Development of a tiered screening strategy for a molecular-initiating event: thyroperoxidase inhibition

Katie Paul Friedman[1,2], Eric D. Watt[1,4], Joan M. Hedge[2], Kevin M. Crofton[4], Michael W. Hornung[3], Steven O. Simmons[4]

[1]Oak Ridge Institute for Science Education Postdoctoral Fellow;
[2]Integrated Systems Toxicology Division; [3]Mid-Continent Ecology Division, NHEERL; and [4]National Center for Computational Toxicology; ORD, U.S. EPA, RTP, NC and Duluth, MN

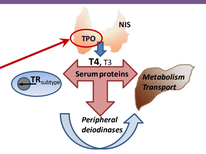
Simmons.Steve@epa.gov.

Abstract

Adverse outcome pathway (AOP) analyses illustrate that some molecular-initiating events (MIEs) for thyroid disruption, including thyroperoxidase (TPO) inhibition, are not evaluated by current ToxCast/Tox21 high-throughput screening (HTS) assays. A novel HTS assay for TPO inhibition was developed by adaptation of the guaiacol oxidation assay with a fluorescent peroxidase substrate (Amplex UltraRed, AUR, LifeTech) in a rat thyroid microsome-based assay optimized to 384-well format. Initial testing of the AUR-TPO assay was conducted using a 21-chemical training set that included a reference chemical, methimazole (MMI), known TPO inhibitors, and negative controls. The AUR-TPO assay signal was stable (30-120 min), the dynamic range and Z' score with MMI were 11-fold and 0.93, and the IC_{50} for MMI was 0.025 μM (compared to 2.20 μM in a guaiacol-based 96-well assay). The ToxCast Phase I & II libraries (1060 chemicals) were tested with the AUR-TPO assay using an initial screen of one high concentration to identify candidate inhibitors. Chemicals that exceeded 20% inhibition were then screened in concentration-response (6 or 8 concentrations for Phase I & II). In addition, 2 parallel screens were conducted: ATP availability in HEK293T cells (24 hr exposure) to indicate cytotoxicity; and, luciferase inhibition to identify nonspecific protein inhibitors. Results demonstrated known and novel chemicals that inhibited TPO at concentrations below cytotoxicity and that did not inhibit luciferase. AOP-based analyses for thyroid screening enabled development of a new HTS assay that allowed screening of ToxCast chemicals for TPO inhibition. This assay provides an additional data stream to support prioritization of chemicals within the Endocrine Disruptor Screening Program. *This abstract does not necessarily reflect the policy of the US EPA.*

Why is thyroperoxidase (TPO) activity screening assay a critical need for integrated thyroid disruptor screening?

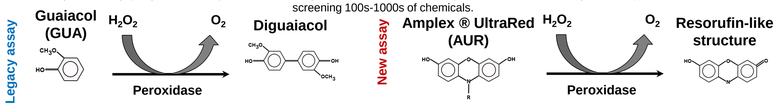
Benzophenone-2, isoflavones, anti-hyperthyroid drugs, coal derivatives, pesticides



- TPO catalyzes TH synthesis within the thyroid follicle
- TPO is the known target of many environment contaminant
- No medium- or high-throughput assays exist for TPO screening

Assay principles

The existing low-throughput guaiacol assay was modified to generate the AUR-TPO assay, which is executed using 384 well-plates and is amenable to screening 100s-1000s of chemicals.



Assay	GUA-TPO	AUR-rTPO
Plate format	3-8 wells of a 96 wp	384 wp
Dynamic range (mean ± SE)	127 ± 80	11.5 ± 2.4
Z' (mean ± SE)	0.62 ± 0.16	0.93 ± 0.021
CV (%)	92	16.1
MMI IC50 (μM)	2.20	0.025
N	3	3
Wells or tests per rat	8.3	83.3
Reaction	Kinetic, 60 s	Endpoint,stable 30 min – 2hr
Detection	Spectrophotometric, 450-470nm	Fluorescent, 544/590

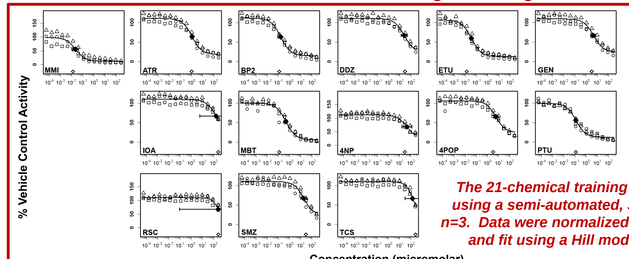
#	Anticipated positive chemicals	Chemical Name	Abbreviation
1		Methimazole	MMI
2		3-Amino-1,2,4-Triazole (Amitrole)	ATR
3		2,2',4,4'-Tetrahydroxy-benzophenone	BP2
4		Daidzein	DDZ
5		Ethylene thiourea	ETU
6		Genistein	GEN
7		2-mercaptobenzothiazole	MBT
8		4-nonylphenol	4NP
9		4-propoxyphenol	4POP
10		6-propylthiouracil	PTU
11		Resorcinol	RSC
12		Sulfmethazine	SMZ

#	Anticipated negative chemicals	Chemical Name	Abbreviation
1		2-hydroxy-4-methoxybenzophenone	BP3
2		Dibutylphthalate	DBP
3		Diethylhexylphthalate	DEHP
4		Diethylphthalate	DEP
5		3,5-Dimethylpyrazole-1-methanol	DPM
6		Iopanoic Acid	IOA
7		Methyl 2-methyl-benzoate	MMB
8		Sodium-perchlorate	NaPER
9		Triclosan	TCS

Hypothesis I: The AUR substrate can be used in a 384-well, semi-automated assay to detect TPO inhibitors (see Paul *et al.*, 2014, Chemical Research in Toxicology).

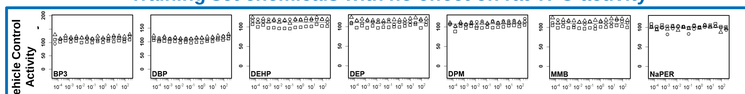
Results from the 21-chemical training set

TPO-inhibitors from the training set using rat TPO

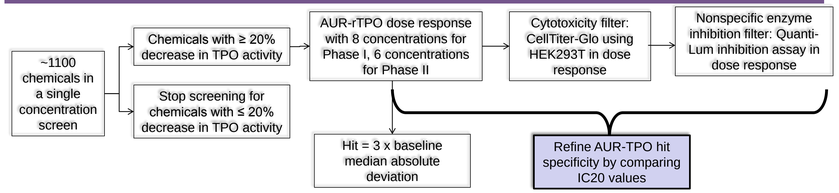


The 21-chemical training set was evaluated using a semi-automated, 384-wp format, with n=3. Data were normalized to % vehicle control, and fit using a Hill model using R.2.15.1.

Training set chemicals with no effect on rat TPO activity



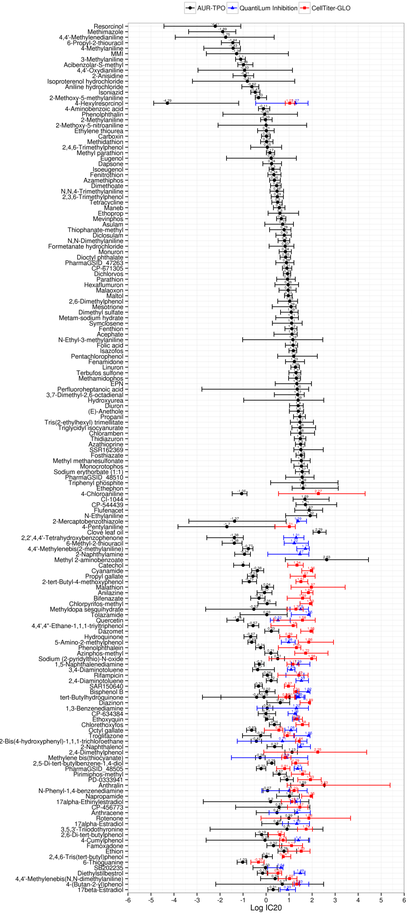
Hypothesis II: The AUR-rTPO assay can be used to screen the ToxCast Phase I and II chemical libraries using a tiered approach.

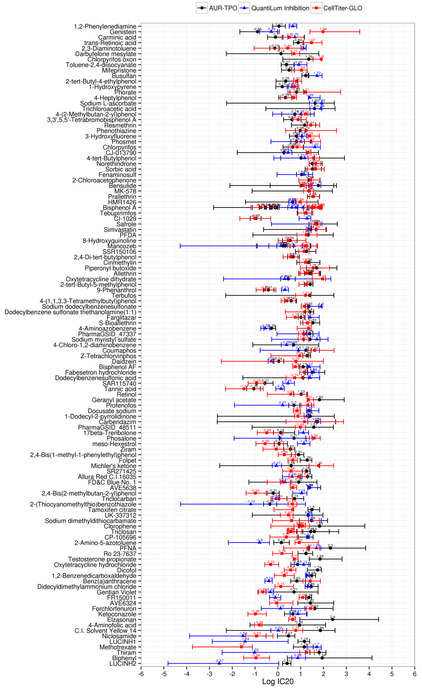


Conclusions

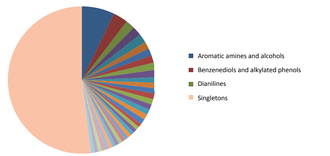
- The 384-well plate AUR-TPO screening using rat thyroid microsomes confers the following advantages:
  - Stable, fluorescent signal for 30 min – 2 hr
  - High reproducibility and adequate dynamic range to identify TPO inhibitors
  - 10-fold reduction in animal tissue required per well tested
  - 500x increase in sample throughput compared to guaiacol assay
- The 21-chemical training set demonstrated that the AUR-TPO assay may be used to specifically discern TPO inhibitors. The assay demonstrated a 100% true positive rate and a 0% false negative rate. Two out of 21 chemicals were unexpected positives when compared to previous work with guaiacol.
- Use of the AUR-rTPO assay in a tiered screening strategy may increase knowledge regarding how many chemicals may inhibit TPO, and may also provide valuable endocrine disruptor screening information while supporting efforts to reduce animal use in testing.
- With ~1100 chemicals screened to date in this new AUR-TPO assay, including parallel non-specific enzyme inhibition and cytotoxicity assays, this work represents the largest screening effort to date for TPO inhibition. AUR-TPO inhibitors were structurally diverse, but many AUR-TPO inhibitors may be nonspecific; thus, use of parallel measures of specificity may provide context for further prioritization decisions.

Specificity: comparison of AUR-TPO IC20 with cytotoxicity and nonspecific protein inhibition.





Chemical structure binning of 250 TPO hits with >30% inhibition *(Tanimoto cut-off =0.5, ChemMine)*



~50% of hits were not structurally related. Largest bins were enriched for anilines and phenols