State of the Science: ToxCast and Tox21 assays and approaches to screening for potential thyroid hormone disruption

Katie Paul Friedman, PhD

Center for Computational Toxicology and Exposure, Office of Research and Development, US Environmental Protection Agency

Research Triangle Park, NC

Email: paul-friedman.katie@epa.gov

The views expressed in this presentation are those of the authors and do not necessarily reflect the views or policies of the U.S. EPA

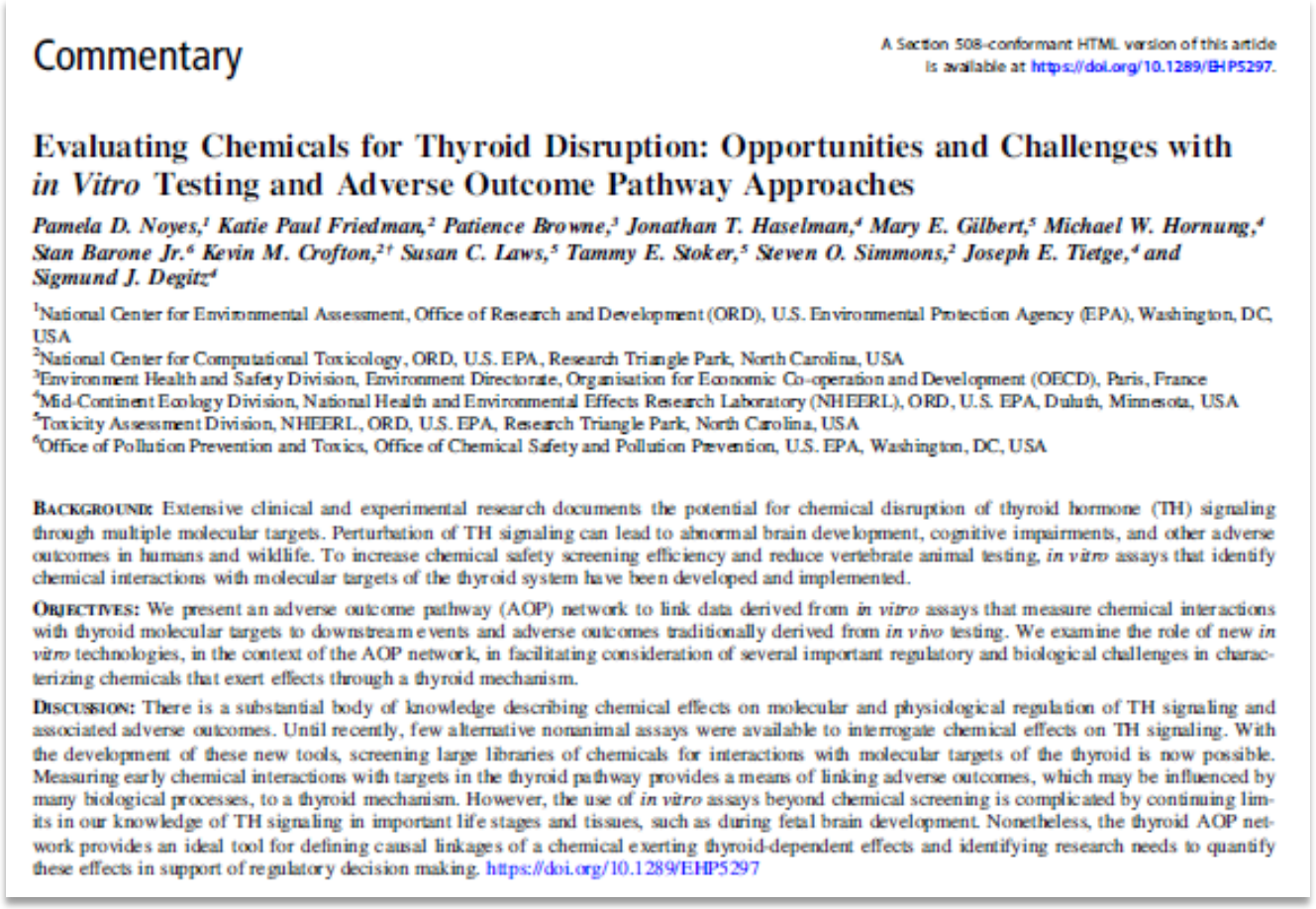
Conflict of Interest Statement

The author declares no conflict of interest.

Overview of this presentation

- A thyroid adverse outcome pathway network as a template for high-throughput screening (HTS) assay development
- Currently available ToxCast/Tox21 HTS assays
- Bringing context to currently available data
- Brief example of a single chemical

A thyroid adverse outcome pathway network as a guide



Commentary

A Section 508-conformant HTML version of this article is available at https://doi.org/10.1289/EHP5297.

Evaluating Chemicals for Thyroid Disruption: Opportunities and Challenges with *in Vitro* Testing and Adverse Outcome Pathway Approaches

Pamela D. Noyes,[1] Katie Paul Friedman,[2] Patience Browne,[3] Jonathan T. Haselman,[4] Mary E. Gilbert,[5] Michael W. Hornung,[4] Stan Barone Jr.[6] Kevin M. Crofton,[2†] Susan C. Laws,[5] Tammy E. Stoker,[5] Steven O. Simmons,[2] Joseph E. Tietge,[4] and Sigmund J. Degitz[4]

[1]National Center for Environmental Assessment, Office of Research and Development (ORD), U.S. Environmental Protection Agency (EPA), Washington, DC, USA
[2]National Center for Computational Toxicology, ORD, U.S. EPA, Research Triangle Park, North Carolina, USA
[3]Environment Health and Safety Division, Environment Directorate, Organisation for Economic Co-operation and Development (OECD), Paris, France
[4]Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory (NHEERL), ORD, U.S. EPA, Duluth, Minnesota, USA
[5]Toxicity Assessment Division, NHEERL, ORD, U.S. EPA, Research Triangle Park, North Carolina, USA
[6]Office of Pollution Prevention and Toxics, Office of Chemical Safety and Pollution Prevention, U.S. EPA, Washington, DC, USA

Background: Extensive clinical and experimental research documents the potential for chemical disruption of thyroid hormone (TH) signaling through multiple molecular targets. Perturbation of TH signaling can lead to abnormal brain development, cognitive impairments, and other adverse outcomes in humans and wildlife. To increase chemical safety screening efficiency and reduce vertebrate animal testing, *in vitro* assays that identify chemical interactions with molecular targets of the thyroid system have been developed and implemented.

Objectives: We present an adverse outcome pathway (AOP) network to link data derived from *in vitro* assays that measure chemical interactions with thyroid molecular targets to downstream events and adverse outcomes traditionally derived from *in vivo* testing. We examine the role of new *in vitro* technologies, in the context of the AOP network, in facilitating consideration of several important regulatory and biological challenges in characterizing chemicals that exert effects through a thyroid mechanism.

Discussion: There is a substantial body of knowledge describing chemical effects on molecular and physiological regulation of TH signaling and associated adverse outcomes. Until recently, few alternative nonanimal assays were available to interrogate chemical effects on TH signaling. With the development of these new tools, screening large libraries of chemicals for interactions with molecular targets of the thyroid is now possible. Measuring early chemical interactions with targets in the thyroid pathway provides a means of linking adverse outcomes, which may be influenced by many biological processes, to a thyroid mechanism. However, the use of *in vitro* assays beyond chemical screening is complicated by continuing limits in our knowledge of TH signaling in important life stages and tissues, such as during fetal brain development. Nonetheless, the thyroid AOP network provides an ideal tool for defining causal linkages of a chemical exerting thyroid-dependent effects and identifying research needs to quantify these effects in support of regulatory decision making. https://doi.org/10.1289/EHP5297

Noyes PD, Paul Friedman K, Browne P, Haselman JT, Gilbert ME, Hornung MW, Barone S, Crofton KM, Laws SC, Stoker TE, Simmons SO, Tietge JE, Degitz SJ. (2019). Evaluating Chemicals for Thyroid Disruption: Opportunities and Challenges with In Vitro Testing and Adverse Outcome Pathway Approaches. Environmental Health Perspectives. DOI: https://doi.org/10.1289/EHP5297

- **Challenge 1:** Health effects guideline studies often measure apical outcomes without measures that indicate relevant molecular initiating event(s) [MIE(s)].

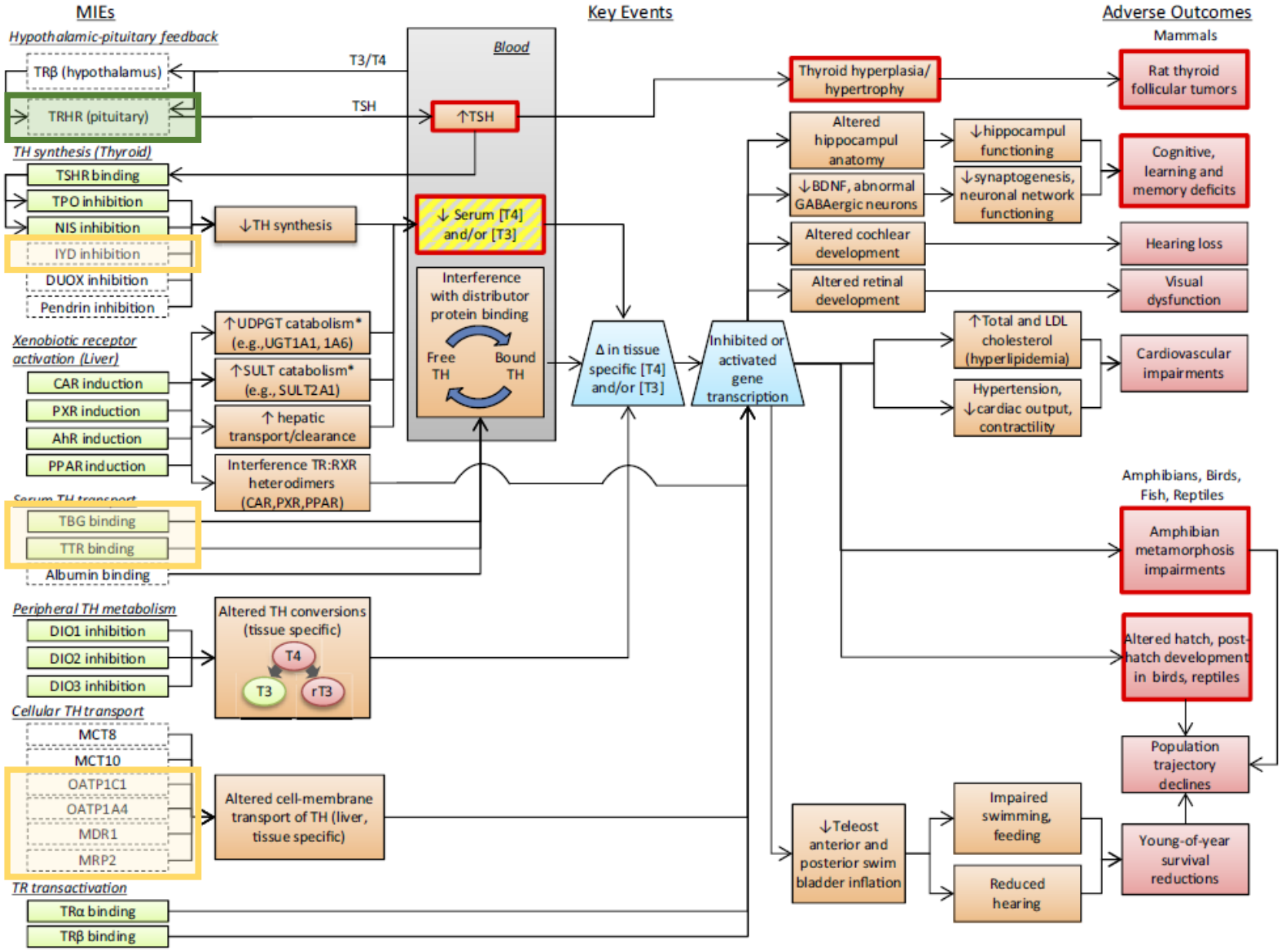
- **Challenge 2:** Screening for thyroid disruption is not centered around the thyroid hormone receptor; indeed there are many other MIEs/KEs that are thought to be more relevant for xenobiotic disruption.

- **Approach:** HTS assays typically target MIEs or key events (KEs), and so a battery of assays will be needed to capture effects observed in *in vivo* models.
- **Progress:** In the last 10 years, more HTS assay development and application have occurred.
- **Outlook:** Many MIE and KE targets still lack confirmatory or orthogonal assay information, and so careful examination of results and additional context will be needed.

A thyroid adverse outcome pathway network as a guide

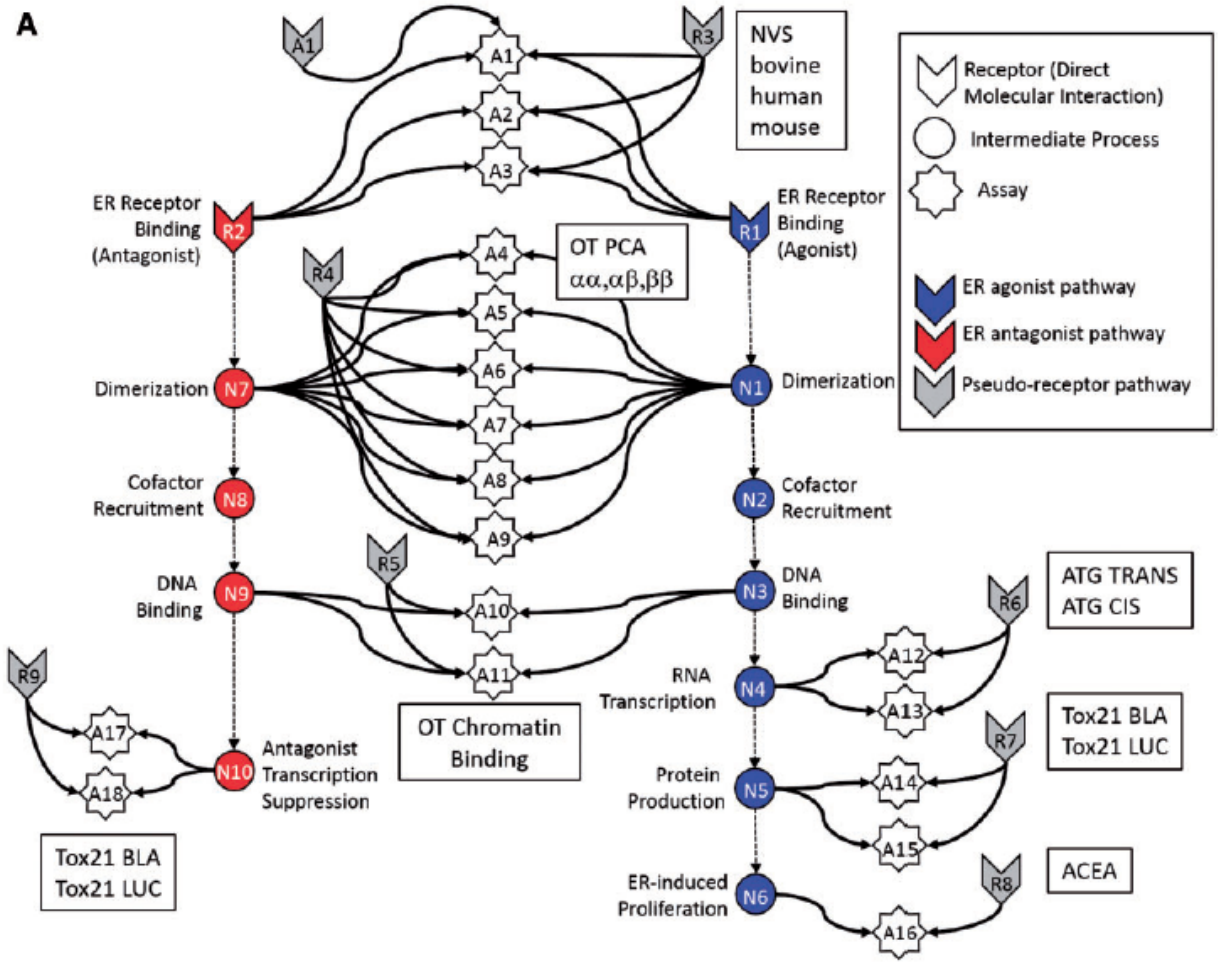
- Green boxes indicate MIEs with HTS data in ToxCast
  - TRHR added since publication;
  - IYD close to publication and in next ToxCast release;
  - Assays exist for TBG and TTR binding, but not in ToxCast;
  - Some indication of liver transporters from HepaRG data recently released (LTEA) and from primary hepatocyte data (CellzDirect).

- *What about the need for redundancy/confirmation at assay targets?*
- *What about quantitative key event relationships?*



In contrast, endocrine-related bioactivity for estrogen and androgen has focused predominantly on their receptors

- Other targets may be important, but the estrogen and androgen receptors themselves are somewhat promiscuous but relevant xenobiotic targets.
- Resources have been spent to establish redundancy of assays for these receptors to enable rationale systems-based modeling of receptor binding, complex formation, translocation, and activity as transcription factors.
- Thyroid hormone receptor (TR) may be less promiscuous for xenobiotics.
- Many more targets may be important for thyroid hormone homeostasis, and so the data integration approaches taken will need to be different.



Contrasting example to thyroid: the ToxCast ER model from Richard Judson and colleagues.

https://doi.org/10.1093/toxsci/kfv168

Assay data available in ToxCast invitrodb v 3.3 (released Aug 2020)

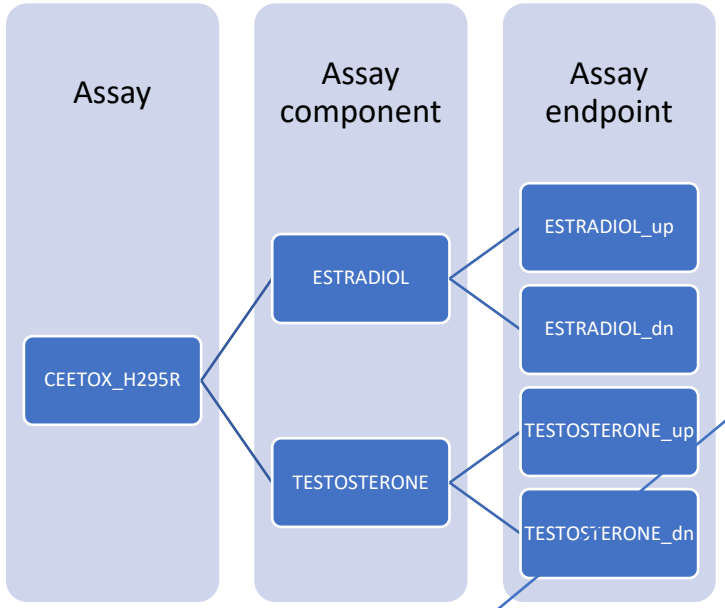
The CompTox Chemicals Dashboard release from July 2020 is now using ToxCast invitrodb version 3.3: **https://doi.org/10.23645/epacomptox.6062479.v5**

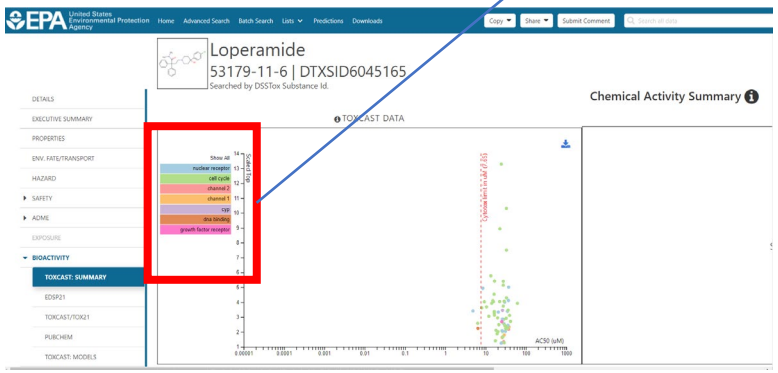
Data downloads for invitrodb and summary files: **https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data**

We anticipate a new ToxCast release in 2021.

A bit about ToxCast assay endpoints and annotation

Example assay annotation hierarchy



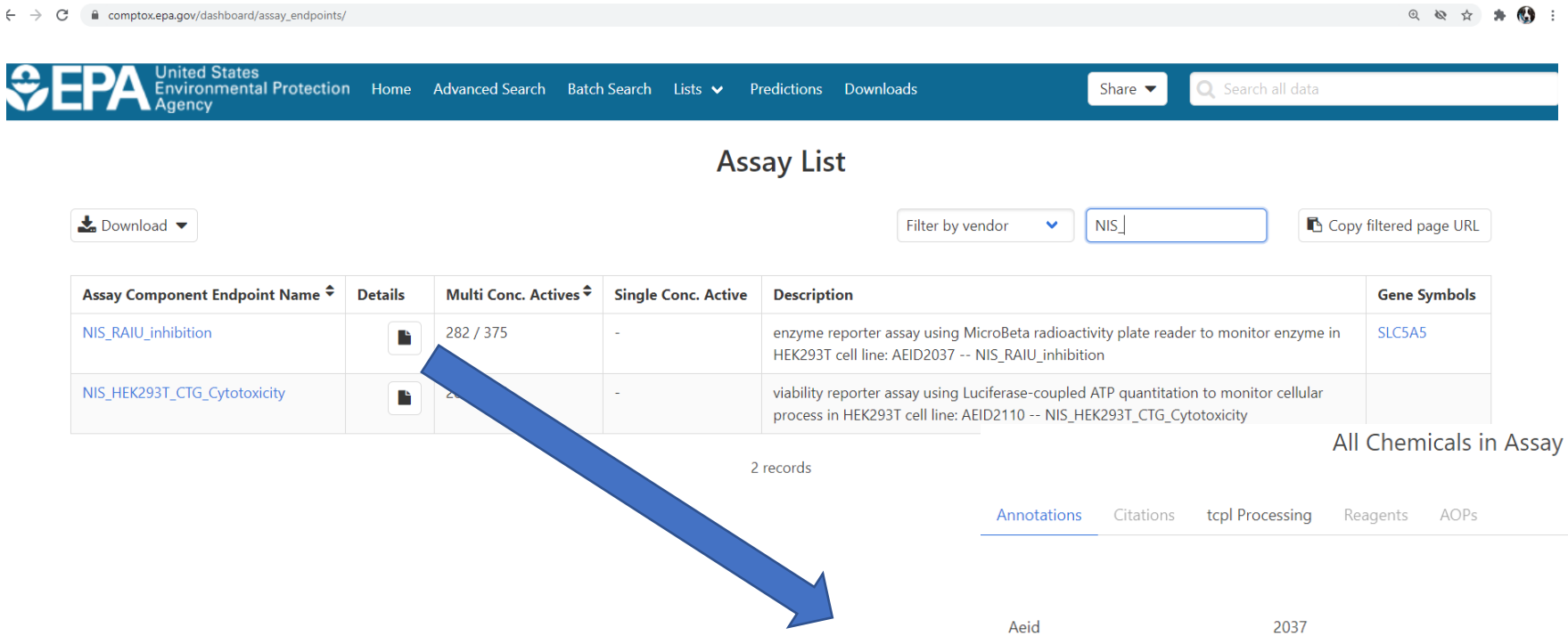


- Many assay endpoints are mapped to a gene, if applicable
- Assay endpoints now cover 1398 unique gene targets in invitrodb version 3.3, in addition to other processes
- Intended target family is one way to understand biological target (incomplete list here):
  - Apolipoprotein
  - Apoptosis
  - Background measurement
  - Catalase
  - Cell adhesion
  - Cell cycle
  - Cell morphology
  - CYP
  - Cytokine
  - Deiodinase
  - DNA binding
  - Esterase
  - Filaments
  - GPCR
  - Growth factor
  - Histones
  - Hydrolase
  - Ion channel
  - Kinase
  - Ligase
  - Lyase
  - Malformation (zebrafish)
  - Membrane protein
  - Metabolite (Stemina metabolomics)
  - Mitochondria
  - Methyltransferase
  - microRNA
  - Mutagenicity response
  - Nuclear receptor
  - Oxidoreductase
  - Phosphatase
  - Protease/inhibitor
  - Steroid hormone
  - Transferase
  - Transporter

https://comptox.epa.gov/dashboard/assay_endpoints/

Download summary information here: https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data

Finding thyroid-related ToxCast assays and additional annotation information



Search by gene (TR, TSHR, TRHR, TPO, SLC5A5, DIO, etc), assay "vendor", or key words on the CompTox Chemicals Dashboard: comptox.epa.gov/dashboard/assay_endpoints.

Can also download INVITRODB SUMMARY file which contains a table that maps assay endpoint to gene.

All Chemicals in Assay Endpoint: NIS_RAIU_inhibition

Excel

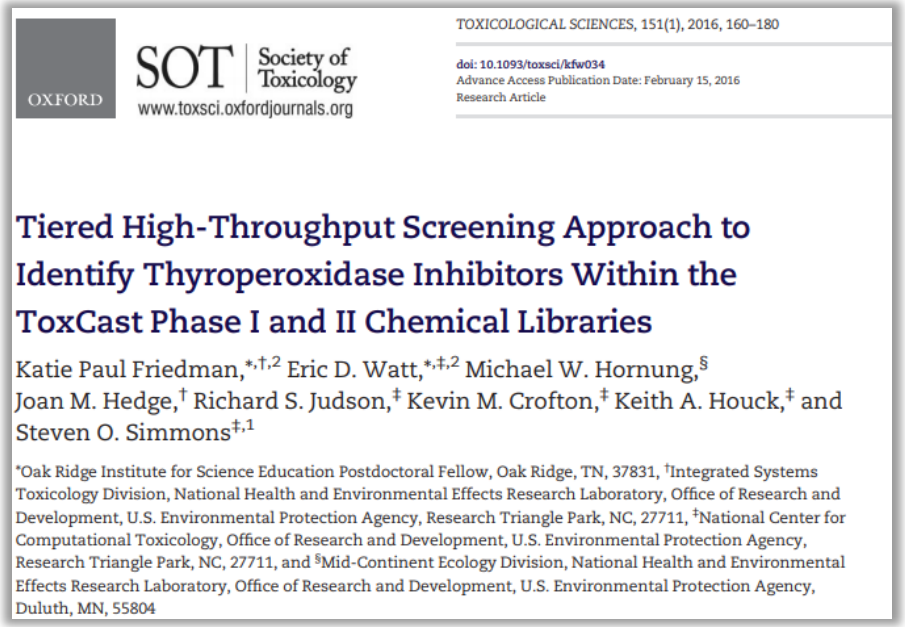
Annotations | Citations | tcpl Processing | Reagents | AOPs

Aeid	2037
Assay Component Endpoint Name	NIS_RAIU_inhibition
Assay Component Endpoint Desc	enzyme reporter assay using MicroBeta radioactivity plate reader to monitor enzyme in HEK293T cell line: AEID2037 -- NIS_RAIU_inhibition
Assay Function Type	binding
Normalized Data Type	percent_activity
Analysis Direction	positive
Burst Assay	false
Key Positive Control	NaClO4
Signal Direction	loss
Intended Target Type	protein

Thyroid hormone synthesis (and peripheral metabolism): thyroperoxidase (TPO), sodium iodide symporter (NIS), and deiodinase inhibition (DIO)

aeid	Assay endpoint name (aenm) (current, changes)
1508	NCCT_TPO_AUR_dn
1509	NCCT_HEK293T_CellTiterGlo
1848	NCCT_QuantiLum_inhib_2_dn
2037	NIS_RAIU_inhibition
2110	NIS_HEK293T_CTG_Cytotoxicity
2309	NHEERL_MED_hDIO1_dn
2532	NHEERL_MED_hDIO2_dn
2533	NHEERL_MED_hDIO3_dn

Assay principle of the current ToxCast Amplex UltraRed TPO (AUR-TPO) inhibition assay



OXFORD | SOT Society of Toxicology
www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 151(1), 2016, 160–180

doi: 10.1093/toxsci/kfw034
Advance Access Publication Date: February 15, 2016
Research Article

Tiered High-Throughput Screening Approach to Identify Thyroperoxidase Inhibitors Within the ToxCast Phase I and II Chemical Libraries

Katie Paul Friedman,*,†,2 Eric D. Watt,*,‡,2 Michael W. Hornung,§ Joan M. Hedge,† Richard S. Judson,‡ Kevin M. Crofton,‡ Keith A. Houck,‡ and Steven O. Simmons‡,1

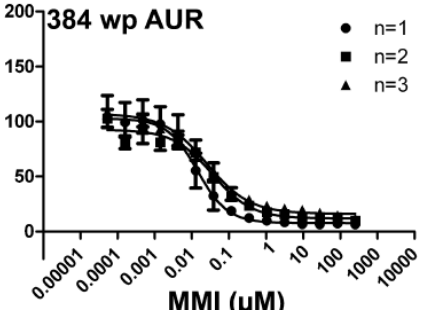
*Oak Ridge Institute for Science Education Postdoctoral Fellow, Oak Ridge, TN, 37831, †Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC, 27711, ‡National Center for Computational Toxicology, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC, 27711, and §Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Duluth, MN, 55804

Thyroid microsome (containing TPO)

Excess H_2O_2

Amplex UltraRed

Amplex UltroxRed (fluorescent)

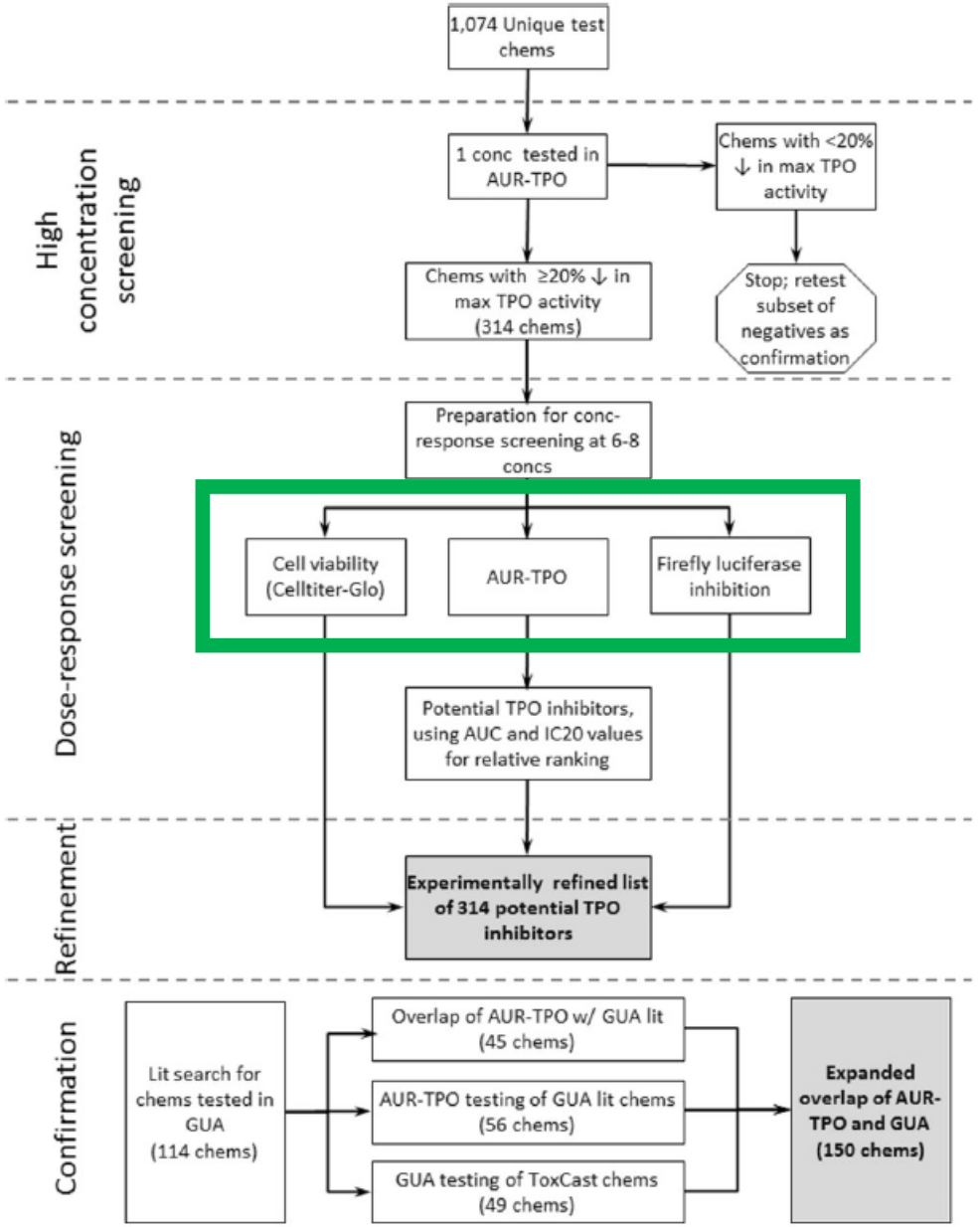


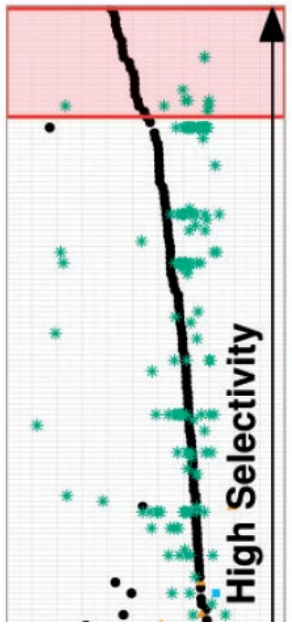
Paul Friedman K, Watt ED, Hornung MW, Hedge JM, Judson RS, Crofton KM, Houck KA, Simmons SO. (2016). Tiered High-Throughput Screening Approach to Identify Thyroperoxidase Inhibitors within the ToxCast Phase I and II Chemical Libraries. Toxicological Sciences. DOI: https://doi.org/10.1093/toxsci/kfw034

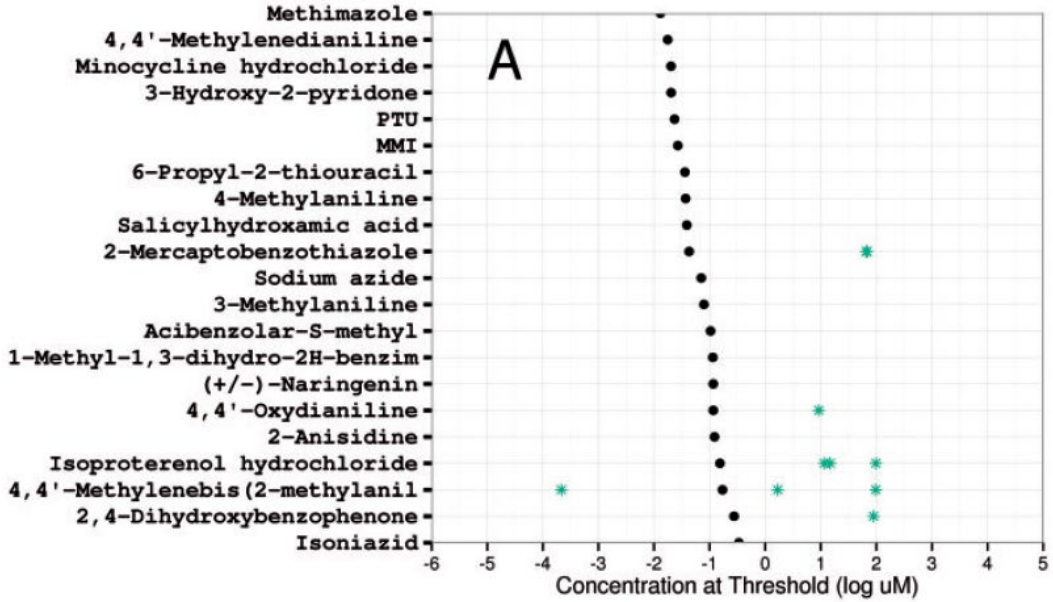
Paul KB, Hedge JM, Rotroff DM, Crofton KM, Hornung MH, Simmons SO. (2014). Development of a thyroperoxidase inhibition assay for medium through-put screening. Chemical Research in Toxicology. https://doi.org/10.1021/tx400310w

- Lead substance: methimazole (MMI)
- Other example positive reference chemicals: 6-propyl-2-thiouracil, dietary isoflavones, malachite green, ethylene bisthiocarbamates
- Also evaluated with a training set of reference chemicals
- Positive rate may approach 30% so context is important for filtering positives (consider sources of interference)
- Loss-of-signal assay

Context for interpretation

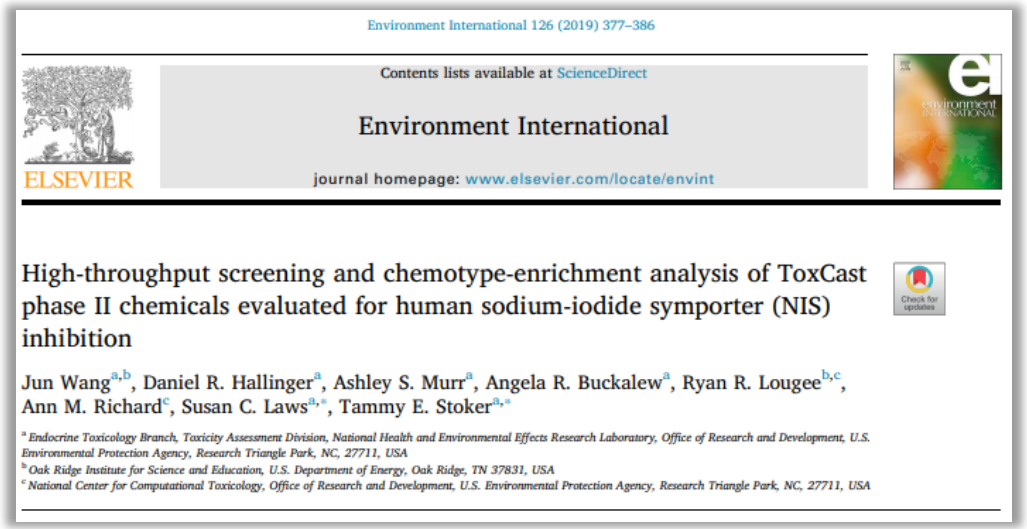






- Consider “selectivity”: is the potency of TPO inhibition distinguishable from potency of nonspecific protein inhibition or cell viability (as an indicator of chemical reactivity/pertinent concentration range?
- This was a tiered screening – most of the chemicals screened in single concentration first.
- Consider lead mode-of-action for these substances?

Assay principle of the ToxCast NIS inhibition assay



Environment International 126 (2019) 377–386

Contents lists available at ScienceDirect

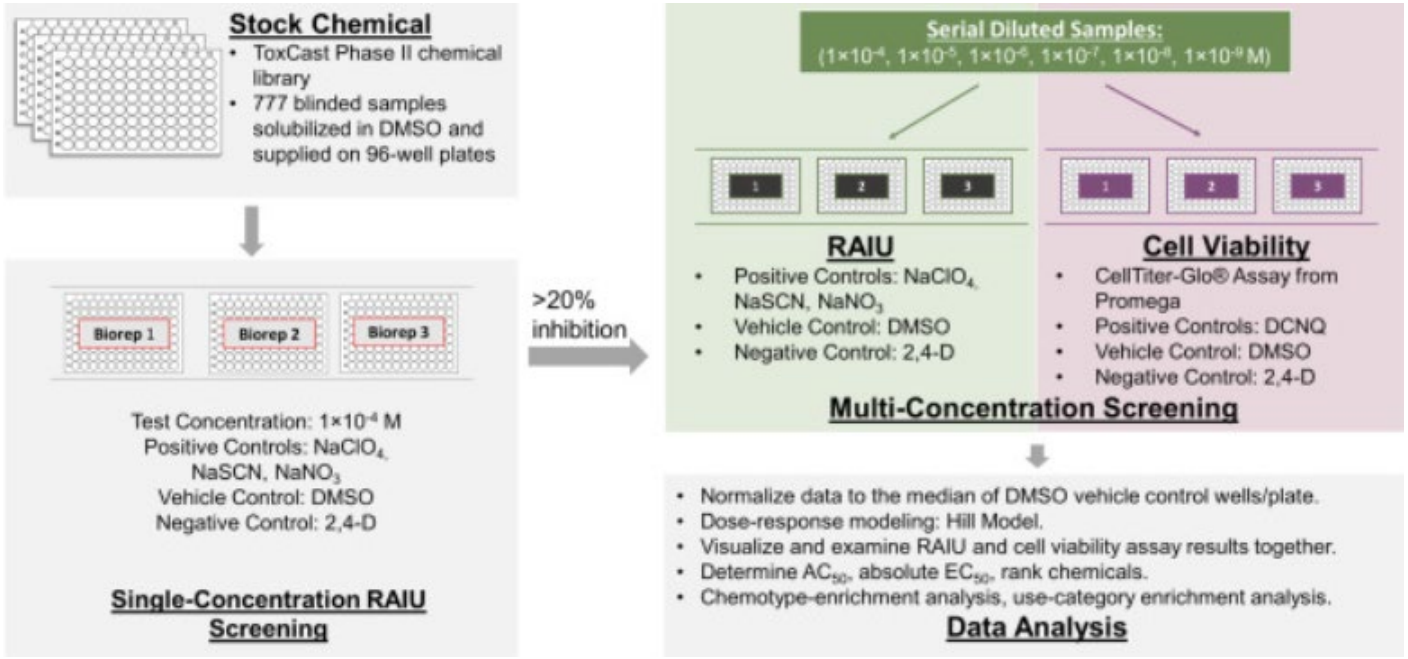
Environment International

journal homepage: www.elsevier.com/locate/envint

High-throughput screening and chemotype-enrichment analysis of ToxCast phase II chemicals evaluated for human sodium-iodide symporter (NIS) inhibition

Jun Wang[a,b], Daniel R. Hallinger[a], Ashley S. Murr[a], Angela R. Buckalew[a], Ryan R. Lougee[b,c], Ann M. Richard[c], Susan C. Laws[a,*], Tammy E. Stoker[a,*]

[a] Endocrine Toxicology Branch, Toxicity Assessment Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC, 27711, USA
[b] Oak Ridge Institute for Science and Education, U.S. Department of Energy, Oak Ridge, TN 37831, USA
[c] National Center for Computational Toxicology, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC, 27711, USA

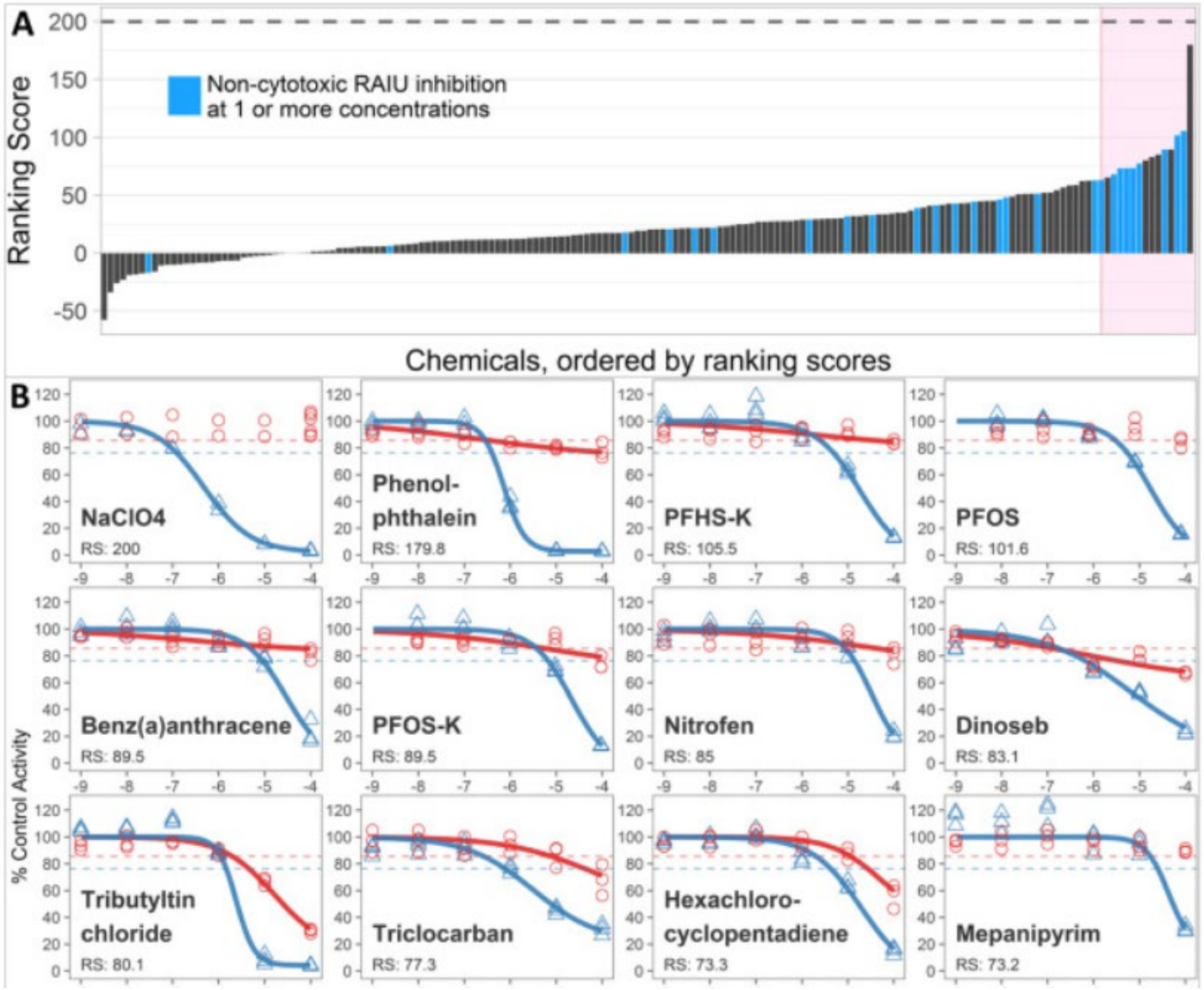


Wang J, Hallinger DR, Murr AS, Buckalew AR, Lougee RR, Richard AM, Laws SC, Stoker TE. (2019). High-throughput screening and chemotype-enrichment analysis of ToxCast phase II chemicals evaluated for human sodium-iodide symporter (NIS) inhibition. https://doi.org/10.1016/j.envint.2019.02.024

Wang J, Hallinger DR, Murr AS, Buckalew AR, Simmons SO, Laws SC, Stoker TE. (2018). High-throughput screening and quantitative chemical ranking for sodium-iodide symporter inhibitors in ToxCast Phase I chemical library. 10.1021/acs.est.7b06145

- Positive rate may approach 30-50% depending on the chemical library screened
- In Screening ToxCast Phase 2, only 25 substances were considered selective

Context for interpretation



From Wang et al. 2019

- Tiered screening (single concentration screening followed by selected multi-concentration screening).
- Also a loss-of-signal assay with high hit-rate.
- Cytotoxicity may be a source of interference.
- Lead modes of action for these substances that may appear selective?

Lecat-Guillet N et al. 2008 identified organics that inhibited NIS beyond perchlorate and other monovalent anions

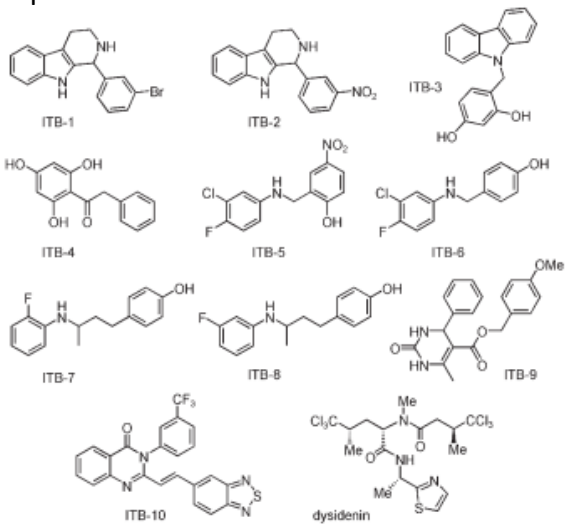


Figure 2. Structures of the most potent iodide uptake inhibitors; dysidenin is also shown.

Assay principle of the DIO inhibition assays

OXFORD SOT Society of Toxicology
www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 168(2), 2019, 430–442

doi: 10.1093/toxsci/kfy302
Advance Access Publication Date: December 18, 2018
Research Article

Screening the ToxCast Phase 1, Phase 2, and e1k Chemical Libraries for Inhibitors of Iodothyronine Deiodinases

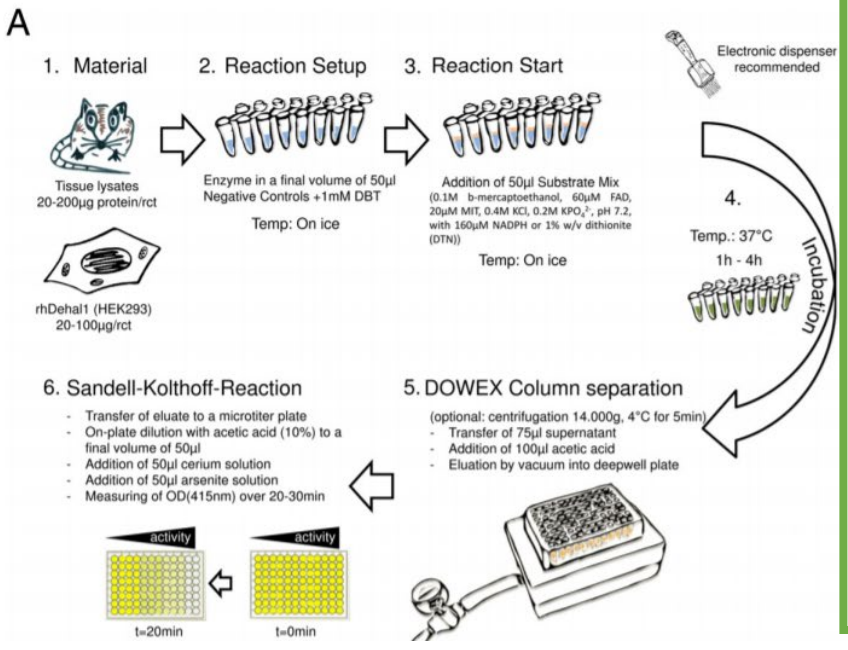
Jennifer H. Olker,[*,†,‡,§,1] Joseph J. Korte,[*,†,‡,§] Jeffrey S. Denny,[*,†,‡,§] Phillip C. Hartig,[*,†,‡,¶] Mary C. Cardon,[*,†,‡,¶] Carsten N. Knutsen,[‖] Paige M. Kent,[‖] Jessica P. Christensen,[‖] Sigmund J. Degitz,[*,†,‡,§] and Michael W. Hornung[*,†,‡,§]

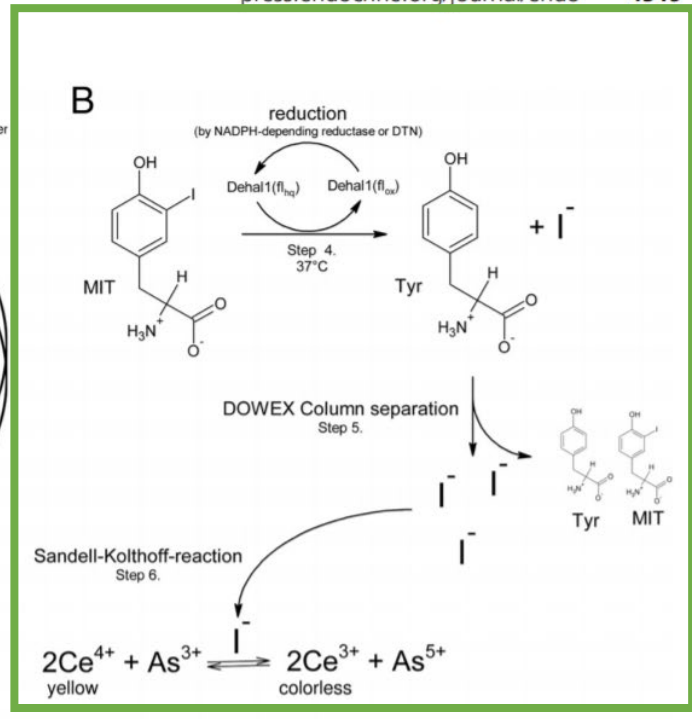
[*]U.S. Environmental Protection Agency; [†]Office of Research and Development; [‡]National Health and Environmental Effects Research Laboratory; [§]Mid-Continent Ecology Division, Duluth, Minnesota 55804; [¶]Toxicity Assessment Division, Research Triangle Park, North Carolina 27709; [‖]Mid-Continent Ecology Division, Student Services Contractor to the U.S. EPA, NHEERL, Duluth, Minnesota 55804; and [‖‖]Mid-Continent Ecology Division, ORAU Student Services Contractor to the U.S. EPA, NHEERL, Duluth, Minnesota 55804

[1]To whom correspondence should be addressed at U.S. EPA, Office of Research and Development, National Health and Environmental Effects Research Laboratory, 6201 Congdon Blvd, Duluth, MN 55804. Fax: (218) 529-5003. E-mail: olker.jennifer@epa.gov.

- HEK293 cell lysates overexpressing DIO1, DIO2, DIO3
- Method similar to Renko et al. 2016 (below) to detect excess iodide
- Examples: DIO1: genistein, PTU, iopanoic acid

doi: 10.1210/en.2016-1549

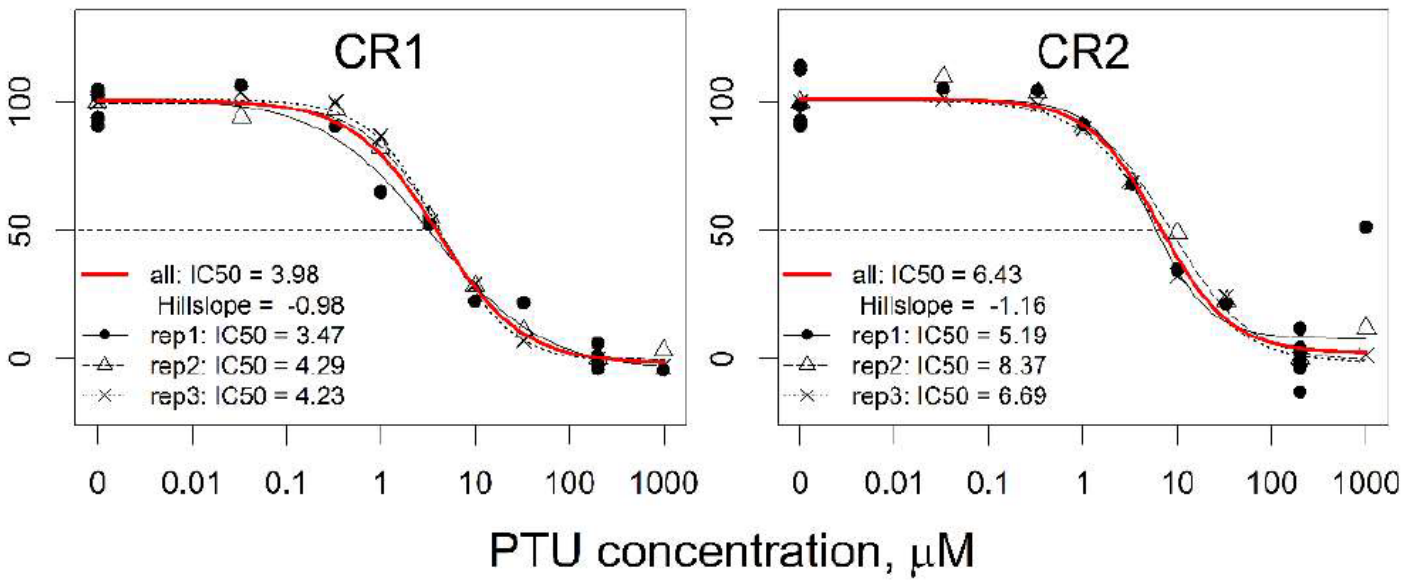




Olker JH, Korte JJ, Denny JS, Hartig PC, Cardon MC, Knutsen CN, Kent PM, Christensen JP, Degitz SJ, Hornung MW. (2019). Screening the ToxCast Phase 1, Phase 2, and e1k Chemical libraries for Inhibitors of Iodothyronine Deiodinases doi: 10.1093/toxsci/kfy302

Hornung MW, Korte JJ, Olker JH, Denny JS, Knutsen C, Hartig PC, Cardon MC, Degitz SJ. (2018). Screening the ToxCast Phase 1 Chemical Library for Inhibition of Deiodinase Type 1 Activity. 10.1093/toxsci/kfx279

Context for interpretation



Example highly reproducible PTU inhibition of DIO1 (from Hornung et al. 2018 Supp Figs)

- Hit rates are a bit lower than the TPO and NIS assays for 20% inhibition (~10-20%)
- Interference from surfactants or chemicals that disrupt membranes/nonspecific protein inhibition
- Iodine-containing substances are not amenable to the Sandell-Kolthoff chemistry
- Lead modes of action again might be considered

Indicators of hepatic catabolism

aeid	aenm
806	TOX21_AhR_LUC_Agonist
807	TOX21_AhR_LUC_Agonist_viability
116	ATG_CAR_TRANS_up
712	NVS_NR_hCAR_Agonist
713	NVS_NR_hCAR_Antagonist
1405	ATG_CAR_TRANS_dn
2047	TOX21_CAR_Agonist
2048	TOX21_CAR_Agonist_viabillity
2049	TOX21_CAR_Antagonist
2050	TOX21_CAR_Antagonist_viability
103	ATG_PXRE_CIS_up
135	ATG_PXR_TRANS_up
721	NVS_NR_hPXR
1474	ATG_PXRE_CIS_dn
1475	ATG_PXR_TRANS_dn
2362	TOX21_PXR_viability
2363	TOX21_PXR_Agonist

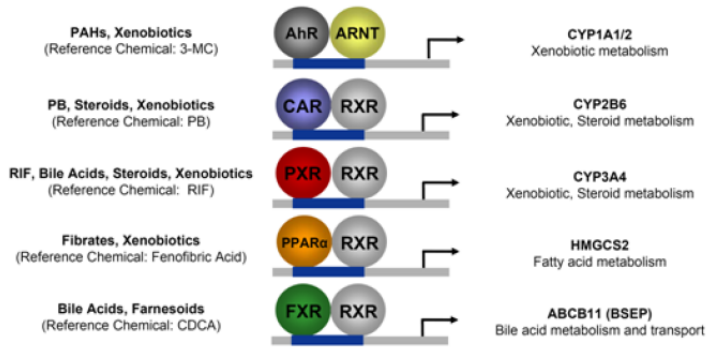
ToxCast/Tox21 is so rich with assays to examine nuclear receptors and hepatic catabolism, but not all substances that activate these receptors and downstream metabolism cause thyroid effects *in vivo* (research/data gap).

The list of nuclear receptor related assays is still growing…

ToxCast liver-related models contain indicators of Phase I and II metabolism and transporters

CellzDirect (CLD): fewer genes, ToxCast Phase I only

- ToxCast 320 Chemical Library
- Fresh Primary Human Hepatocytes
- 2 human donors
- 6 Reference Chemicals (Rif, PB, 3-MC, Fenofibric Acid, CDCA, CITCO)
- 5 receptors targets (AhR, CAR, PXR, PPARα, FXR)
- 2 endogenous control gene targets (GAPDH, Actin)
- 14 relevant gene targets
- 3 Time Points (6,24,48 hours)
- 5 Concentrations (.004, .04,0.4, 4, 40 µM)

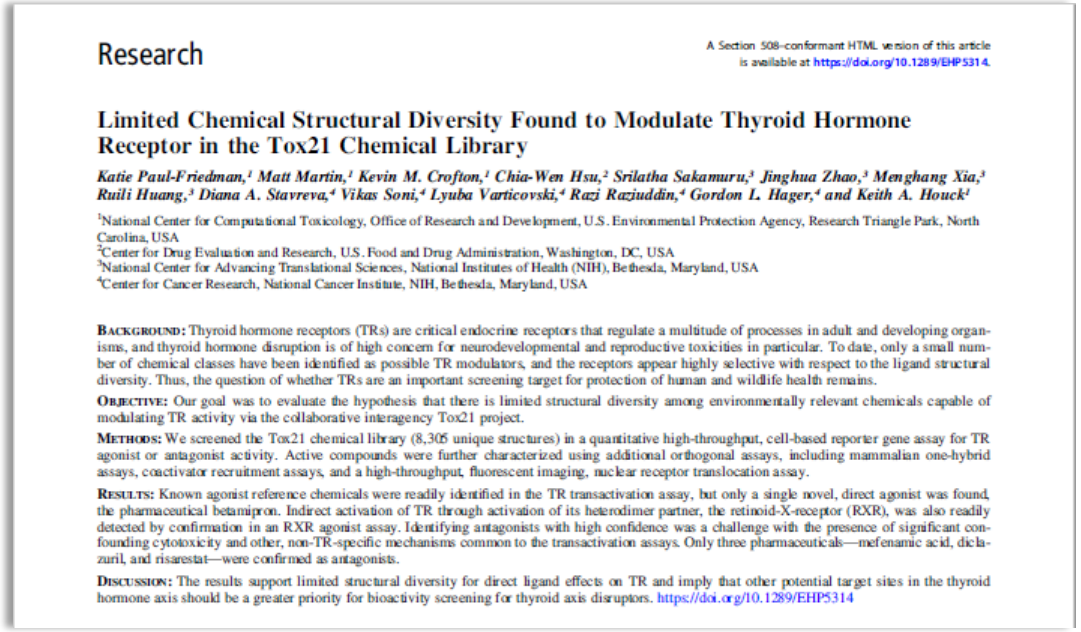


LifeTech Expression Analysis (LTEA): HepaRG cells, 1060 substances

- Newly released in invitrodb version 3.3
- ToxCast Phase I and Phase II Chemical library
- 189 assay endpoints, including ~93 genes: biotransformation, transporters, cell cycle, disease state markers (inc microRNA), etc.
- Paper forthcoming from Wambaugh and colleagues

Thyroid-relevant receptors: thyroid hormone receptor (TR), thyroid-stimulating hormone receptor (TSHR), and thyrotrophin-releasing hormone receptor (TRHR)

Evaluating the hypothesis that the thyroid hormone receptor is less promiscuous than other steroid hormone receptors



Research

A Section 508-conformant HTML version of this article is available at https://doi.org/10.1289/EHP5314.

Limited Chemical Structural Diversity Found to Modulate Thyroid Hormone Receptor in the Tox21 Chemical Library

Katie Paul-Friedman,[1] Matt Martin,[1] Kevin M. Crofton,[1] Chia-Wen Hsu,[2] Srilatha Sakamuru,[3] Jinghua Zhao,[3] Menghang Xia,[3] Ruili Huang,[3] Diana A. Stavreva,[4] Vikas Soni,[4] Lyuba Varticovski,[4] Razi Raziuddin,[4] Gordon L. Hager,[4] and Keith A. Houck[1]

[1]National Center for Computational Toxicology, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina, USA
[2]Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Washington, DC, USA
[3]National Center for Advancing Translational Sciences, National Institutes of Health (NIH), Bethesda, Maryland, USA
[4]Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland, USA

Background: Thyroid hormone receptors (TRs) are critical endocrine receptors that regulate a multitude of processes in adult and developing organisms, and thyroid hormone disruption is of high concern for neurodevelopmental and reproductive toxicities in particular. To date, only a small number of chemical classes have been identified as possible TR modulators, and the receptors appear highly selective with respect to the ligand structural diversity. Thus, the question of whether TRs are an important screening target for protection of human and wildlife health remains.

Objective: Our goal was to evaluate the hypothesis that there is limited structural diversity among environmentally relevant chemicals capable of modulating TR activity via the collaborative interagency Tox21 project.

Methods: We screened the Tox21 chemical library (8,305 unique structures) in a quantitative high-throughput, cell-based reporter gene assay for TR agonist or antagonist activity. Active compounds were further characterized using additional orthogonal assays, including mammalian one-hybrid assays, coactivator recruitment assays, and a high-throughput, fluorescent imaging, nuclear receptor translocation assay.

Results: Known agonist reference chemicals were readily identified in the TR transactivation assay, but only a single novel, direct agonist was found, the pharmaceutical betamipron. Indirect activation of TR through activation of its heterodimer partner, the retinoid-X-receptor (RXR), was also readily detected by confirmation in an RXR agonist assay. Identifying antagonists with high confidence was a challenge with the presence of significant confounding cytotoxicity and other, non-TR-specific mechanisms common to the transactivation assays. Only three pharmaceuticals—mefenamic acid, diclazuril, and risarestat—were confirmed as antagonists.

Discussion: The results support limited structural diversity for direct ligand effects on TR and imply that other potential target sites in the thyroid hormone axis should be a greater priority for bioactivity screening for thyroid axis disruptors. https://doi.org/10.1289/EHP5314

- Hypothesis: TR modulators represent limited structural diversity.
  - X-ray crystallography of TR isoforms suggests the need for high homology to thyroid hormone.
  - Few known TRβ therapeutic selective agonists and antagonists and with limited diversity.
  - Some *in vitro* reports of TR modulation, possibly via interaction with recruitment of corepressors/coactivators to the receptor complex.
  - Examples in the literature: OH-PCBs, OH-PBDEs, BPA and TBBPA.

Integrating multiple assay endpoints: agonism and antagonism of thyroid hormone receptor (TR) occurs with a limited number of substances

We tested the hypothesis that TR has a more restrictive ligand-binding pocket than estrogen and androgen receptors using Tox21 screening and follow-up assays.

Table 1. Assay names (aenm) and assay end point identification (aeid) values used in the text and invitrodb database together with mode and purpose of assay.

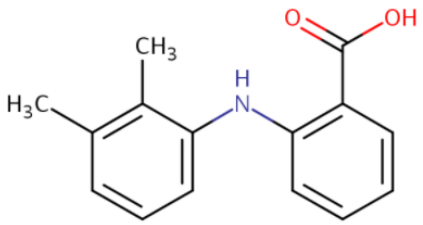
Assay short name	invitrodb: aenm	invitrodb: aeid	Cell line	Assay mode	Function
GH3-TRE-Ag	TOX21_TR_LUC_GH3_Agonist	803	GH3-TRE-Luc	Agonist	Primary qHTS
GH3-TRE-Antag	TOX21_TR_LUC_GH3_Antagonist	804	GH3-TRE-Luc	Antagonist	Primary qHTS
GH3-TRE-Via	TOX21_TR_LUC_GH3_Antagonist_viability	805	GH3-TRE-Luc	Viability	Cytotoxicity
GH3-TRE-Ag-Followup	TOX21_TR_LUC_GH3_Agonist_Followup	2226	GH3-TRE-Luc	Agonist	Confirmation
GH3-TRE-Antag-Followup	TOX21_TR_LUC_GH3_Antagonist_Followup	2227	GH3-TRE-Luc	Antagonist	Confirmation
TRb-bla	TOX21_TRB_BLA_Antagonist_Followup_ratio	2240	TRβ-UAS-bla HEK 293T	Antagonist	Specificity
RXRa-bla-Ag	TOX21_TR_RXR_BLA_Agonist_Followup_ratio	2253	RXRα-UAS-bla HEK 293T	Agonist	Specificity
RXRa-bla-Antag	TOX21_TR_RXR_BLA_Antagonist_Followup_ratio	2257	RXRα-UAS-bla HEK 293T	Antagonist	Specificity
RXRa-Via	TOX21_TR_RXR_BLA_Antagonist_Followup_viability	2258	RXRα-UAS-bla HEK 293T	Viability	Cytotoxicity
TRa-coa	TOX21_TRA_COA_Agonist_Followup_ratio	2230	NA	Agonist	Orthogonal
TRb-coa	TOX21_TRB_BLA_Agonist_Followup_ratio	2236	NA	Agonist	Orthogonal
GFP-GR-TRb	NA	NA	GFP-GR-TRβ MCF7	Agonist and antagonist	Orthogonal

Note: Ag, agonist; Antag, antagonist; bla, beta-lactamase; coa, coactivator; GFP, green fluorescent protein;GH3, rat pituitary cell line; GR, glucocorticoid receptor; HEK 293T, human embryonic kidney cell line; LUC, luciferase; MCF7, human breast cancer cell line; NA, not applicable; qHTS, quantitative high-throughput screen; RXRa, retinoid X receptor alpha; TRa, thyroid hormone receptor alpha; TRb, thyroid hormone receptor beta; TRE, thyroid hormone receptor response element; UAS, upstream activating sequence; Via, viability.

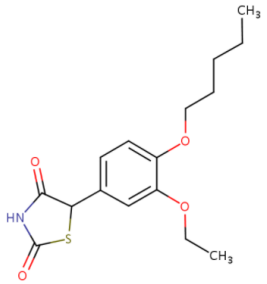
Integrating multiple assay endpoints: agonism and antagonism of thyroid hormone receptor (TR) occurs with a limited number of substances

- 11 chemicals identified of 8,305 unique substances as putative direct TR ligands
  - 8 agonists
    - T3 analogs (see table to right)
  - Additional 9 chemicals, largely pharmaceuticals, that agonize RXR through TR:RXR heterodimer resulting in partial agonism in the transactivation assays (permissive heterodimer effect); no activity when RXR not present
  - 3 antagonists of higher confidence: pharmaceuticals, at concentrations exceeding therapeutic concentrations

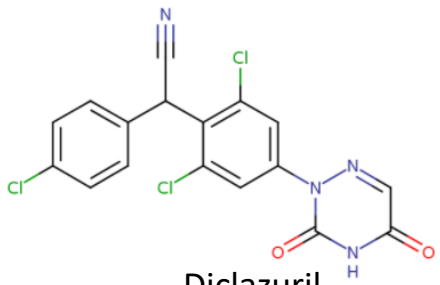
Chemical name
CP-634384
3,5,3′-Triiodothyronine
Levothyroxine
Tetrac
3,3′,5′-Triiodo-L-thyronine
Tiratricol
3,3′,5-Triiodo-L-thyronine sodium salt
Betamipron



Mefenamic acid
(NSAID, some evidence of plasma TH effects in rats)



Risarestat
(aldose reductase inhibitor for hypoglycemia assoc. with diabetes)



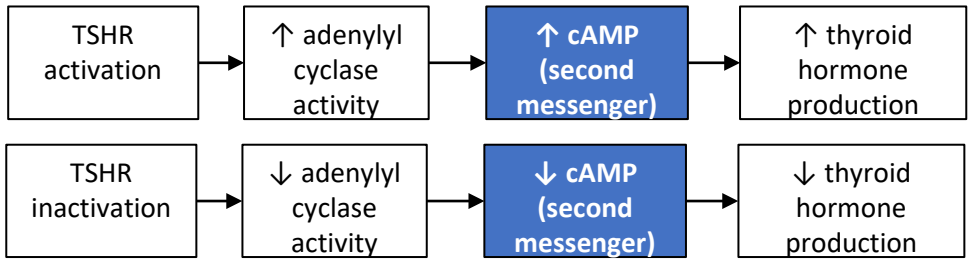
Diclazuril
(anticoccidal used in poultry)

Overall conclusion: work supports the hypothesis that TR is a very selective nuclear receptor.

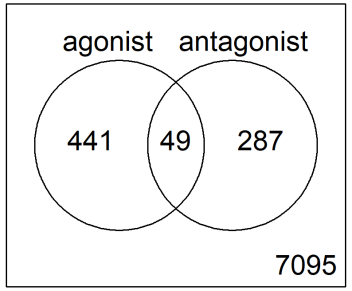
This work used a lot of expert judgment and substances with clear lead MOA were excluded from follow-up.

TOX21 TSHR assay principle

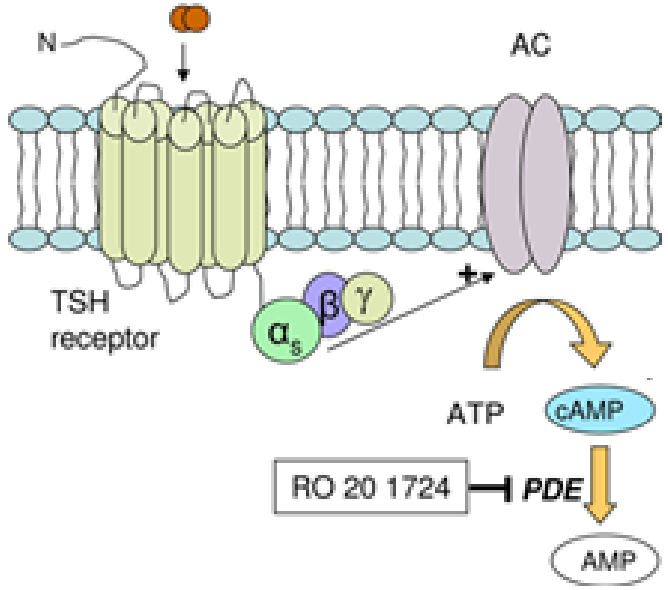
Aeid	Aenm
2040	TOX21_TSHR_HTRF_Agonist_ratio
2043	TOX21_TSHR_HTRF_Antagonist_ratio
2046	TOX21_TSHR_HTRF_wt_ratio



cAMP is the signal measured in this assay platform

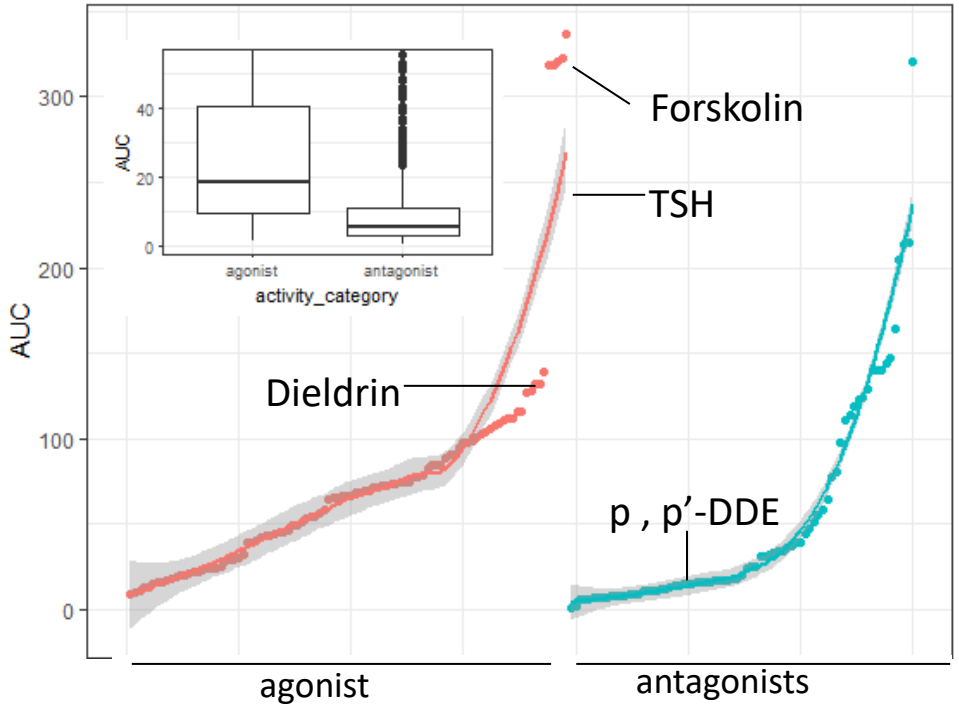


- TSHR is a GPCR with a few known agonists or antagonists.
- This assay measures agonism or antagonism for TSHR through the Gs-cAMP pathway.



- Hits from the primary screen need to be confirmed or evaluated with orthogonal information.
- Assay interference may come from cytotoxicity, auto-fluorescent or blue dyes, agonists of other GPCRs may modulate cAMP, (e.g., B-adrenergic receptors) and other activators of adenylyl cyclase.

Context for interpretation and ongoing work



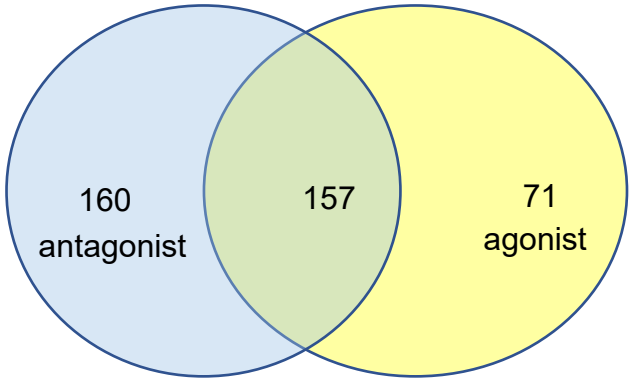
Chemicals from agonist and antagonist modes were ranked according to the area-under-the-curve (AUC) of curve fits from concentration-response modeling using the tcpl package. Figure 1 shows the AUC values of the selected list for follow-up testing. **Forskolin** was expected to increase cAMP production and exhibits high activity, slightly higher than the native agonist **TSH**. **Dieldrin**, a suggested inverse agonist from the literature, shows activity in the 90th percentile of potential activators for TSHR.

Approach: Use area under the curve, information from the null/wildtype assay, selectivity (cytotoxicity), and chemical structure to select ~90 substances for confirmation follow-up of agonist and antagonist responses in a TSH-responsive model of biological complexity by Chad Deisenroth (US EPA).

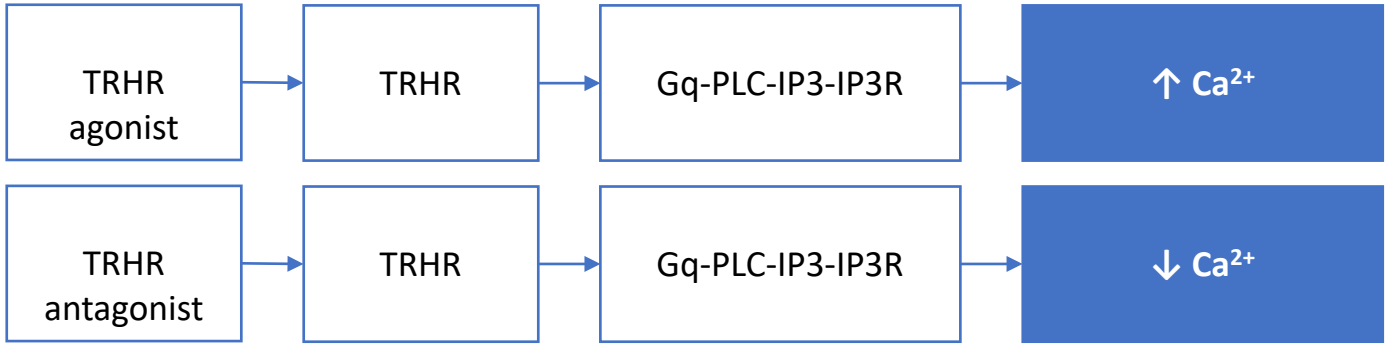
Bottom-line: review the available TOX21 TSHR data with an eye to context until more confirmation is available.

TOX21 TRHR assay principle

Aeid	Aenm
2364	TOX21_TRHR_HEK293_Agonist
2365	TOX21_TRHR_HEK293_Antagonist



388 Total Hits



Calcium is detected using a fluorescence detection kit

- Hits from the primary screen need to be confirmed or evaluated.
- Potential sources of interference: auto-fluorescence, nonspecific calcium interference, nonspecific GPCR activity, etc.
- Ongoing work to contextualize these results using molecular docking approaches.
- View these hits as putative until additional confirmation can be used.

Pharmacophore modeling approach for TRHR

Analysis based on reference dataset

Generation of 3D conformations

Molecular alignment of binders

Generation & validation of pharmacophore models

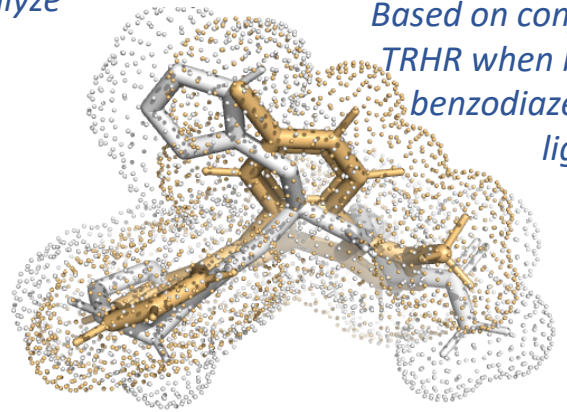
Apply to all tested chemicals

Querying structure database

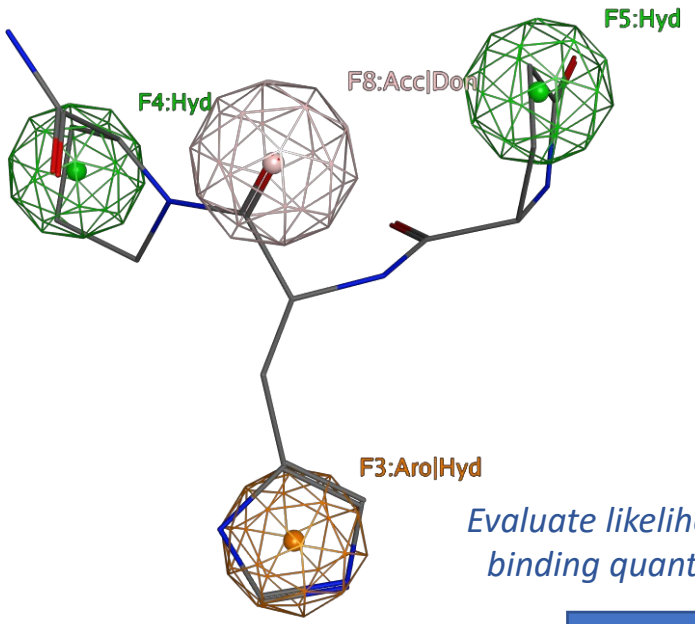
Analysis of model results

Uses MOE software to analyze possible 3D binding

MOE



Based on conformation of the TRHR when bound to TRH or benzodiazepines (known ligand)



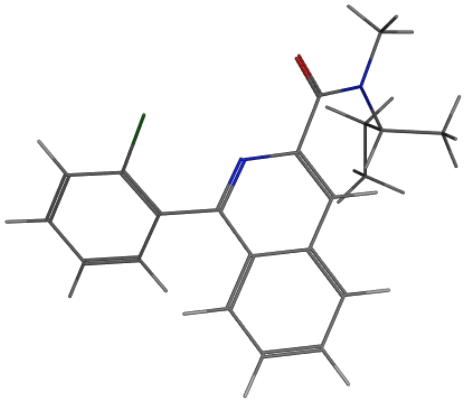
Evaluate likelihood of 3D binding quantitatively

mol	rmsd
	0.4799
	0.2784
	0.2636
	0.5092
	0.2334
	0.3147
	0.4857
	0.2842
	0.3047
	0.3550
	0.2474
	0.3806
	0.3041
	0.2422
	0.2404
	0.2885
	0.4383
	0.5112
	0.2485
	0.3482

Unpublished; adapted from work in progress by Dr. Mahmoud Shobair

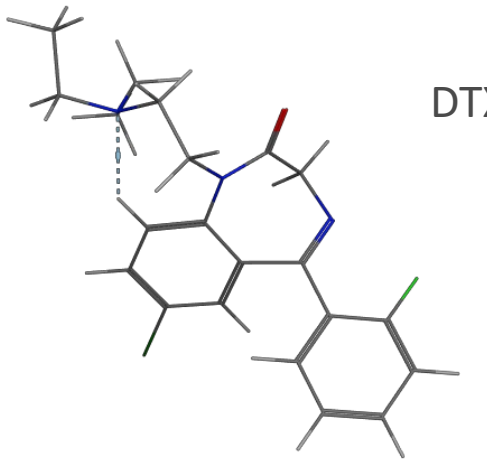
Example predicted TRHR inhibitor: PK-11195 (Moderate binder)

isoquinoline carboxamide binds selectively to the peripheral benzodiazepine receptor (PBR)

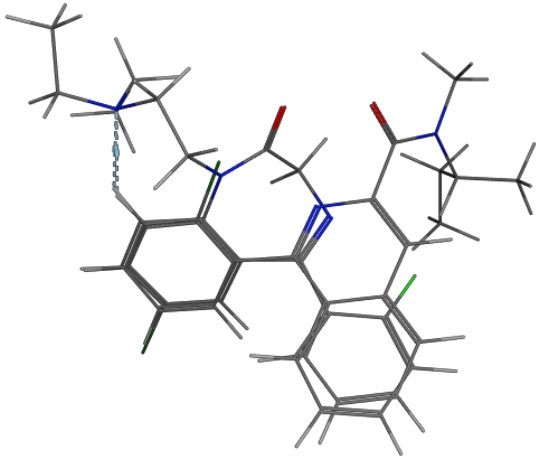


DTXSID7041097

Midazolam, known TRHR binder



DTXSID8047846



PK-11195 aligned to Midazolam

A limitation of this work is that the 3D modeling assumes the TRHR binding pocket in the native conformation.

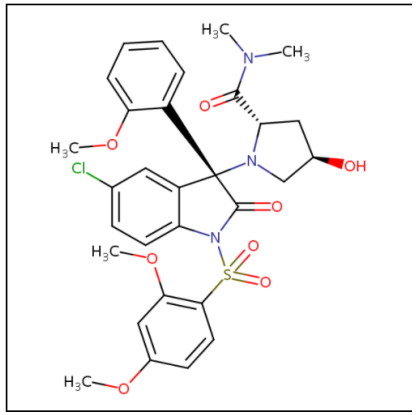
Unpublished; adapted from work in progress by Dr. Mahmoud Shobair

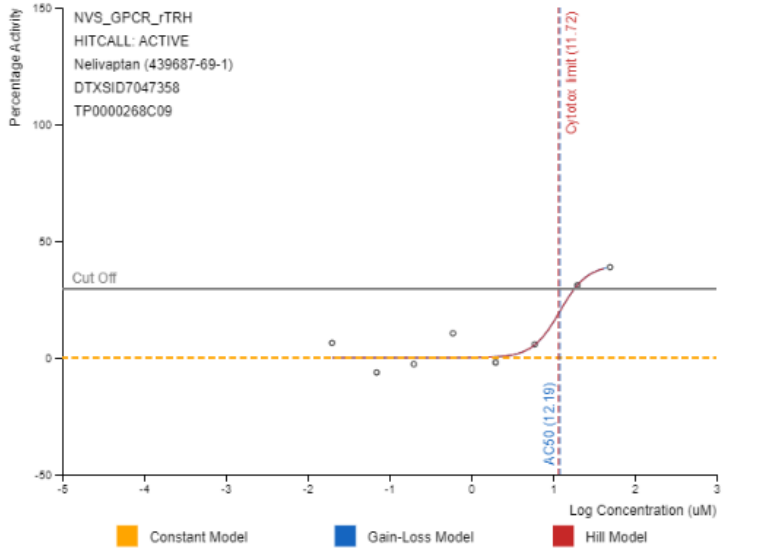
Assay principle of the NVS TRHR assay

aeid	aenm
683	NVS_GPCR_rTRH

- Measures changes in scintillation (radioactivity) counts from [[3H]-(3-methylHis[2])-TRH] binding to rat TRHR.
- TRHR from rat forebrain membranes.
- 1000 substances screened in multi-concentration– limited overlap in the screen with the TOX21 TRHR screen, and nearly no overlap in hits.
- 35/1000 are hitcall=1; some clear interference from organometallic substances and detergents; borderline or noisy activity; possibly other GPCR modulators. Most of these hits seem easy to dismiss when inspecting the curves.

Nelivaptan
439687-69-1 | DTXSID7047
Searched by DSSTox Substance Id.





Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	70.66	18.36	-	-	-
	Gain-Loss	60.69	5.03	39.7	12.19	2.59
✓	Hill	56.69	5.03	39.7	12.19	2.59

Nelivaptan is one of the only credible putative hits, but it has clear PXR activity at lower concentrations. This drug was developed for another GPCR, vasopressin receptor V1B in the anterior pituitary gland that works to release ACTH, prolactin, endorphins.

Examining a single substance using a weight-of-evidence approach

A generic workflow is illustrated here. For putative thyroid-related bioactivity, we might consider:

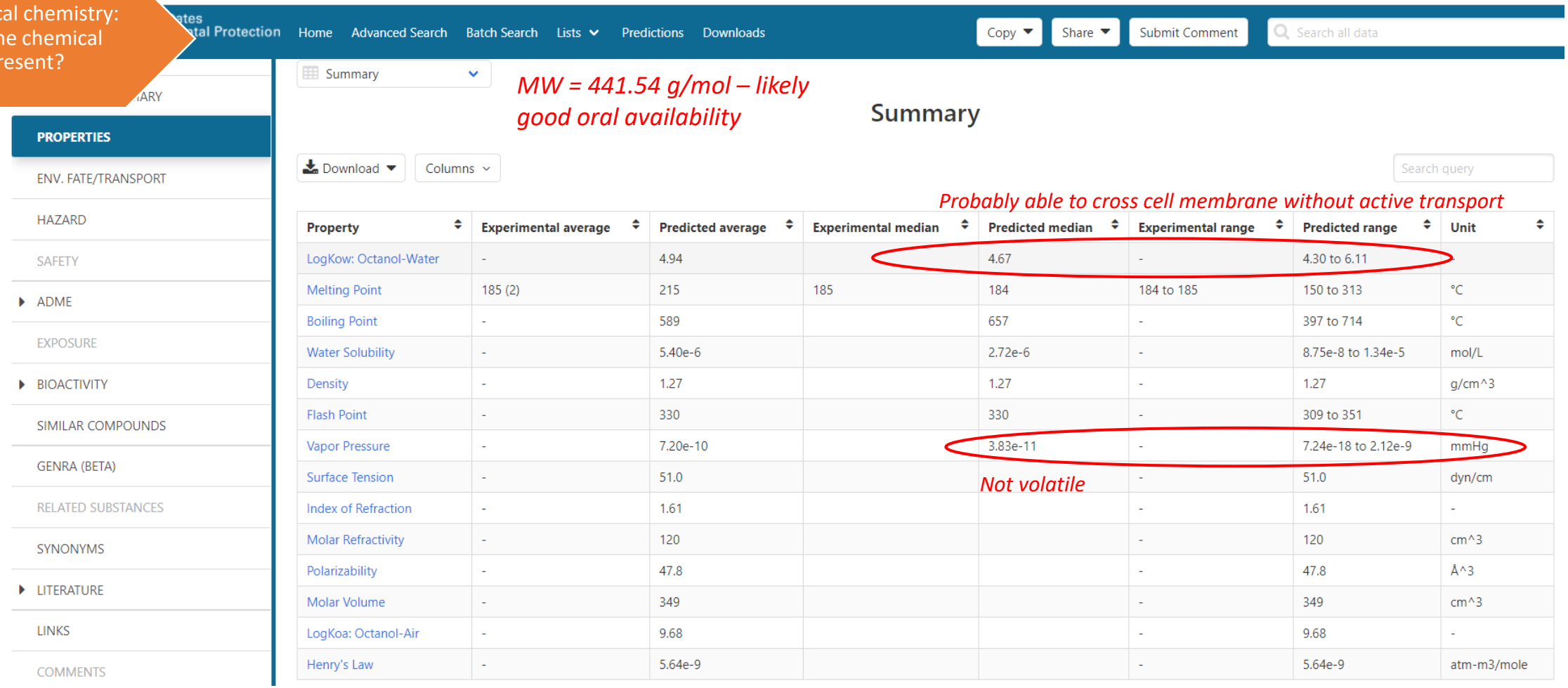
- the amenability of the substance for HTS screening and sample quality;
- Models or single assays available; and,
- Whether the activity is likely to be selective or not.

Troglitazone

- Troglitazone
- Treatment for Type II diabetes, works primarily by activating PPARγ
  - Also involved in immune response via decrease in NF-KB
- Drug removed from market due to DILI, with several proposed mechanisms, including:
  - Mitochondrial toxicity [Electron transport chain inhibitor (Complex I) at low micromolar concentrations]
  - Inhibits of bile acid transport/cholestatic effects (e.g., BSEP)
  - Apoptosis
  - Formation of reactive metabolites/oxidative stress

Troglitazone: in domain of current screening?

Analytical chemistry: was the chemical present?



Property	Experimental average	Predicted average	Experimental median	Predicted median	Experimental range	Predicted range	Unit
LogKow: Octanol-Water	-	4.94		4.67	-	4.30 to 6.11	
Melting Point	185 (2)	215	185	184	184 to 185	150 to 313	°C
Boiling Point	-	589		657	-	397 to 714	°C
Water Solubility	-	5.40e-6		2.72e-6	-	8.75e-8 to 1.34e-5	mol/L
Density	-	1.27		1.27	-	1.27	g/cm^3
Flash Point	-	330		330	-	309 to 351	°C
Vapor Pressure	-	7.20e-10		3.83e-11	-	7.24e-18 to 2.12e-9	mmHg
Surface Tension	-	51.0			-	51.0	dyn/cm
Index of Refraction	-	1.61			-	1.61	-
Molar Refractivity	-	120			-	120	cm^3
Polarizability	-	47.8			-	47.8	Å^3
Molar Volume	-	349			-	349	cm^3
LogKoa: Octanol-Air	-	9.68			-	9.68	-
Henry's Law	-	5.64e-9			-	5.64e-9	atm-m3/mole

Troglitazone seems to fit into the domain of screening based on chemistry

Analytical chemistry: was the chemical present?

States ental Protection | Home | Advanced Search | Batch Search | Lists | Predictions | Downloads | Copy | Share | Submit Comment | Search all data

Searched by DSSTox Substance Id.

PROPERTIES
ENV. FATE/TRANSPORT
HAZARD
SAFETY
ADME
EXPOSURE
BIOACTIVITY
TOXCAST: SUMMARY
EDSP21
TOXCAST/TOX21

ToxCast/Tox21

Select samples that were analyzed (the chemical in DMSO stock) are high purity and confirmed

QC Data ID	Grade	Description
Tox21_112119	Pass	Purity>90% and MW confirmed
Tox21_112119_1	Pass	Purity>90% and MW confirmed
Tox21_300470	Pass	Purity>90% and MW confirmed

Assay Selection 0 Selected | A Single Assay Can Have Multiple Charts | Representative Samples Only | Bioactivity Summary | Number of Charts: 0

Active | Inac

Filter assays

Tanguay Lab (0 of 19 s

Tox21/NCGC (0 of 235

NHEERL Mid-Continent

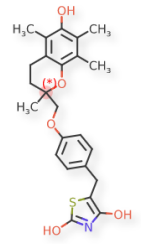
tripod.nih.gov/tox21/samples/Tox21_112119

Apps | Files - OneDrive | toxrefdb - OneDrive | Google Scholar | Altmetric it! | ORD Graphics and... | ORD@Work | Request Research H... | Travel Authorizatio... | Chemtrack | Toxicity Data Collec...

Tox21 | Structure Search | Search...

Home / Tox21 Samples / Tox21_112119

Seems stable under screening sample conditions (DMSO, room temp, 0-4 months)



QC Grade

T0	A	MW Confirmed, Purity > 90%
T4	A	MW Confirmed, Purity > 90%

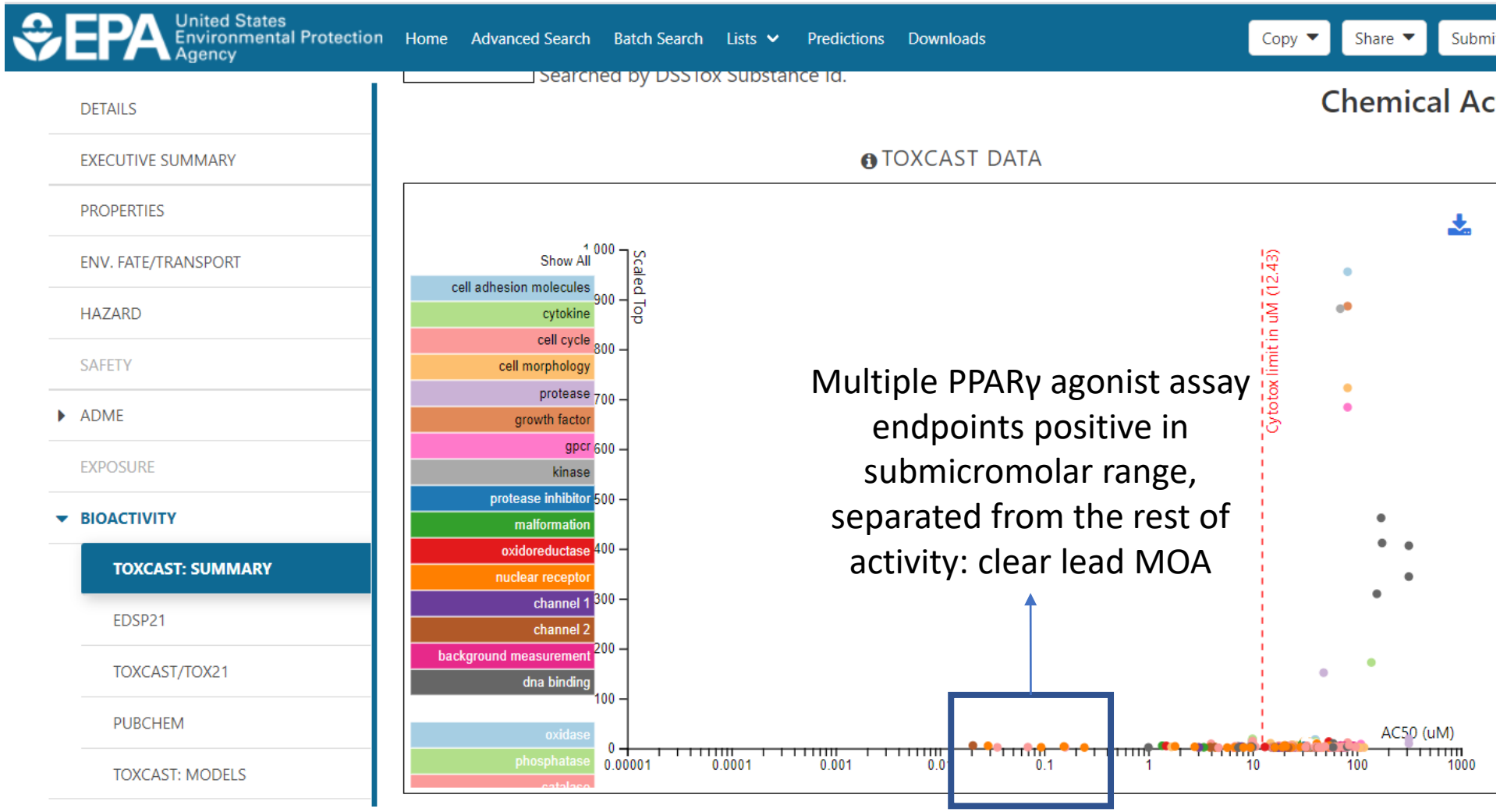
Identifiers

Tox21	Tox21_112119
NCATS	NCGC00159457-01
CAS	
PubChem	

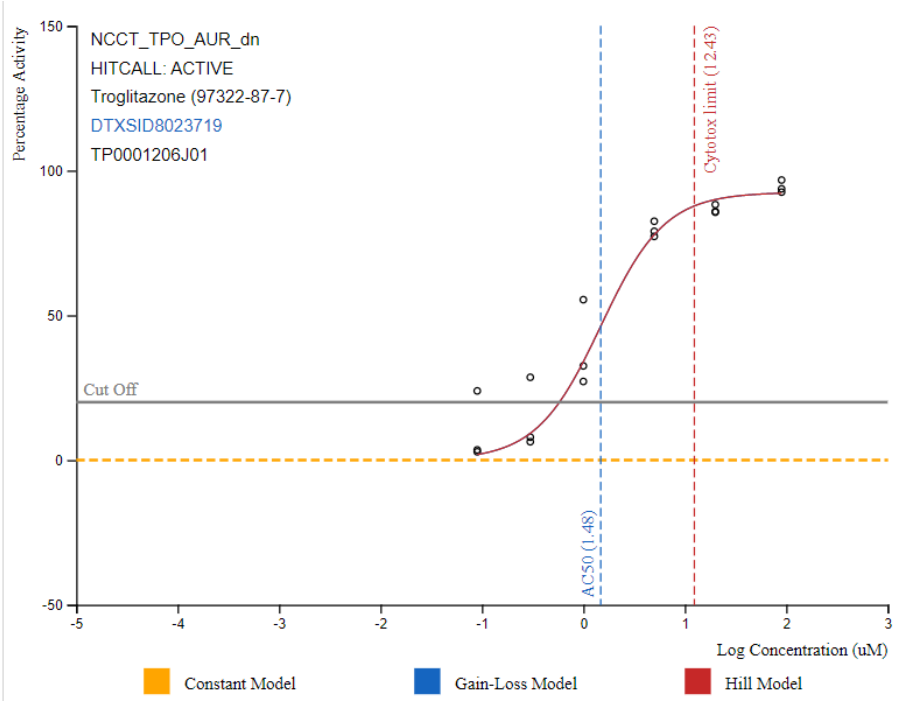
But what bioactivity does troglitazone have?

Predictive or integrated models available? If not, single assays?

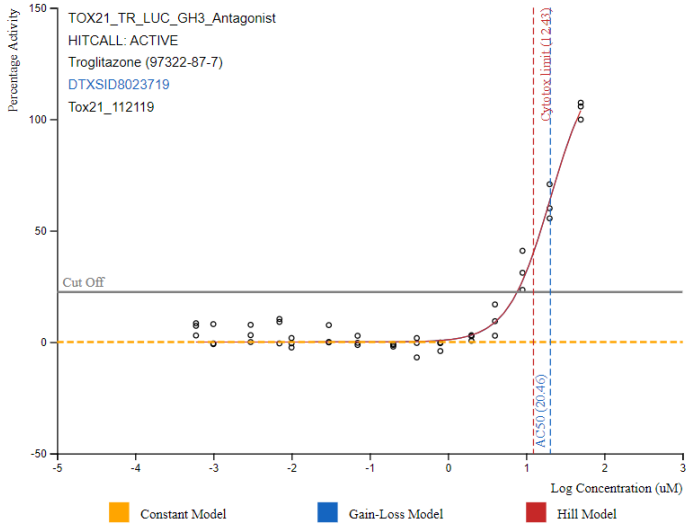
Selective or non-selective?

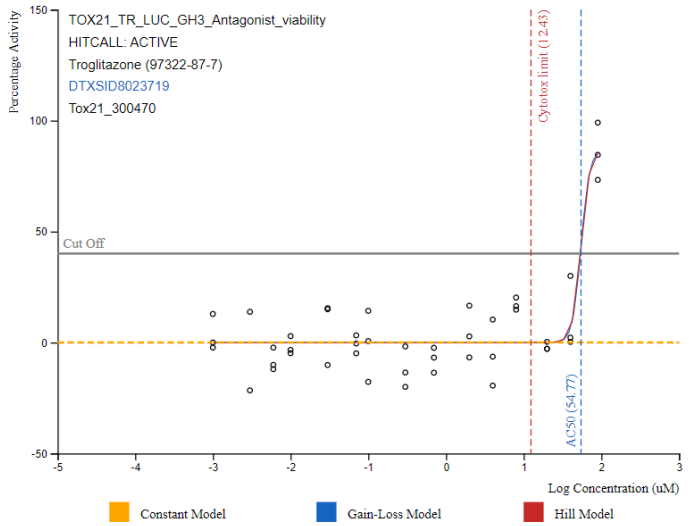


It does have some putative thyroid-related bioactivity, but at concentrations that appear to exceed its lead MOA



The TPO inhibition curve is of a high quality and seems well separated from positive hits on cytotoxicity and nonspecific protein inhibition. But the concentration that TPO inhibition occurs at is 10-100x the PPARg AC50s that range 0.02-0.2 from TOX21 and ATG assays.





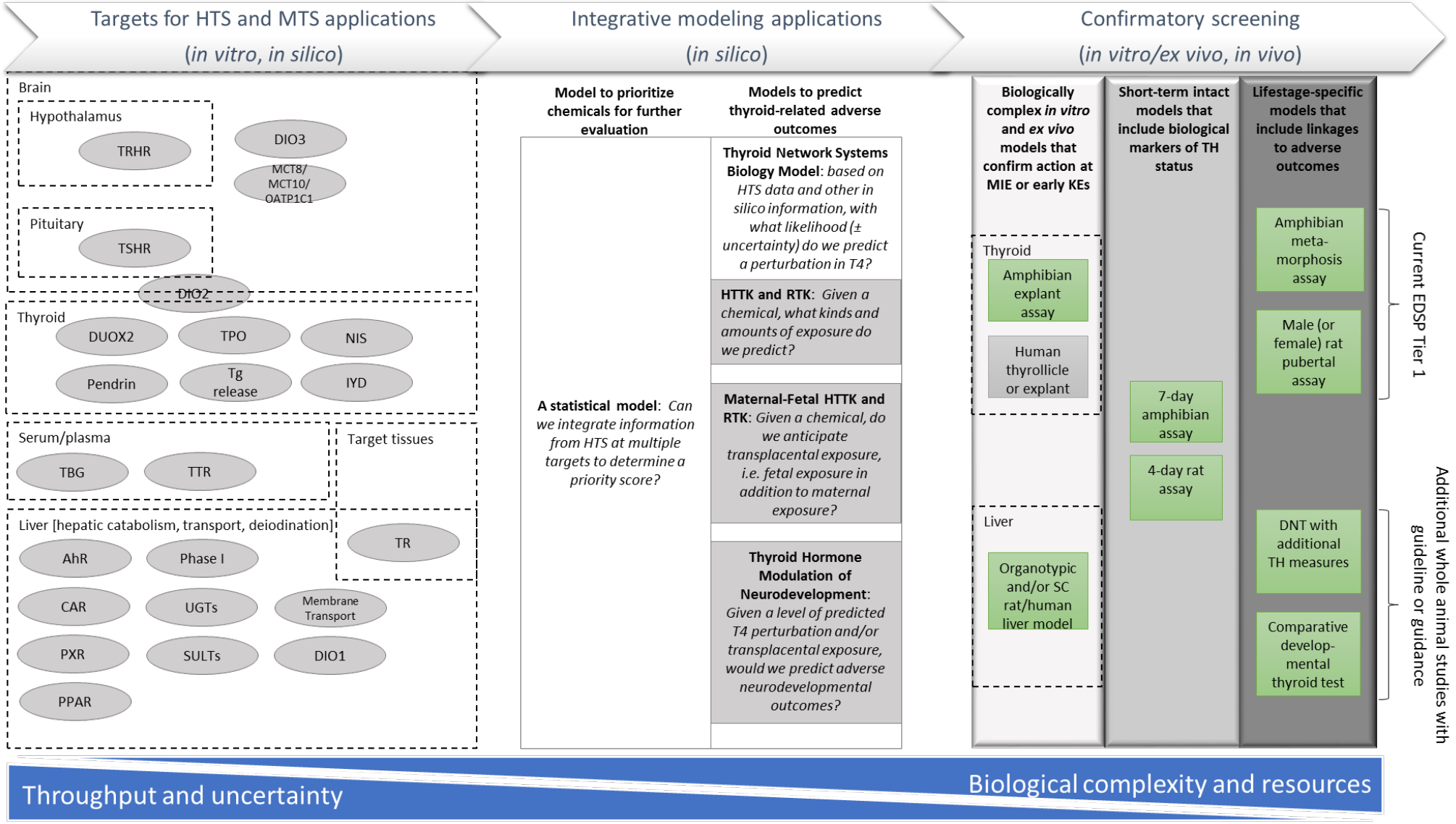
There is a credible TR antagonist response with AC50 ~20 micromolar, versus cytotoxicity at ~50 micromolar. But this possible antagonism is at 100-1000X the PPARg AC50s that range 0.02-0.2 from TOX21 and ATG assays.

Looking forward to data integration

- Multiple MIE and KE targets will need consideration.
- Several different integration and modeling approaches are possible.
- Distinguishing signal from assay interference or other confounders is critical for interpreting the data from thyroid-related HTS assays.
  - Importance of verifying “selectivity” even for cell-free assays
  - Use of multiple assays together to understand thyroid-relevant outcomes (versus other modes of action)

Future: HTS, model development, and confirmatory screening

Many of the MIE targets have MTS and HTS assays, <u>but efforts to evaluate the screening sensitivity and specificity of those screens are still in progress (e.g., TR, TRHR, TSHR).</u>



Acknowledgements

A huge team of people have contributed to the development, screening, and analysis of thyroid-related ToxCast and Tox21 data and thyroid-related projects.

Key EPA ORD contributors:
Jason Brown
Madison Feshuk
Mahmoud Shobair
Ann Richard
Keith Houck
Richard Judson
Jennifer Olker
Jonathan Haselman
Michael Hornung
Sig Degitz
Jun Wang
Tammy Stoker
Susan Laws
Ashley Murr
Angela Buckalew
Daniel Hallinger
Steve Simmons
Chad Deisenroth
Katie Paul Friedman
Pamela Noyes
Eric Watt
And more…

Collaborators
Menhang Xia and Ruili Huang and others at National Center for Advancing Translational Sciences

Center for Computational Toxicology and Exposure (CCTE)
Office of Research and Development (ORD)
US Environmental Protection Agency