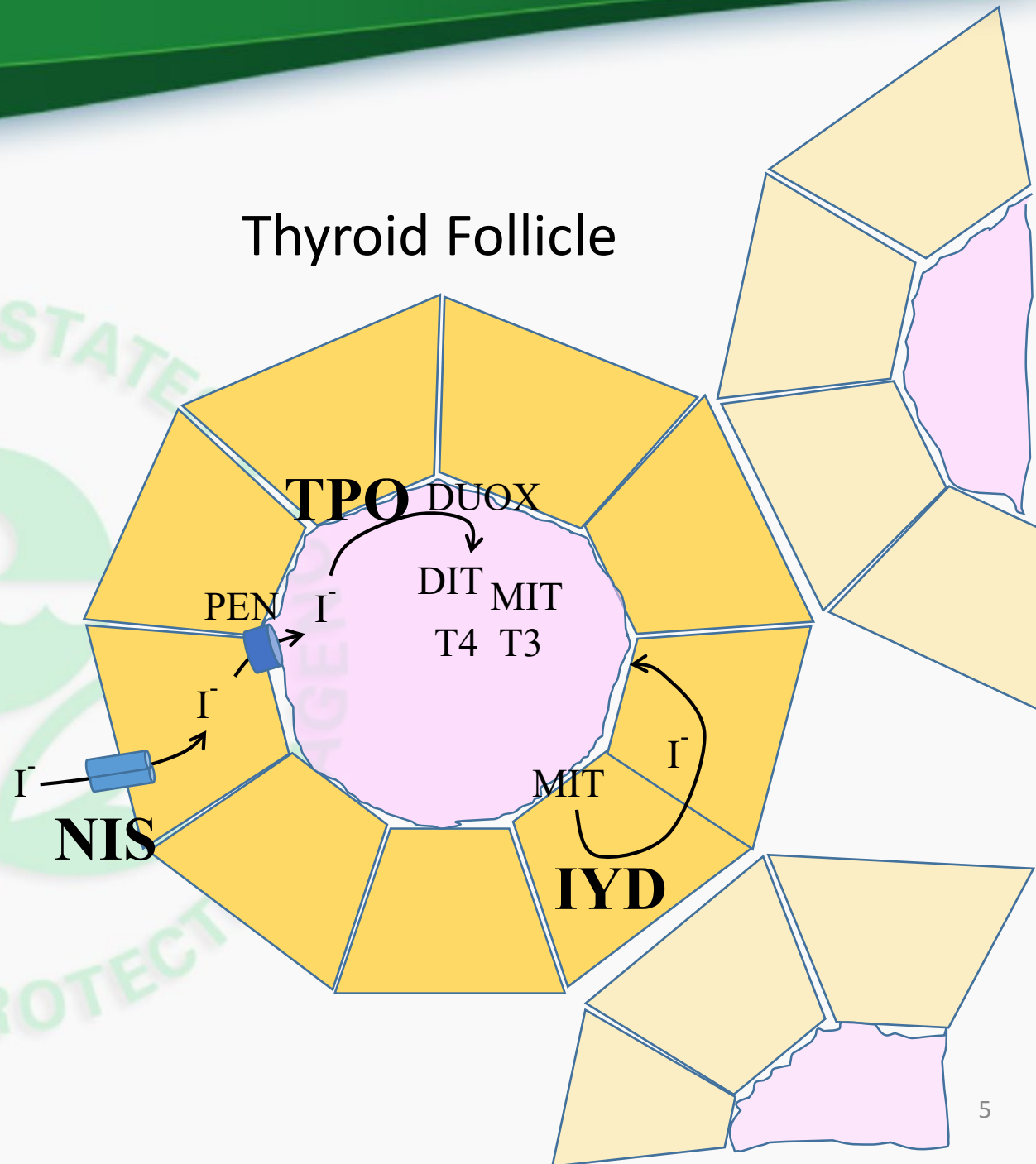
<u>Tyrosyl Iodination and Coupling</u>

Thyroperoxidase (TPO)

Dual Oxidase (DUOX)

<u>Iodide Recycling</u>

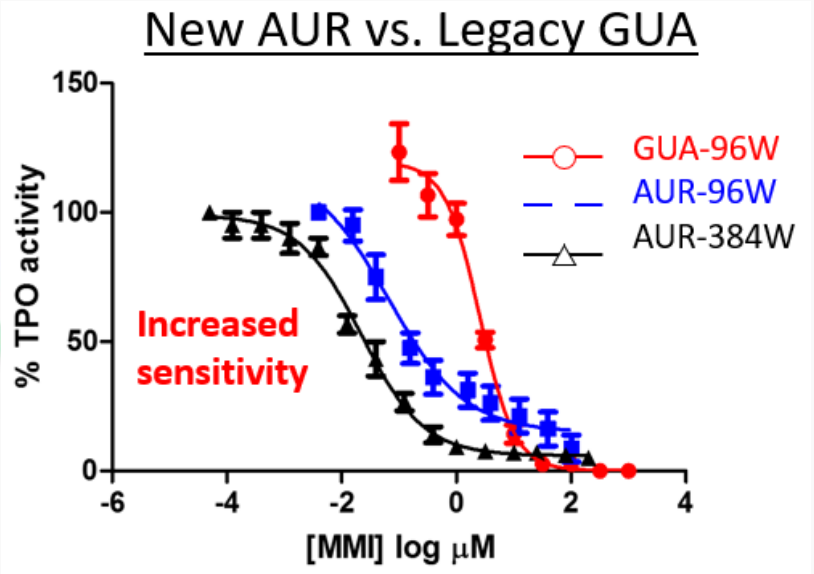
Iodotyrosine Deiodinase (IYD)

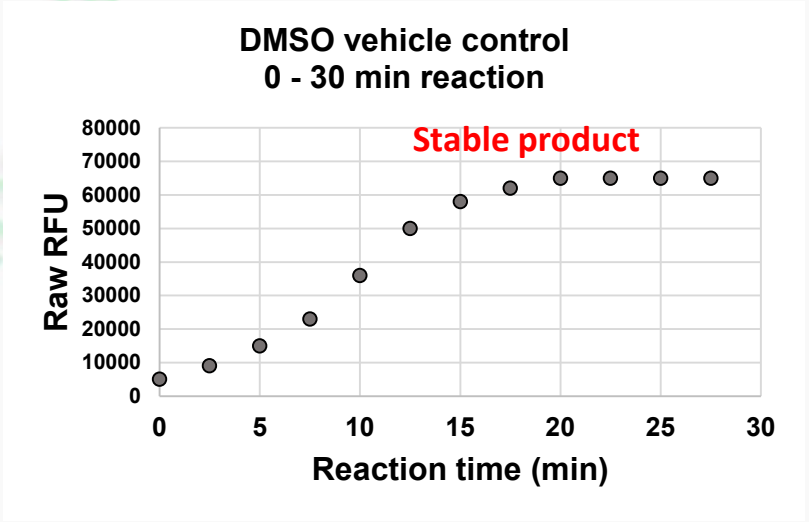


Thyroid Hormone Synthesis - TPO

Thyroperoxidase Inhibition

- TPO in the thyroid gland catalyzes tyrosine iodination & coupling in synthesis of T4 & T3.
- Evaluated commercially-available peroxidase substrates to find a substitute for guaiacol that was HTS-amenable:
    - **Amplex UltraRed (AUR)** met criteria needed for a HTS substrate
- Rat thyroid gland microsomes were source of TPO for this screening
- Recombinant TPO source is promising

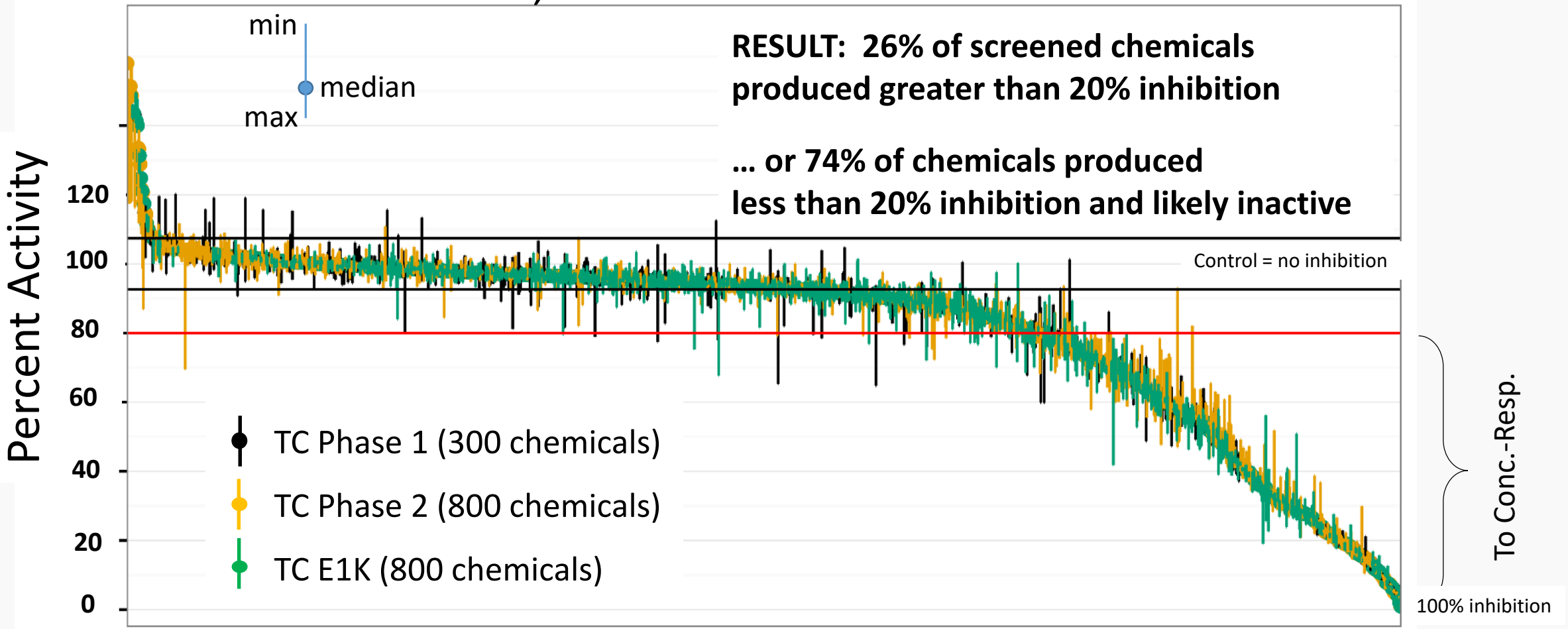




Thyroid Hormone Synthesis - TPO

Single-concentration screen for TPO inhibition activity:

1,900 ToxCast chemicals

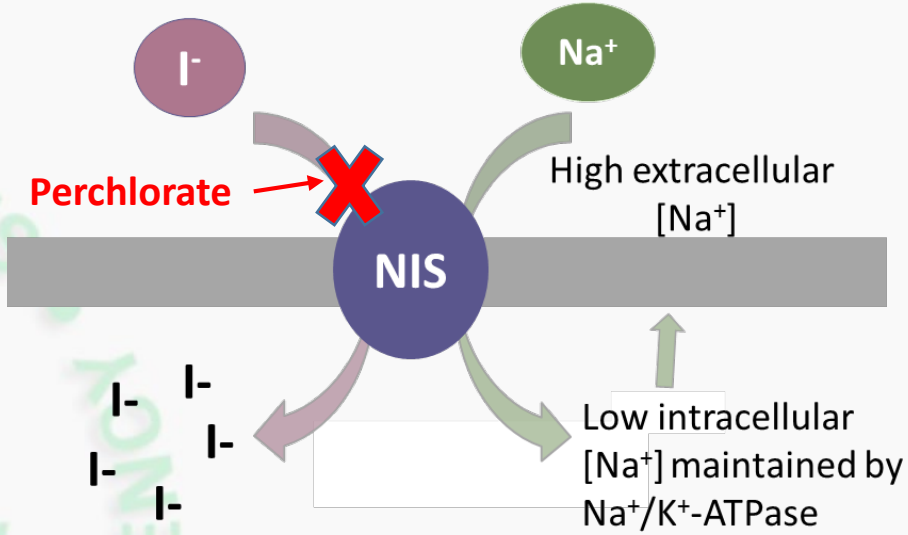


Thyroid Hormone Synthesis - NIS

Iodide Uptake Inhibition

NIS = Sodium/iodide (Na^+/I^-) symporter

- Mediates thyroid gland iodide uptake
- Known target of environmental contaminants (ex. Perchlorate, ClO_4^-)
- Limited knowledge for more structurally diverse chemicals



Radioactive Iodide Uptake Assay (RAIU)

Cell Culture
hNIS-HEK293T-EPA

→

Radioactive ^{125}Iodide Uptake
X
Cell Viability

→

Measure ^{125}I uptake
X
Cell Viability by ATP

NIS Screening – Single concentration results

Radioactive Iodide Uptake Inhibition

ToxCast phase 1, 2, and e1K libraries

Single-Concentration Screening

1896 Samples
RAIU assay (100 µM)

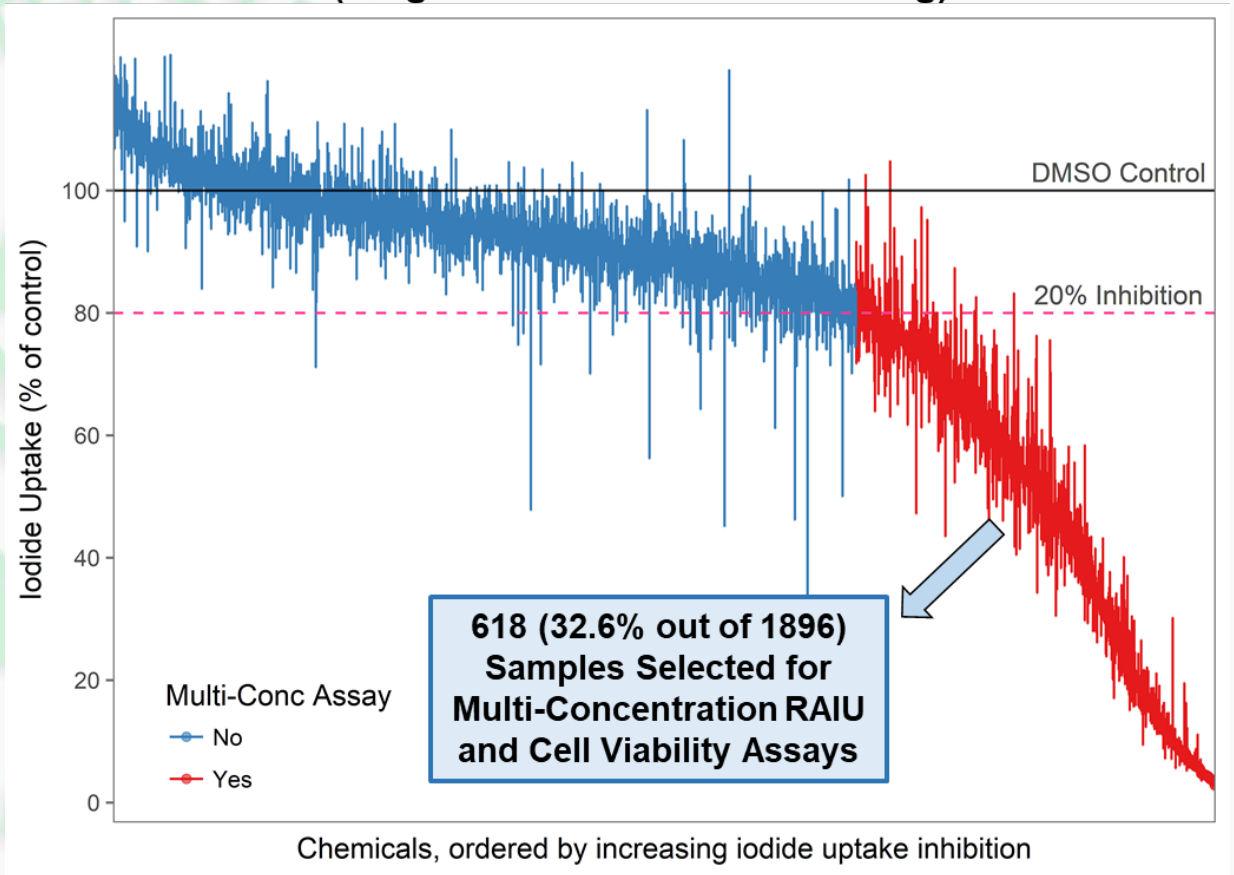
Selection

> 20% inhibition

Multi-Concentration Screening

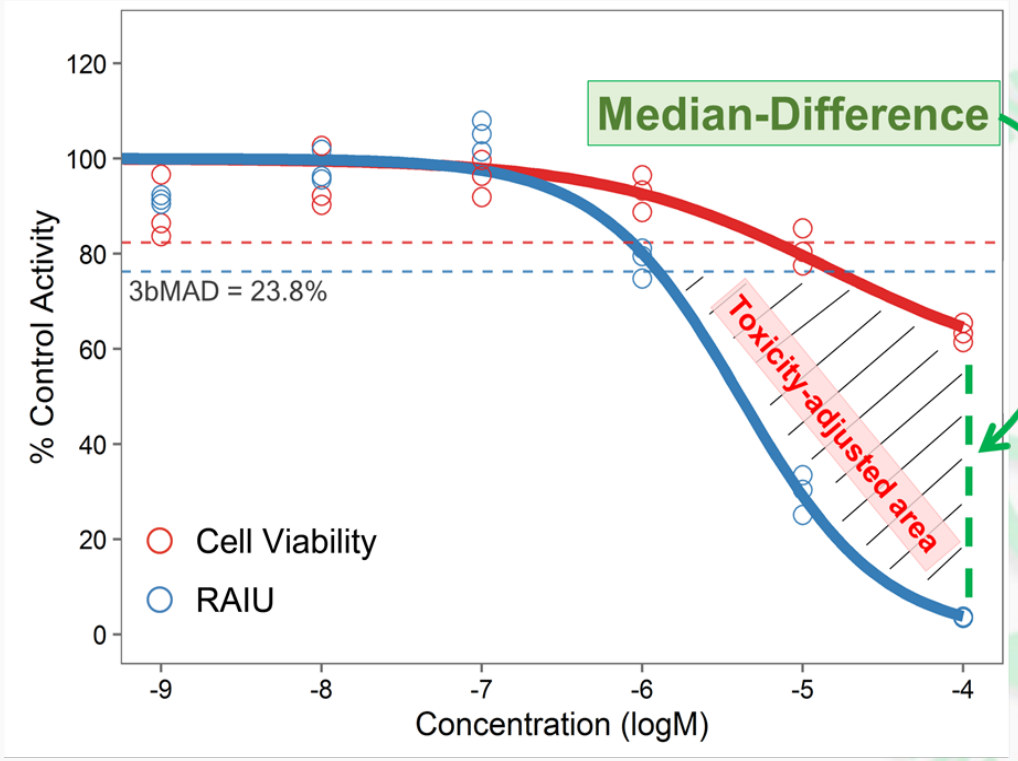
618 Samples
RAIU & Cytotox Assays
(0.001 - 100 µM)

Median and Range of Test Chemical Responses (Single Concentration Screening)



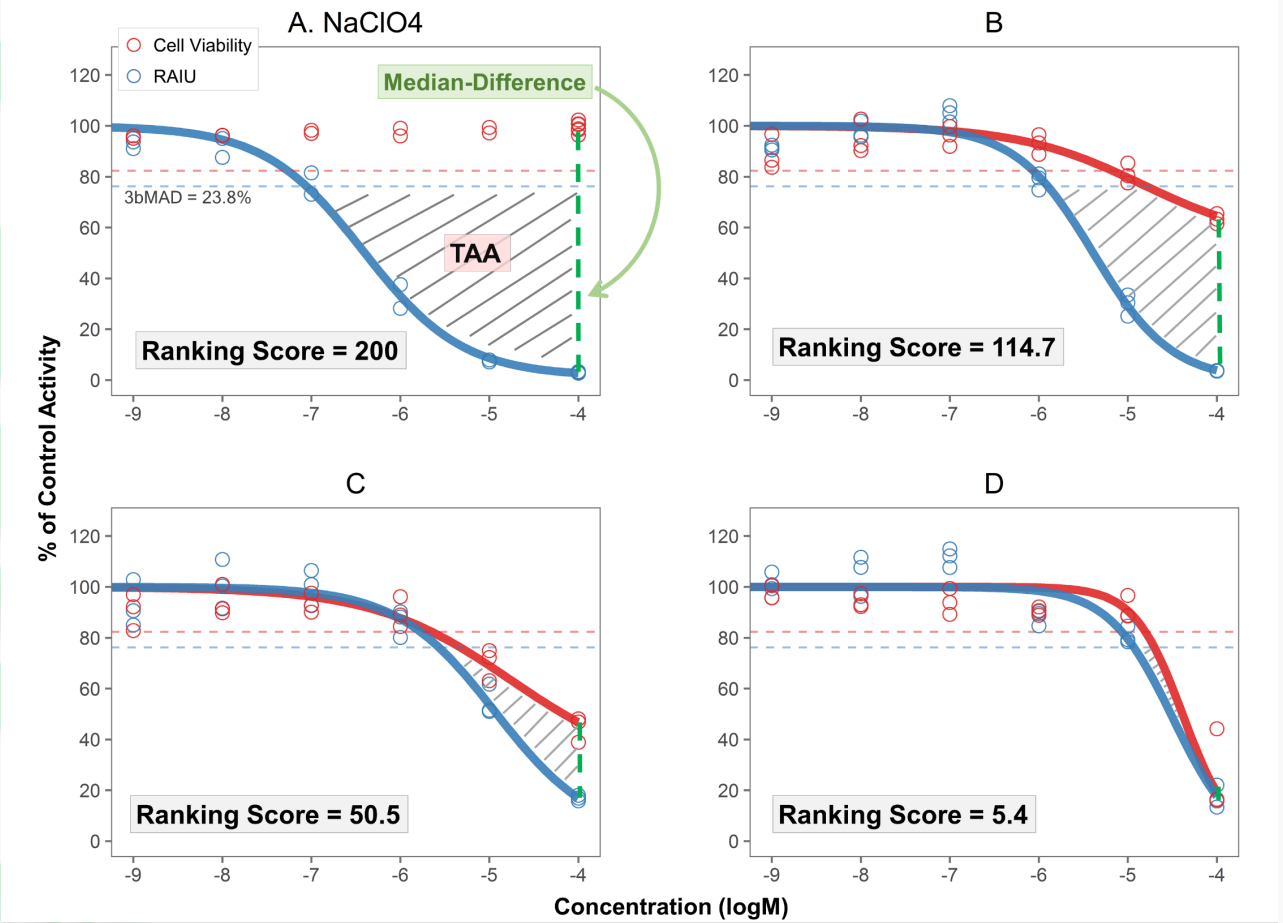
NIS Screening – Multi-concentration results

Quantitative Chemical Ranking for NIS Inhibitors



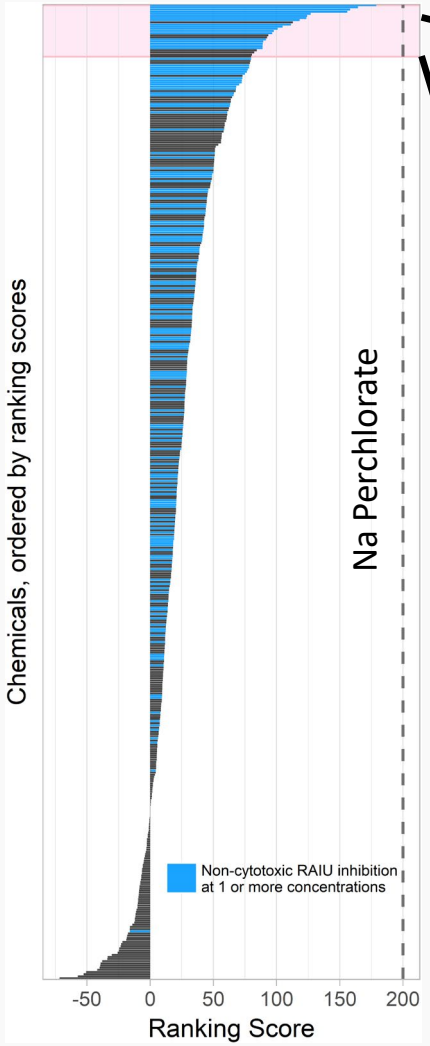
Toxicity-adjusted area + Median-Difference = Ranking Score

Example Curves and Ranking Scores

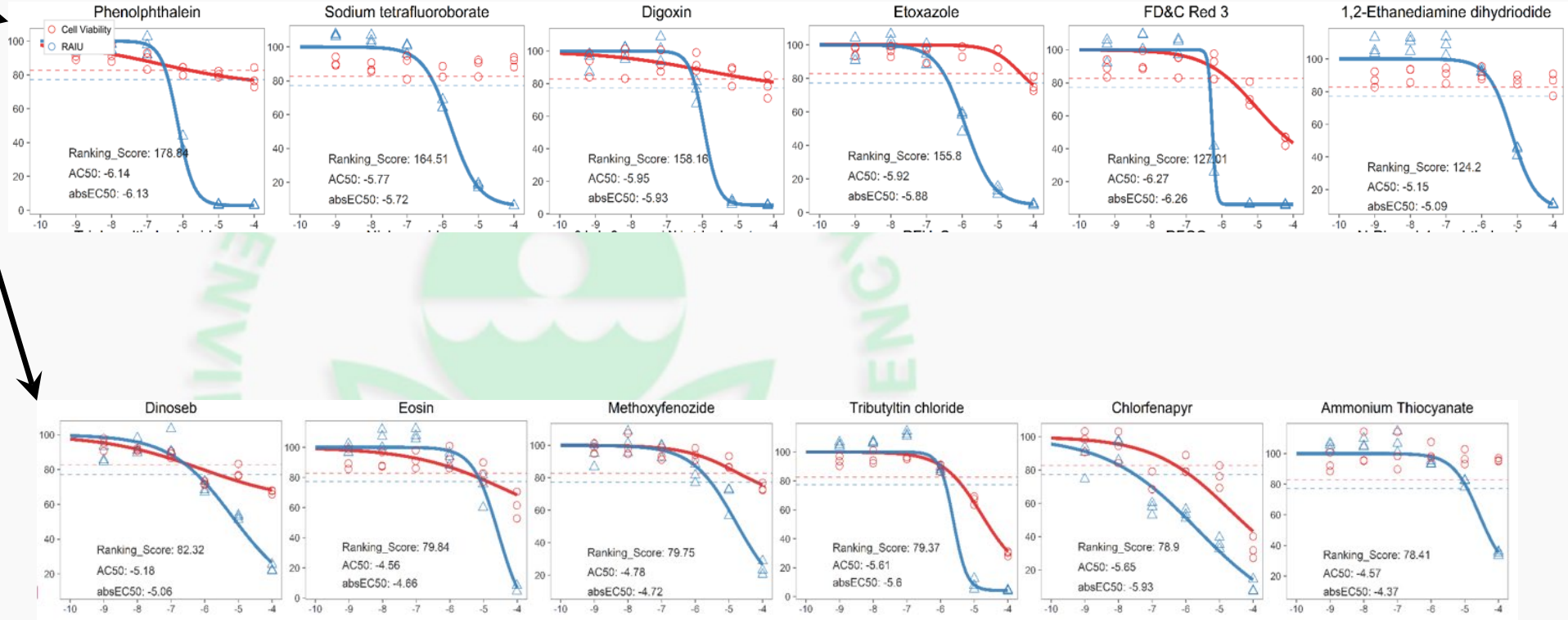


NIS Screening – Multi-concentration results

Ranking Scores



Conc.-resp. curves for example chemicals with high ranking scores



IYD Inhibition Assay

Recombinant human IYD (hIYD) Enzyme Production

Produced with baculovirus system in insect cells

Xenopus IYD (xIYD) Enzyme Source

Liver microsomal fractions from *Xenopus laevis* tadpoles

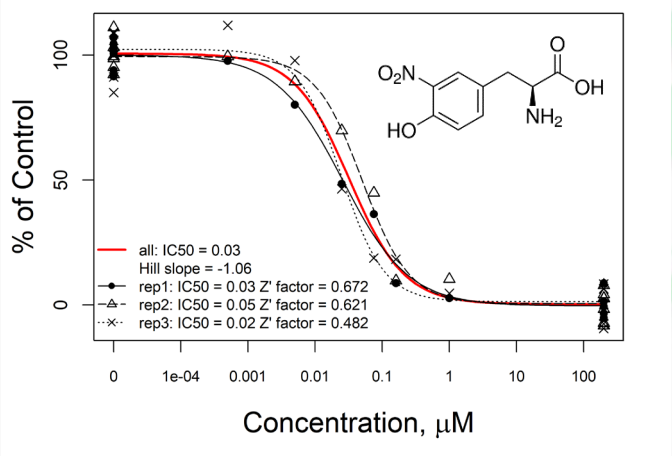
IYD Enzyme Assay

In 96-well plates, 3 h incubation

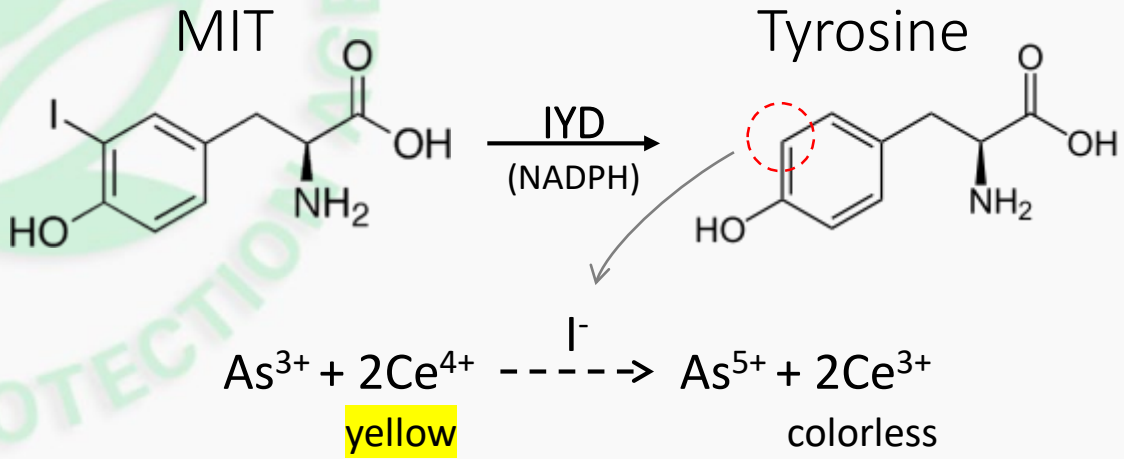
Measure Detection Signal

Sandell-Kolthoff (SK) reaction to detect free iodide

3-Nitro-L-tyrosine (MNT) as positive control



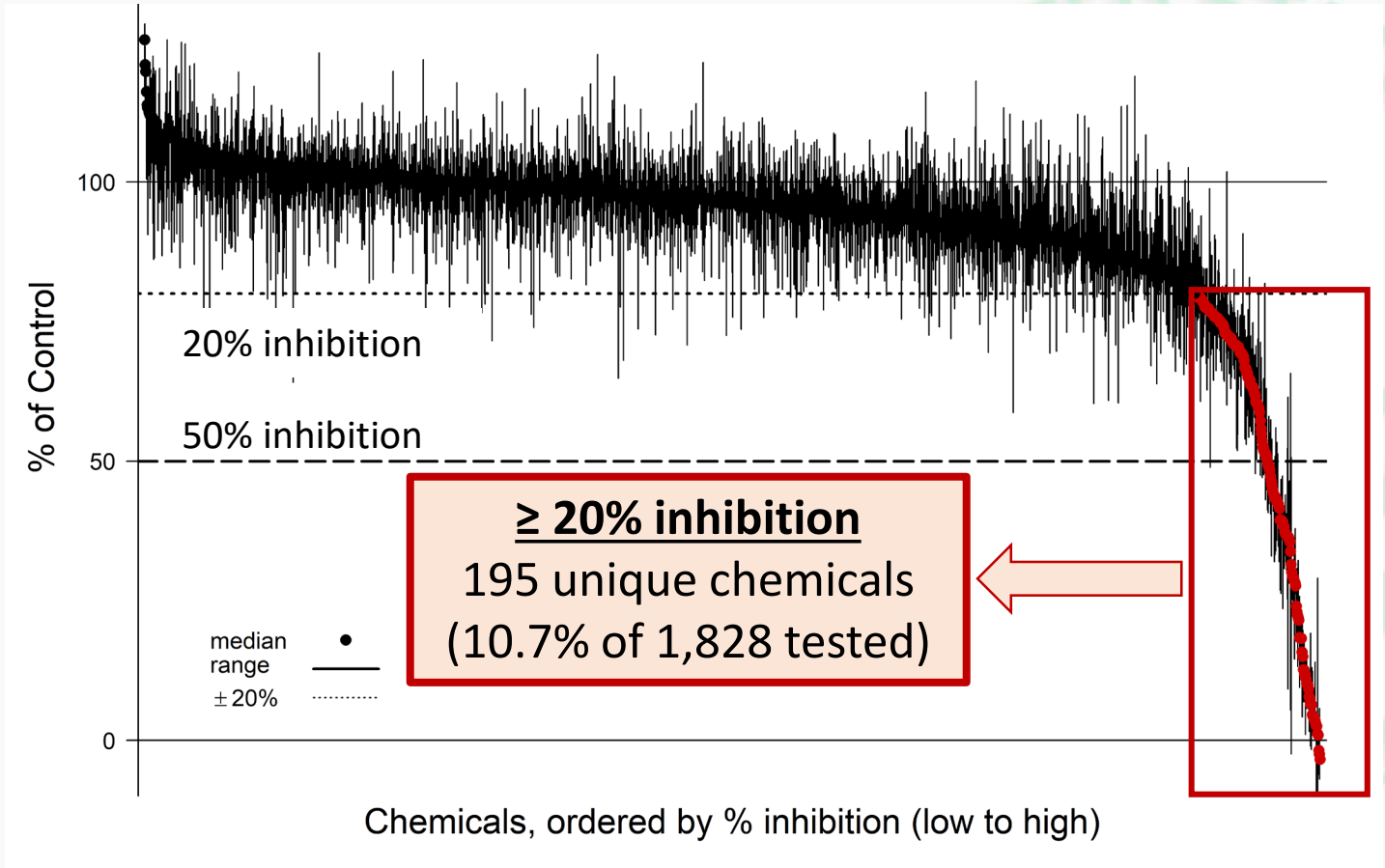
Iodotyrosine deiodinase (IYD) reaction scheme



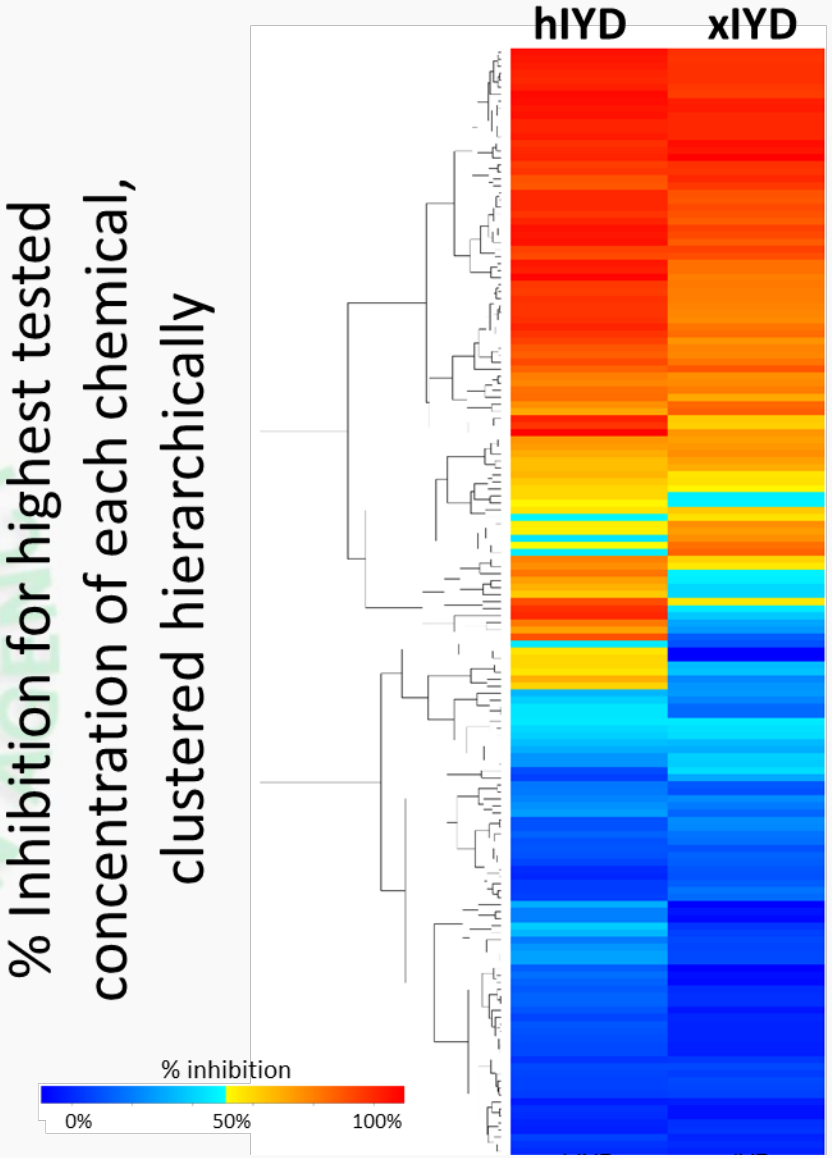
IYD Inhibition Assay Screening

Human IYD Screening

ToxCast ph1_v2, ph2, and e1K chemicals across all plates

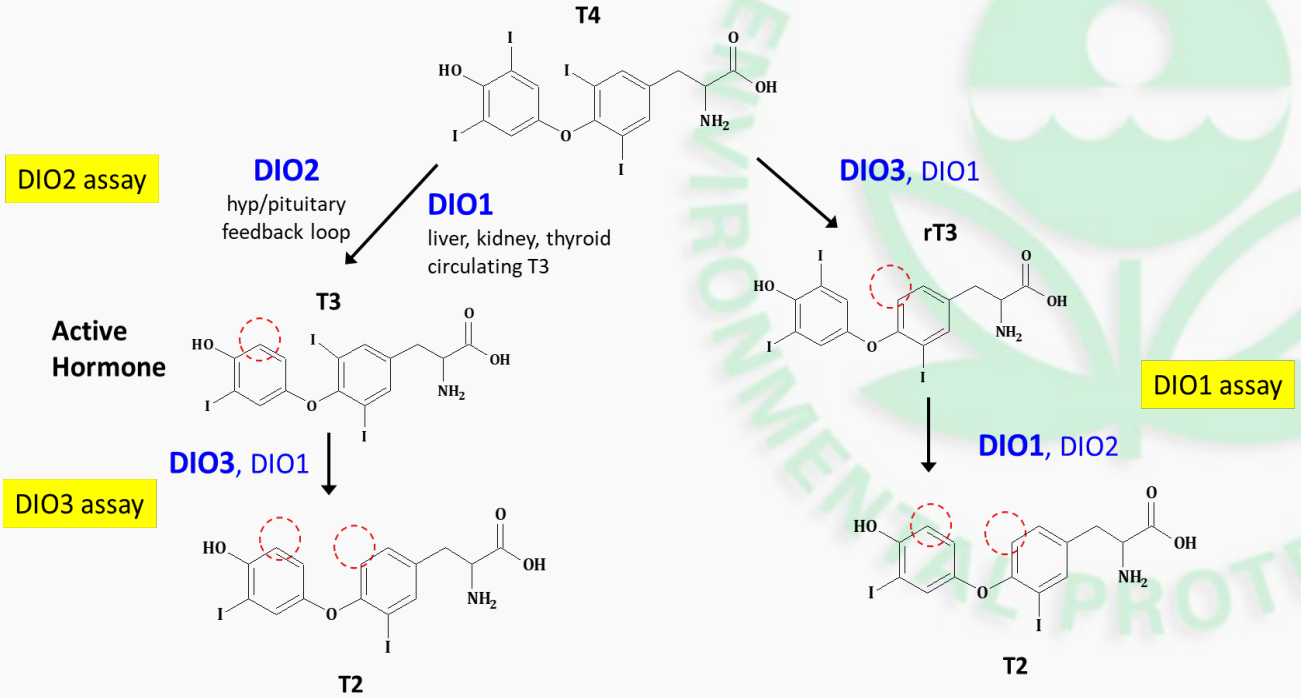


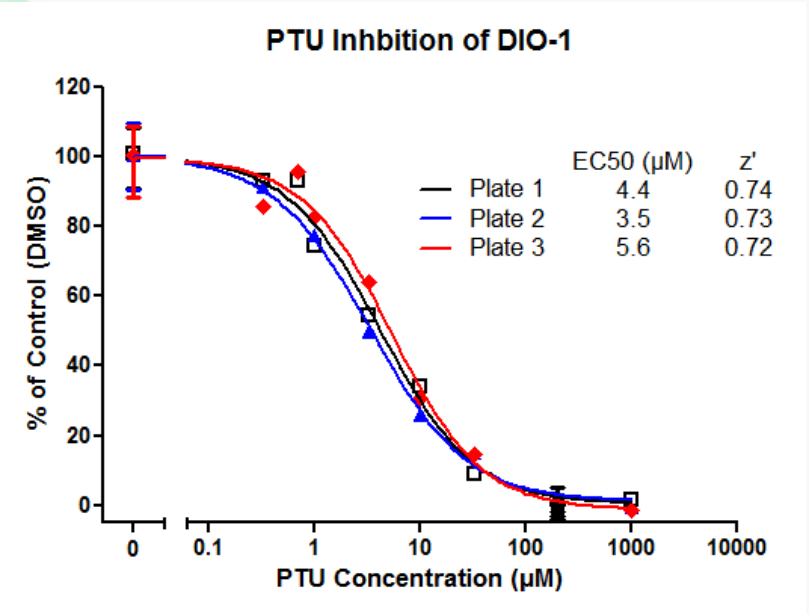
% Inhibition for highest tested concentration of each chemical, clustered hierarchically



Deiodinase Inhibition Assay

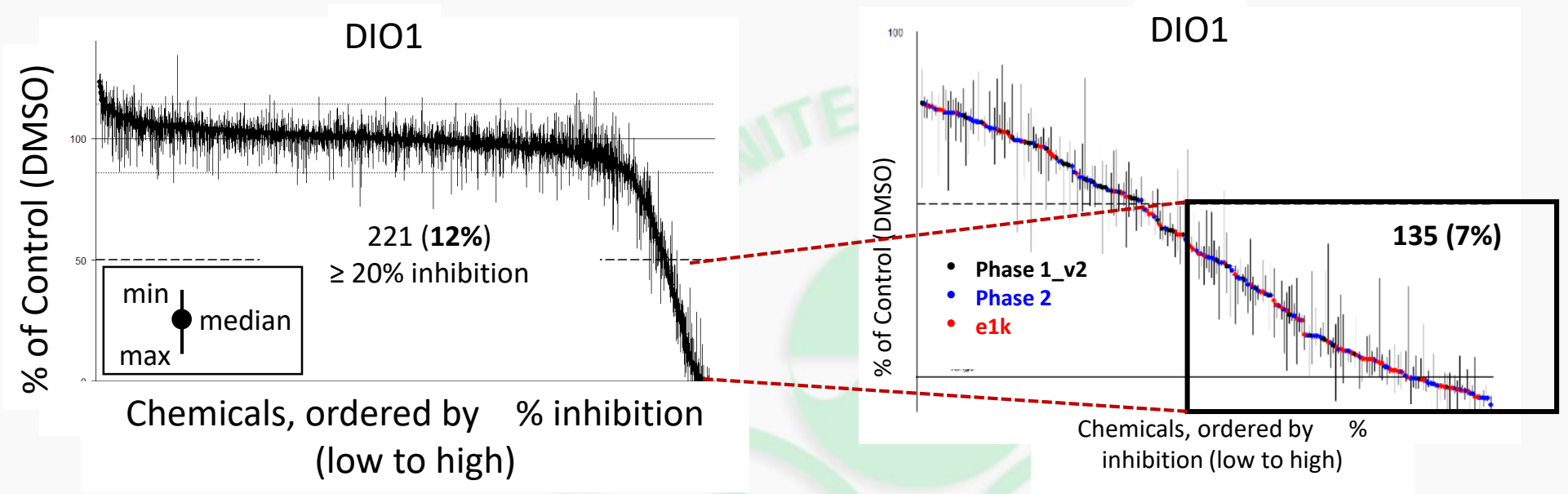
- Use adenovirus expression system in HEK293 cells to obtain active DIO1, 2, and 3 enzymes.
- Deiodinase assay incubated in 96-well plate followed by 96-well solid phase Dowex columns to extract and isolate Iodide
- Sandell-Kolthoff reaction used to quantify iodide (same as for IYD assay).
- Inhibition of the DIO reduces amount of iodide, resulting in reduced rate of absorbance change.
- Assay modified from Renko et al. 2012 *Endocrinology*, **153**, 2506-2513. Renko et al. 2015 *Thyroid*, **25**, 1-7.





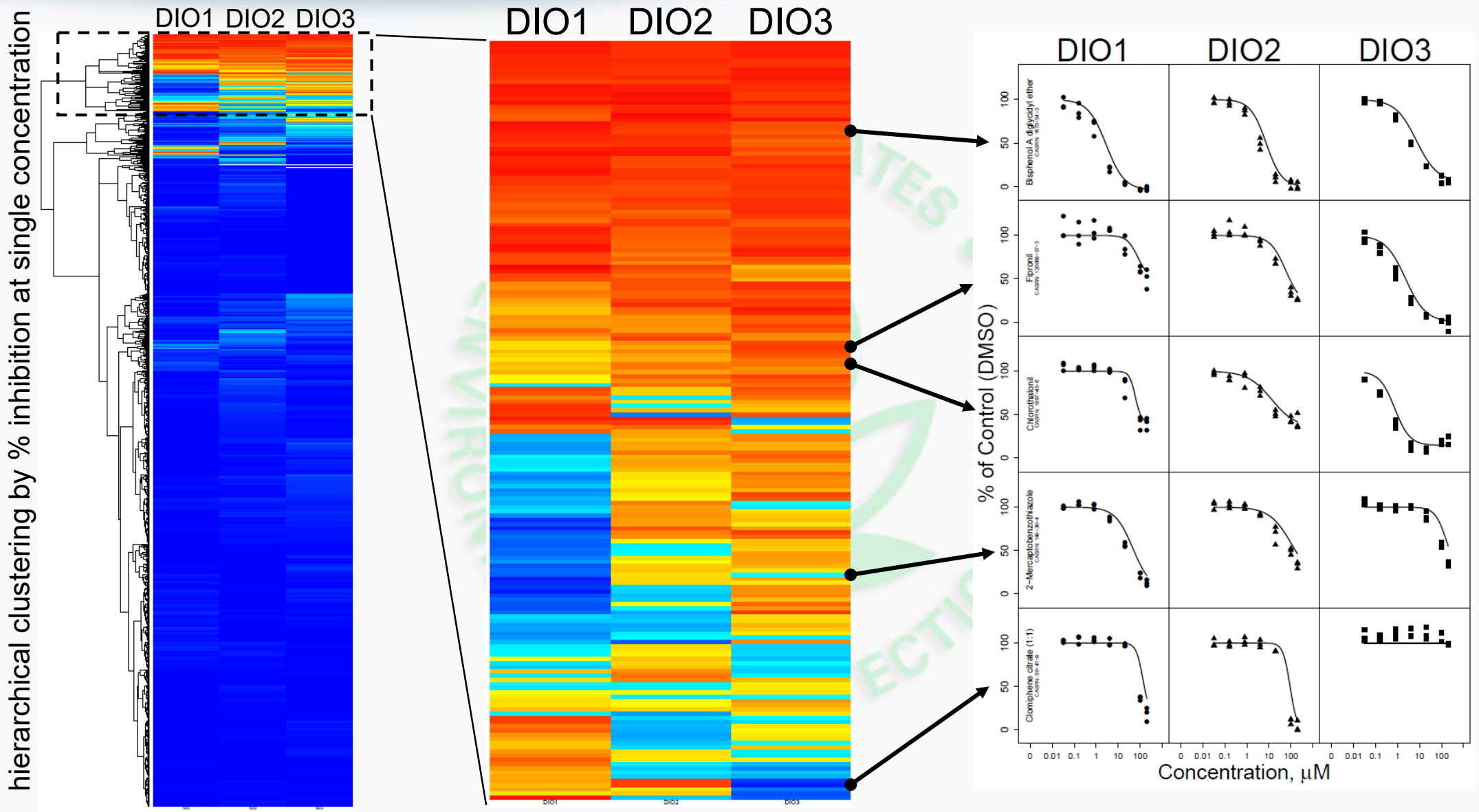
Hormone Activation / Inactivation

- Deiodinase Inhibition Assay



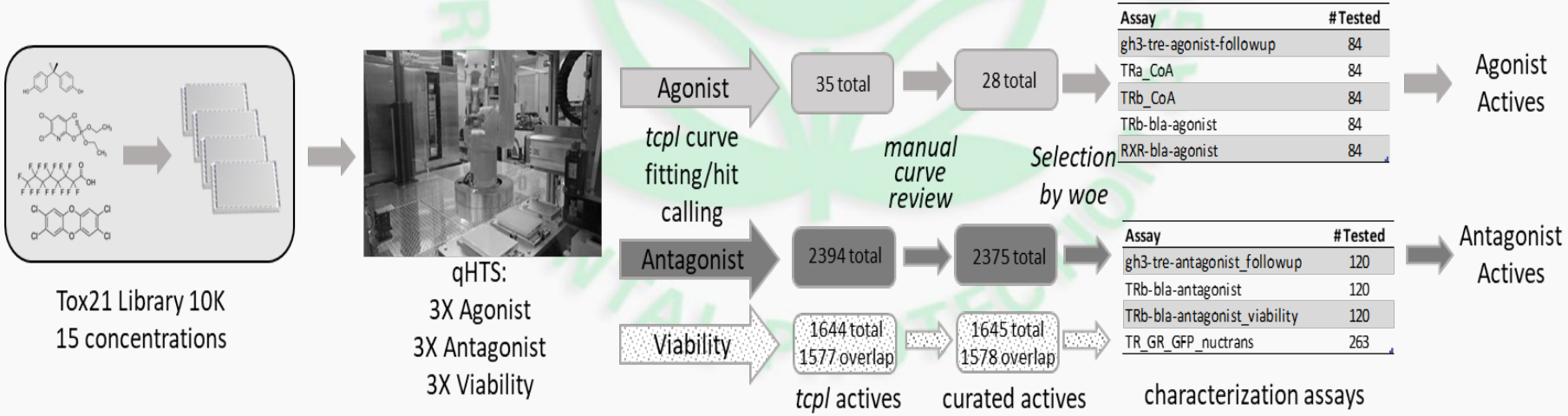
		Deiodinase Type 1		Deiodinase Type 2		Deiodinase Type 3	
Chemical Library	# chemicals tested*	# with ≥ 20% inhibition	% with ≥ 20% inhibition	# with ≥ 20% inhibition	% with ≥ 20% inhibition	# with ≥ 20% inhibition	% with ≥ 20% inhibition
ToxCast p1_v2	290	49	16.9 %	54	18.6 %	57	19.7 %
ToxCast p2	748	95	12.7 %	126	16.8 %	117	15.6 %
ToxCast e1K	781	77	9.9 %	123	15.8 %	133	17.1 %
Total	1,819	**221**	**12.1 %**	**303**	**16.7 %**	**307**	**16.9 %**

hDIO Inhibition



Thyroid Hormone Receptor Transactivation

- Primary screen in GH3-TRE-Luc cell line (A. Murk)
- 8300 unique structures screened in Tox21 library
- Assays run in agonist and antagonist mode
- Confirmatory and orthogonal assays run to verify actives
  - TR:Coactivator recruitment assays
  - GAL4-LBD (human) reporter gene assay
  - RXR reporter gene assay
  - TR-GR-GFP nuclear translocation assay (G. Hagar lab)
  - Cytotoxicity assays



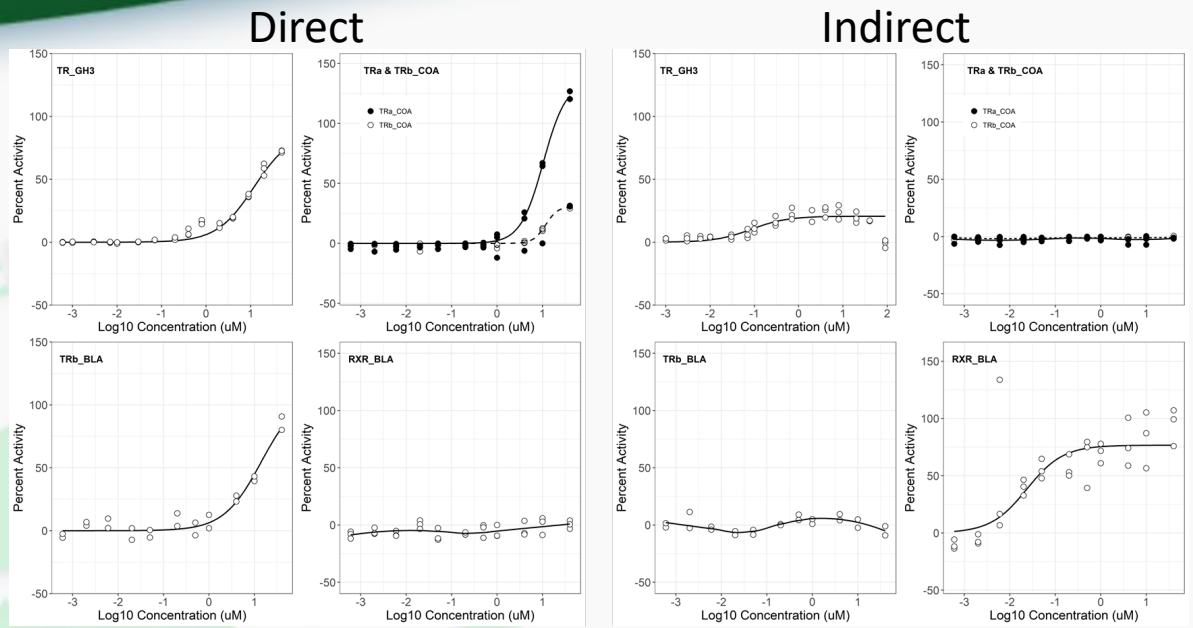
Receptor Screening Results

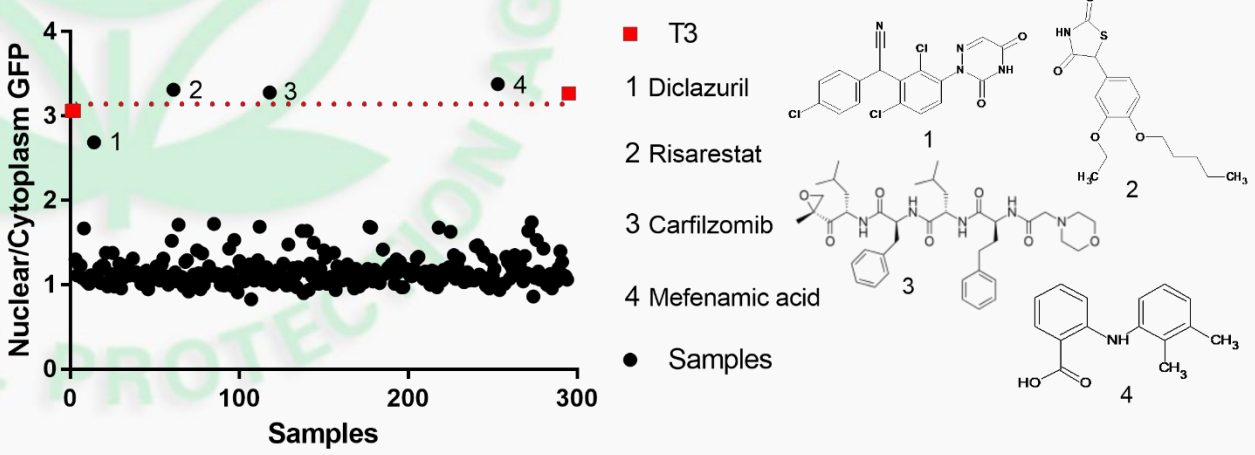
Agonists

- Direct agonists--T3 analogs
- Indirect agonists—RXR ligands

Antagonists

- Only 3 high confidence antagonists, all pharmaceutical class compounds
- Suspected indirect antagonists such as proteasome inhibitors also identified
- Nuclear translocation assay very useful as orthogonal assay





Summary

MIE	# Chemicals Tested	Data Status	References
NIS Radioactive Iodide Uptake Inhibition	>1,800 (ToxCast ph1v2, ph2, e1k)	multi-concentration data for ph1v2 and ph2 available online	Hallinger et al. 2017 Wang et al. 2018 Wang et al. 2019
Thyroperoxidase Inhibition	>1,800 (ToxCast ph1v2, ph2, e1k)	multi-concentration data available online	Paul-Friedman et al. 2016
Iodothyronine Deiodinase Type 1	>1,800 (ToxCast ph1v2, ph2, e1k)	multi-concentration data available online in next update of InVitroDB	Hornung et al. 2018 Olker et al. 2019
Iodothyronine Deiodinase Type 2	>1,800 (ToxCast ph1v2, ph2, e1k)	multi-concentration data available online in future update of InVitroDB	Olker et al. 2019
Iodothyronine Deiodinase Type 3	>1,800 (ToxCast ph1v2, ph2, e1k)	multi-concentration data available online in future update of InVitroDB	Olker et al. 2019
Iodotyrosine Deiodinase	>1,800 (ToxCast ph1v2, ph2, e1k)	Analysis in progress, to be submitted to InVitroDB early 2020	Olker et al. *In prep*.
Thyroid Hormone Receptor Transactivation	8,300	Source data available on InVitroDB	Paul-Friedman et al. 2019

Summary

- Nearly two dozen molecular initiating events (MIE) have been identified/proposed for thyroid axis adverse outcome pathways; about half have high-throughput screening assay available or being developed for the MIE.
- Future efforts needed to translate *in vitro* activity to *in vivo* responses to verify Adverse Outcomes.
- Incorporate in vitro screening date for MIE and AOP into framework for use in risk assessment for chemical disruption of thyroid hormones
  - prioritization near-term goal
  - ultimately inform & develop quantitative AOP to predict toxicity

Recent commentary on this effort.

Noyes et al. 2019. Evaluating Chemicals for Thyroid Disruption: Opportunities and Challenges with In Vitro Testing and Adverse Outcome Pathway Approaches. *Environ. Health Perspect*. 127(9) Sept 2019. https://doi.org/10.1289/EHP5297

Publicly available data

iCSS ToxCast dashboard

https://comptox.epa.gov/dashboard

Invitrodb database

https://doi.org/10.23645/epacomptox.6062623.v1

Data related to published papers also available on www.data.gov

Publications

TPO

- Paul et al. 2014. Development of a thyroperoxidase inhibition assay for high-throughput screening. *Chemical Research in Toxicology* 27(3) 387-399.
- Paul-Friedman et al. 2016. Tiered High-Throughput Screening Approach to Identify Thyroperoxidase Inhibitors within the ToxCast Phase I and II Chemical Libraries. *Toxicol. Sci.* 151(1): 160–180.

NIS

- Hallinger et al. 2017. Development of a screening approach to detect thyroid disrupting chemicals that inhibit the human sodium iodide symporter (NIS). *Toxicol In Vitro* 40:66-78.
- Wang et al. 2018. High-Throughput Screening and Quantitative Chemical Ranking for Sodium-Iodide Symporter Inhibitors in ToxCast Phase I Chemical Library. *Environ Sci Technol* 52(9): 5417-5426.
- Wang et al. 2019. High-throughput screening and chemotype-enrichment analysis of ToxCast phase II chemicals evaluated for human sodium-iodide symporter (NIS) inhibition. *Environ. Int.* 126: 377-386.

DIO

- Hornung et al. 2018. Screening the ToxCast Phase 1 Chemical Library for Inhibition of Deiodinase Type 1 Activity. *Toxicological Sciences. 162*(2), 570-581.
- Olker et al. 2019 Screening the ToxCast Phase 1, Phase 2, and e1k Chemical Libraries for Inhibitors of Iodothyronine Deiodinases. *Toxicol Sci* 168(2):430-442.

IYD

- Olker et al. 2018. Evaluating Iodide Recycling Inhibition as a Novel Molecular Initiating Event for Thyroid Axis Disruption in Amphibians. *Toxicol. Sci.* 166, 318-331

THR Transactivation

- Paul-Friedman et al. 2019. Limited Chemical Structural Diversity Found to Modulate Thyroid Hormone Receptor in the Tox21 Chemical Library. *Environ. Health Perspect*. 127(9) Sept 2019. https://doi.org/10.1289/EHP5314

Review / Commentary

- Noyes et al. 2019. Evaluating Chemicals for Thyroid Disruption: Opportunities and Challenges with In Vitro Testing and Adverse Outcome Pathway Approaches. *Environ. Health Perspect*. 127(9) Sept 2019. https://doi.org/10.1289/EHP5297