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

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Part I

- Why is thyroperoxidase inhibition a concern?
- "Top-down" assay development strategy.
- Our tiered screening approach to identifying potentially relevant putative TPO inhibitors.
- Results of the screening and future directions.

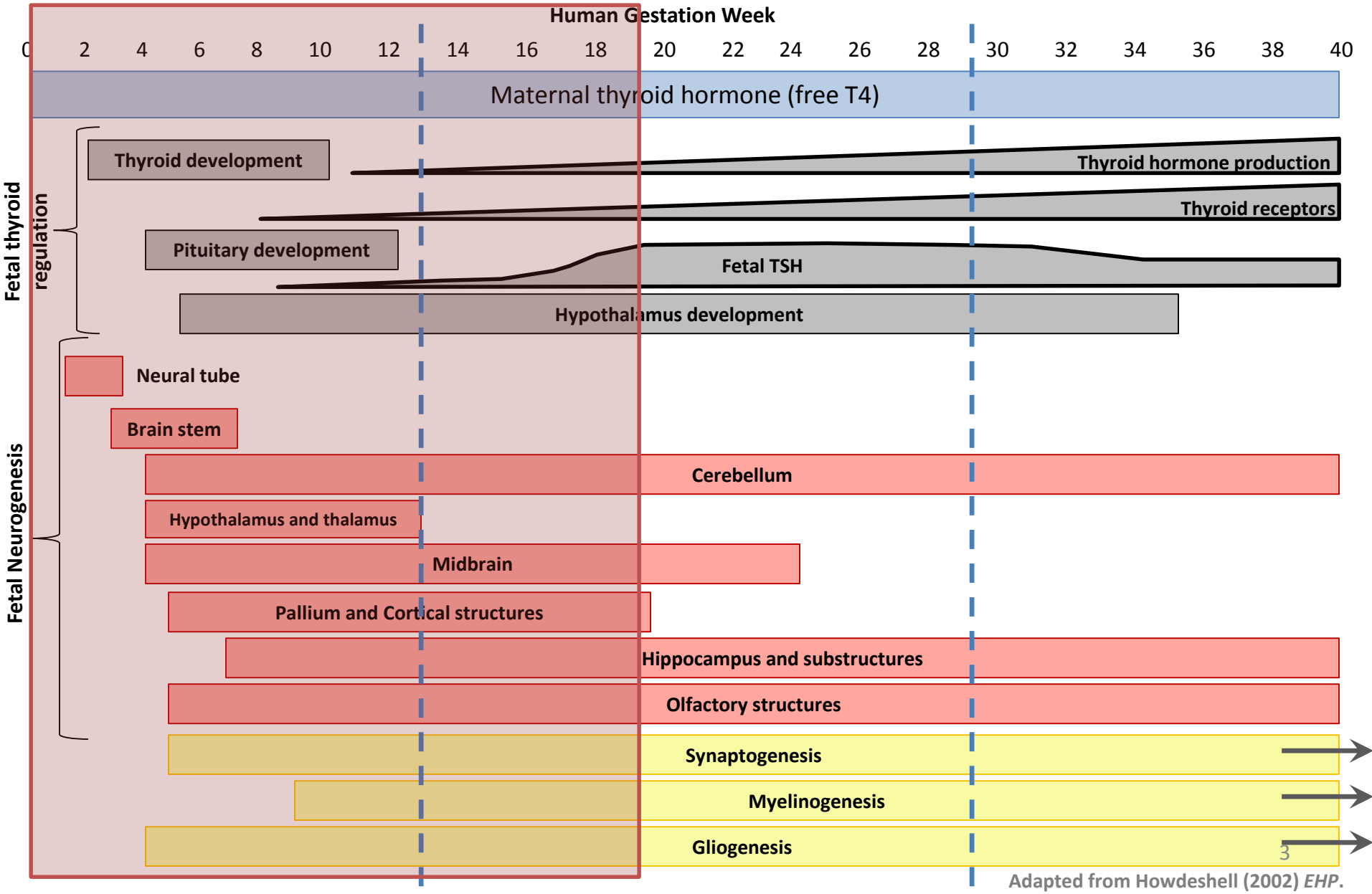
Part II

- Availability of TPO Inhibition Assay Data (the Dashboard and invitrodb_v2).

Key References

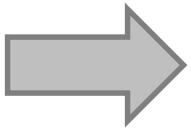
- See our latest publication: Paul Friedman K, Watt ED, Hornung MW, Hedge JM, Judson RS, Crofton KM, Houck KA, Simmons SO. 2016. Tiered High-Throughput Screening Approach to Identify Thyroperoxidase Inhibitors within the ToxCast Phase I and II Chemical Libraries. Toxicological sciences : an official journal of the Society of Toxicology. Feb 15. (http://toxsci.oxfordjournals.org/content/early/2016/02/15/toxsci.kfw034.long)
- See downloadable data set (October 2015): http://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data

Maternal thyroid hormones (THs) modulate human brain development; thus screening for developmental Δ[TH] may identify neurotoxicants.



Adapted from Howdeshell (2002) *EHP*.

Moderate maternal thyroid hormone disruption



Cognitive and brain abnormalities.

(Barone et al., 2000; Berbel et al. 2009; Cuevas et al., 2005; Howdeshell, 2002; Morreale de Escobar et al., 2000; Rice et al., 2000; Zoeller et al., 2000)

Human maternal hypothyroxinemia in the first trimester

- ↓ behavioral response @ **3 wks** (Kooistra et al. 2006);
- ↓ psychomotor development @ **10, 12, 24 mos** (Pop et al., 1999; Pop et al., 2003);
- ↓ motor coordination/socialization at **18 mos** (Berbel et al. 2009);
- Language delays at **18 and 30 mos** (Henrichs et al. 2010);
- Small IQ point ↓ at **7-9 yrs** (Haddow et al. 1999).

Rat models of maternal T4 insufficiency

- **Δ cytoarchitecture** (Auso et al., 2004; Cuevas et al., 2005; Lavado-Autric et al., 2003; Sharlin et al. 2008);
- **Δ synaptic calcium regulation and myelination** (Ibarrola et al., 1997; Iniguez et al., 1996);
- **Gene expression Δ :synaptic calcium/transmission, myelination, and developmental cell adhesion** (Morreale de Escobar et al., 2008; Morreale de Escobar et al., 2000; Morreale de Escobar et al., 2004, Royland et al.,2008).

TPO inhibition is a well-characterized MIE for TH disruption (aopwiki.org)

Currently in revision

Adverse Outcome Pathway Wiki

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Actions

Feedback

Tools

AOP Title

Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals

Short name: TPO Inhibition and Altered Neurodevelopment

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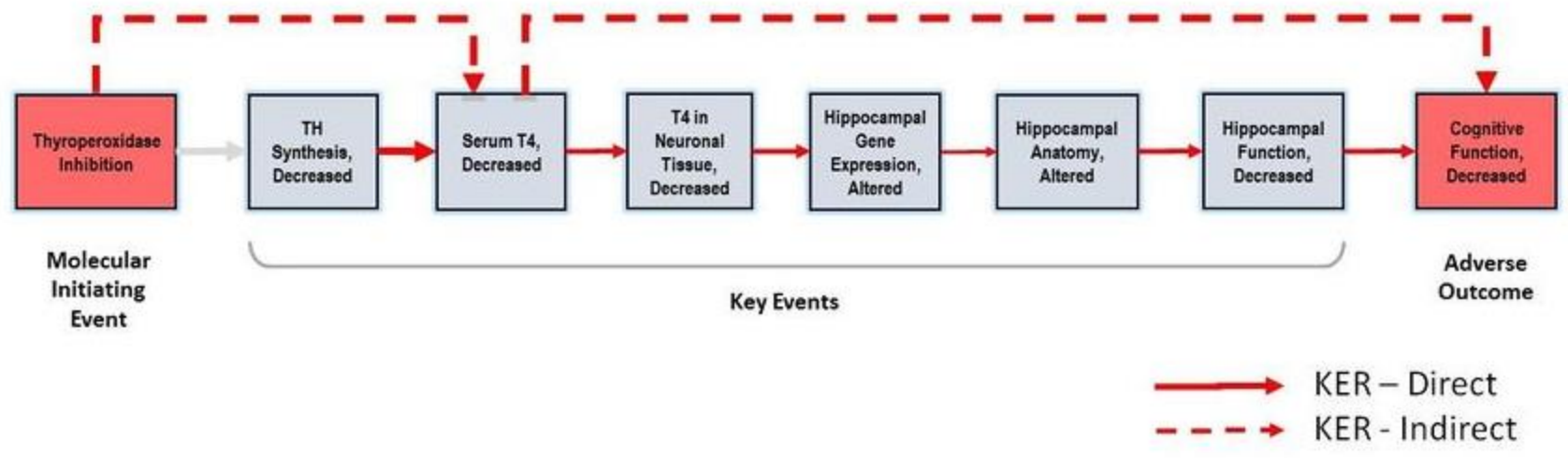
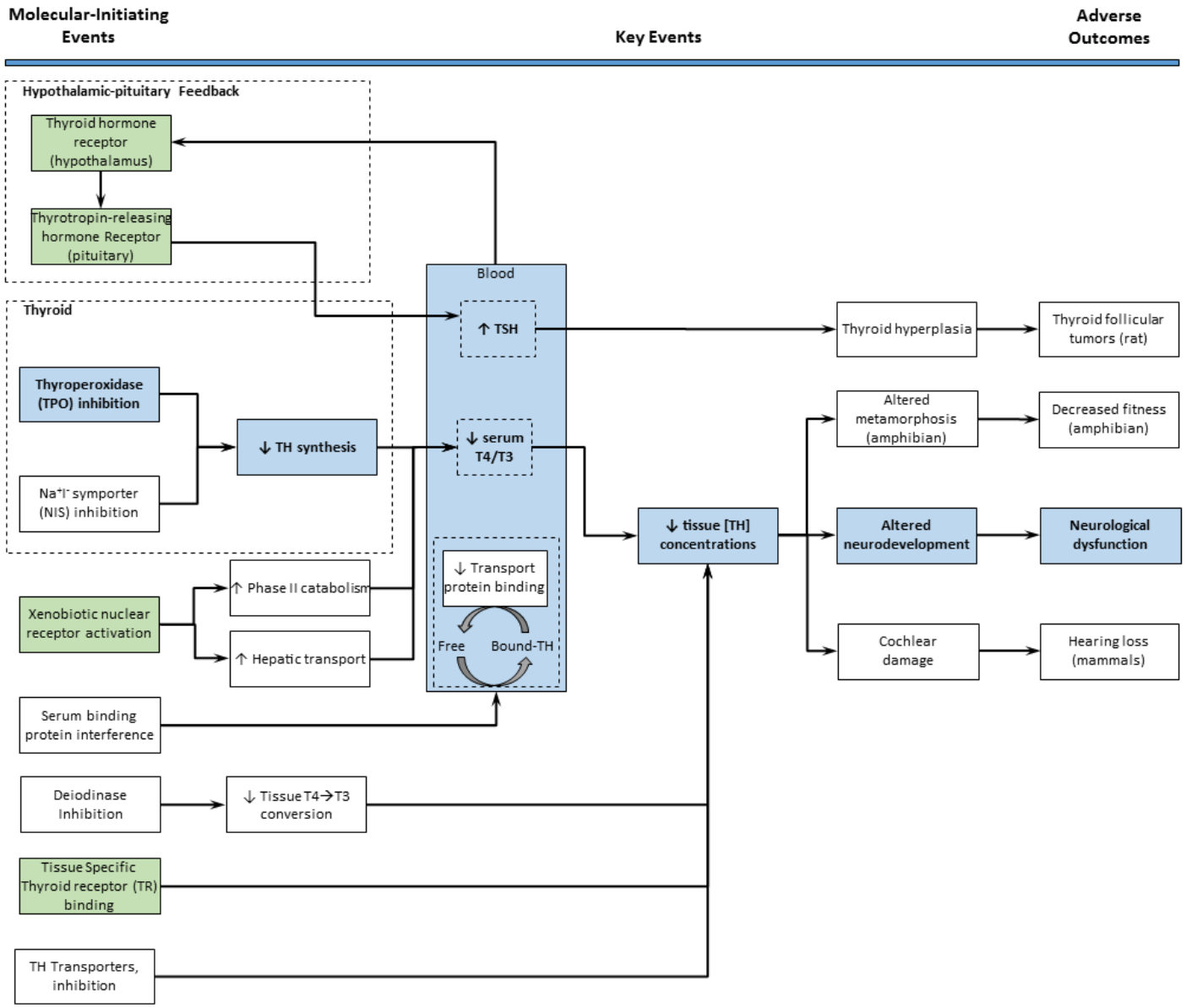
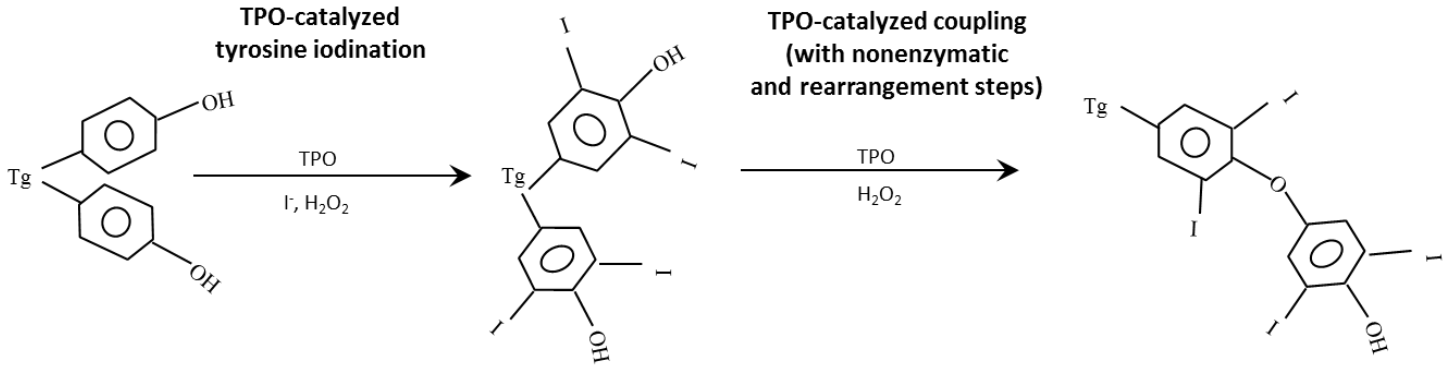




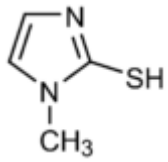
Top-down approach to assay development based on an AOP network for thyroid perturbation.

Figure 1, Paul Friedman, Watt *et al.* 2016, Toxicol Sci.

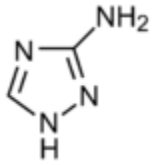
Thyroperoxidase (TPO) mediates TH synthesis in the thyroid gland.



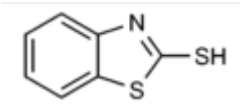
TPO is a known target for drug and chemical agents. Results from TPO inhibition assays reported for ~100 chemicals.



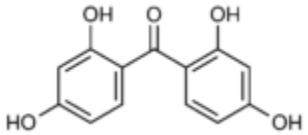
Methimazole:
anti-hyperthyroidism drug



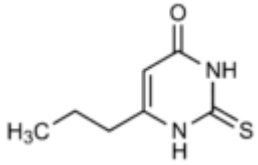
Amitrole:
herbicide



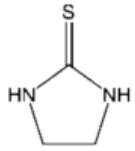
2-mercaptobenzothiazole:
vulcanizing agent, sensitizer



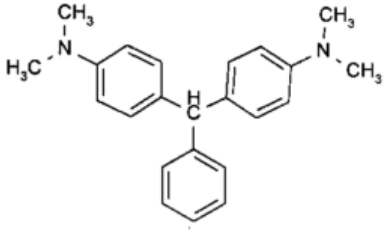
Benzophenone-2:
UV blocker



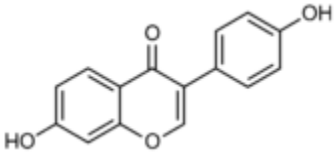
6-propyl-2-thiouracil:
anti-hyperthyroidism drug



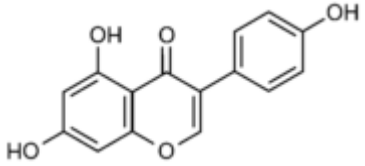
Ethylene thiourea:
EBDC pesticide metabolite



Leucomalachite green:
aquaculture pesticide



Daidzein:
dietary isoflavone

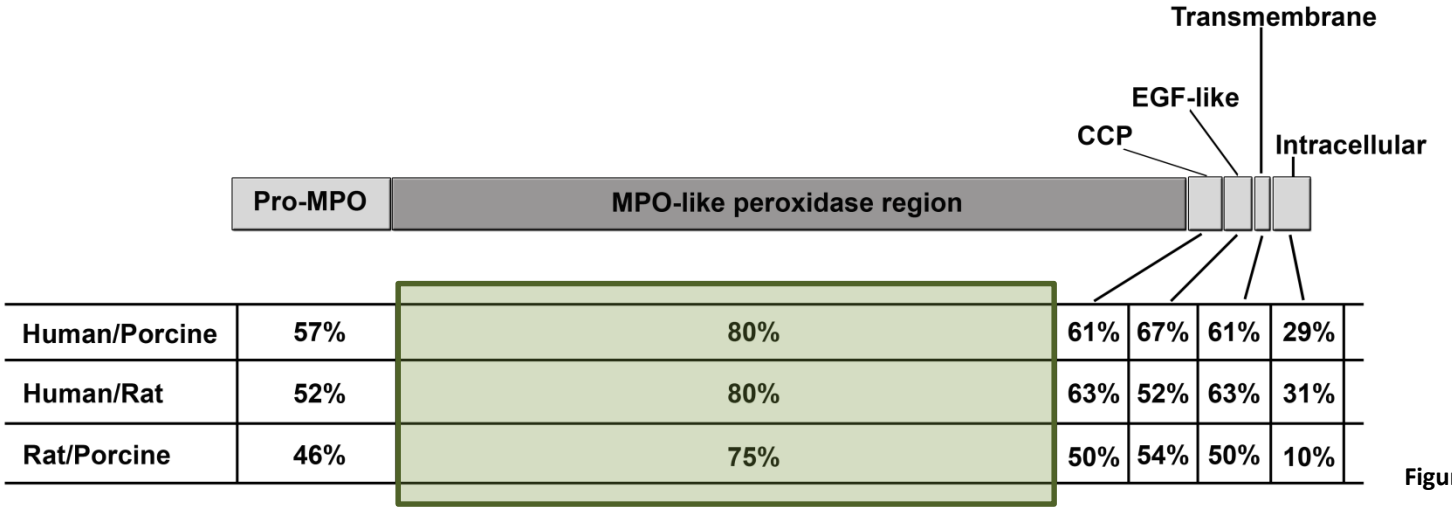


Genistein:
dietary isoflavone

Portions of Table 1, Paul et al., 2014; Figure 1, Doerge et al., 1998

TPO is a relevant cross-species target

Example: Efficacy of anti-hyperthyroidism medication in humans, veterinary applications, and research models.

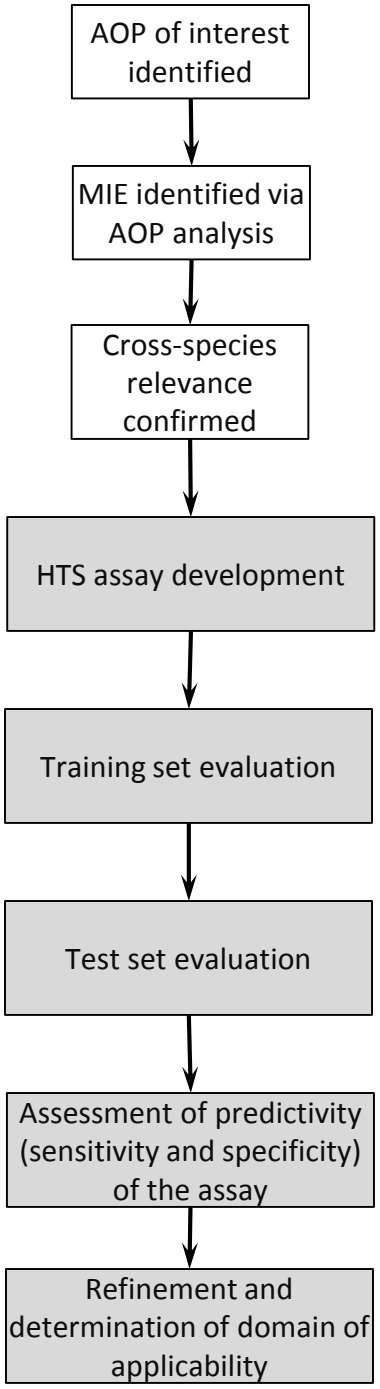


Human/Porcine	57%	80%	61%	67%	61%	29%
Human/Rat	52%	80%	63%	52%	63%	31%
Rat/Porcine	46%	75%	50%	54%	50%	10%

Figure 3, Paul et al., 2013.

Contributions to any divergent responses across species may include :

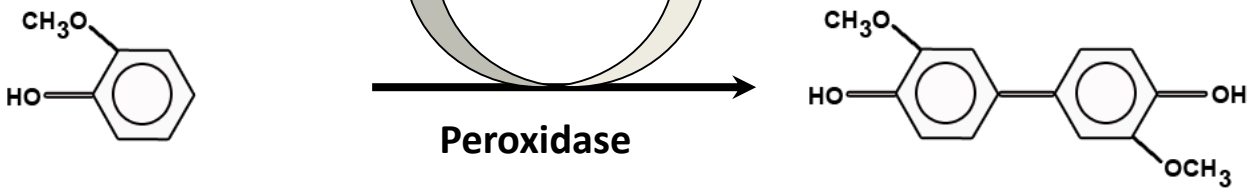
- *Key differences in the kinetics of thyroid hormone homeostasis*
- *Differences in xenobiotic absorption, metabolism, distribution, and excretion*
- *Differences in relative potency at TPO that appear to alter "hit-calls"*
- *Effect of incomplete conservation of catalytic domain*



What assay technology could be employed to screen 100's to 1000's of chemicals for TPO inhibition?

The guaiacol oxidation assay for detecting TPO inhibitors was not practical for HTS.

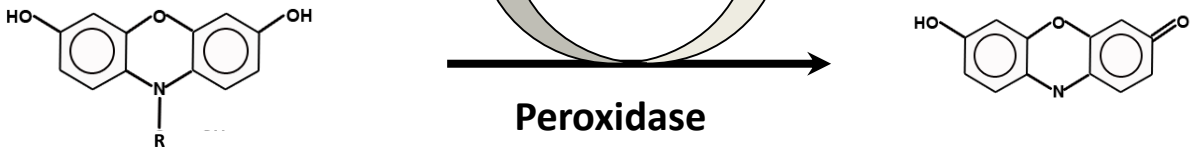
Guaiacol (GUA) **H_2O_2** **O_2** **Diguaiacol**



Kinetic (0-60 sec)
Absorbance (450-470 nm)

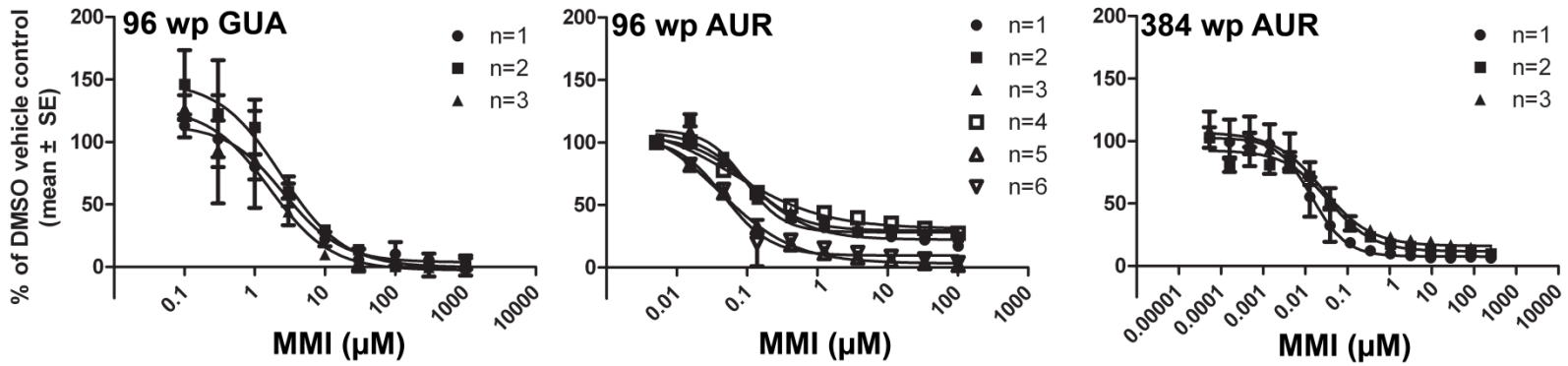
AUR assay technology capitalizes on conservation of peroxidase catalytic domains.

Amplex ® UltraRed (AUR) **H_2O_2** **O_2** **Amplex UltroxRed (Resorufin-like structure)**



Endpoint (30 min – 2 hr)
Fluorescence (544/590)

A step-wise approach to HTS assay development.



Assay Format	96-GUA	96-AUR	384-AUR
Dynamic range (mean ± SE)	127 ± 80	18.2 ± 10	11.5 ± 2.4
Z' (mean ± SE)	0.62 ± 0.16	0.86 ± 0.025	0.93 ± 0.021
Average CV (%)	92.0	6.24	16.1
IC_{50}	2.20	0.093	0.025
N	3	6	3
μg protein/reaction	50	12.5	5

Figure 1, Paul et al., 2014

A 21-chemical training set was employed to evaluate the utility of the assay.

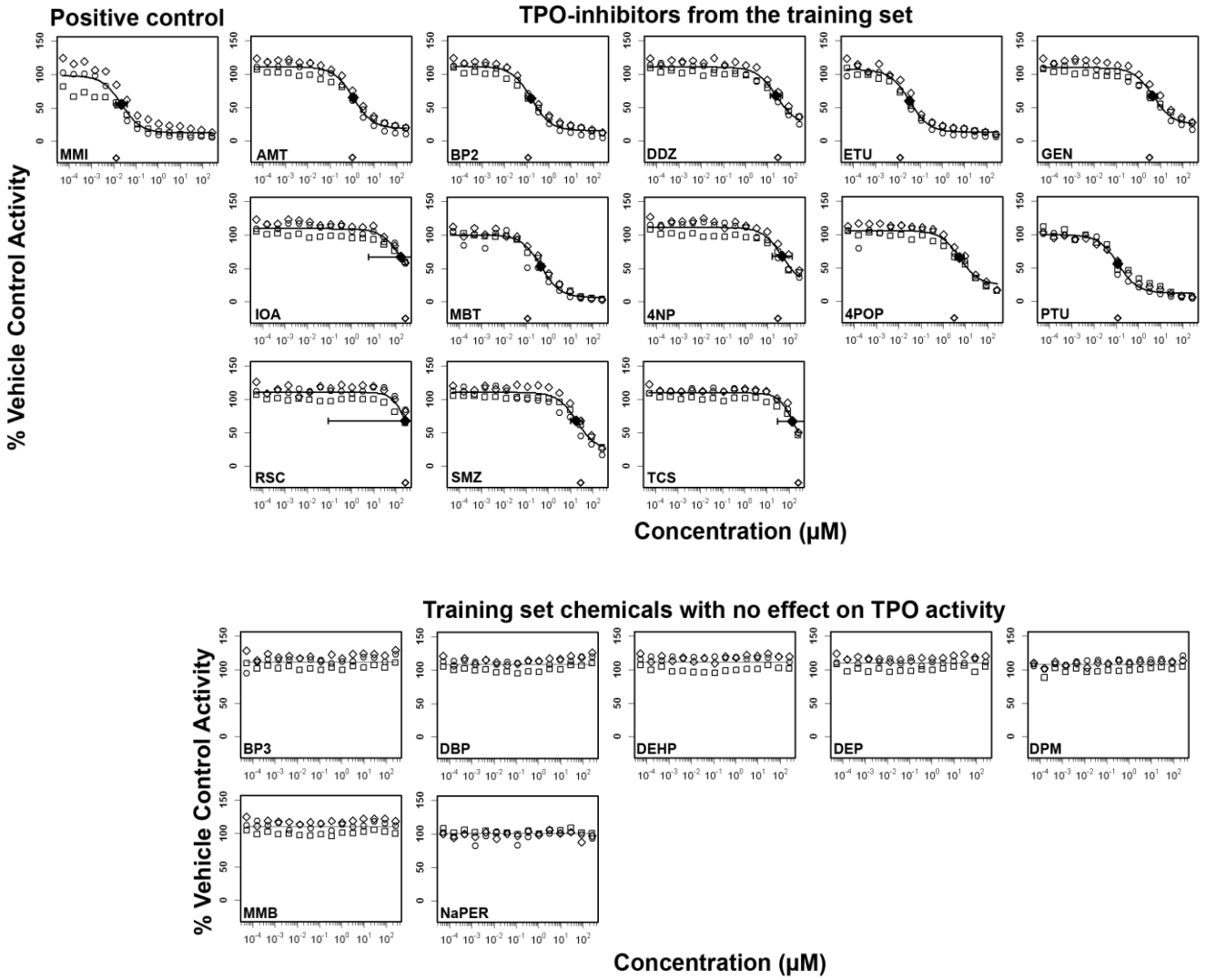
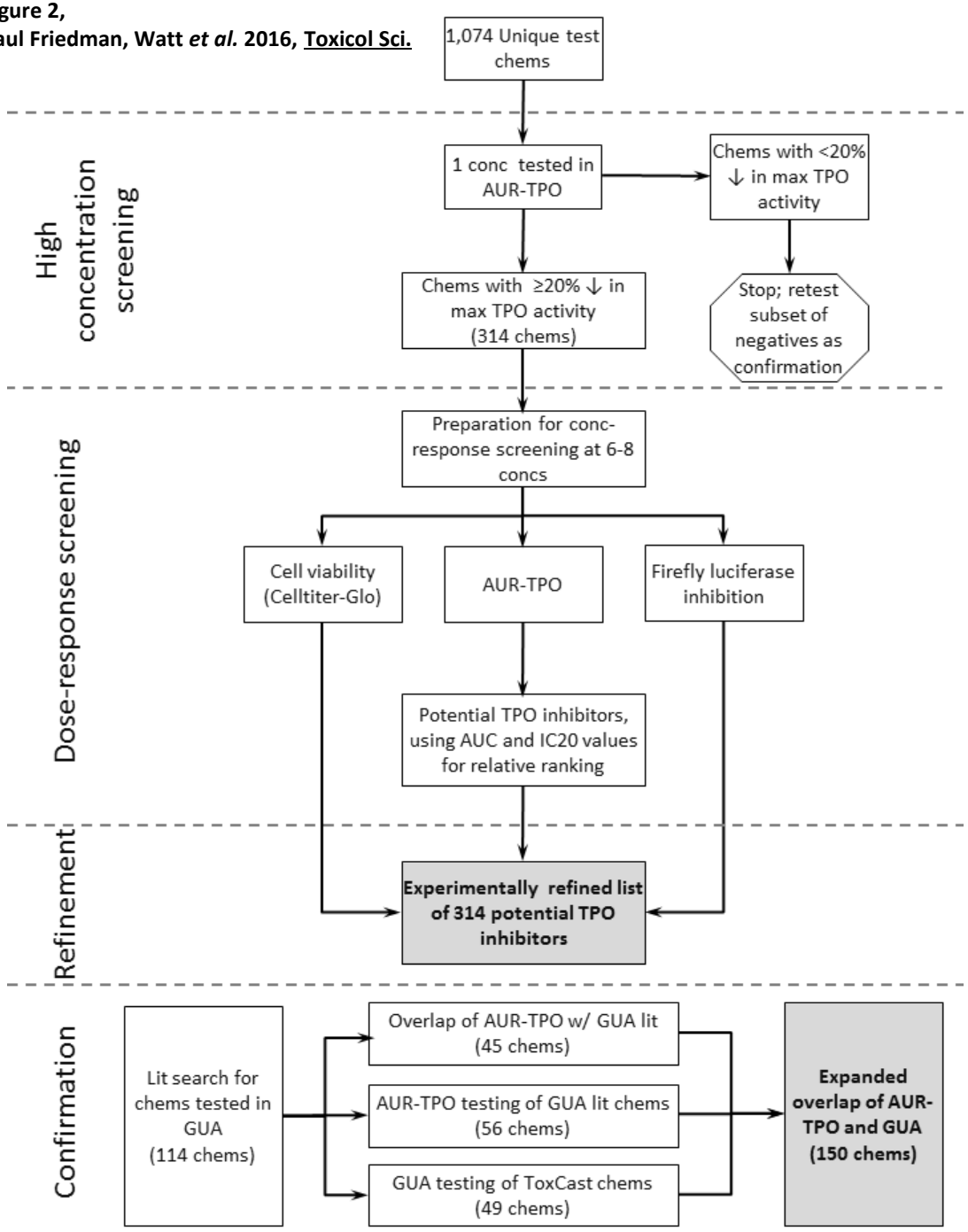


Figure 2, Paul et al., 2014

Figure 2,
Paul Friedman, Watt *et al.* 2016, Toxicol Sci.



Tiered screening with a test set

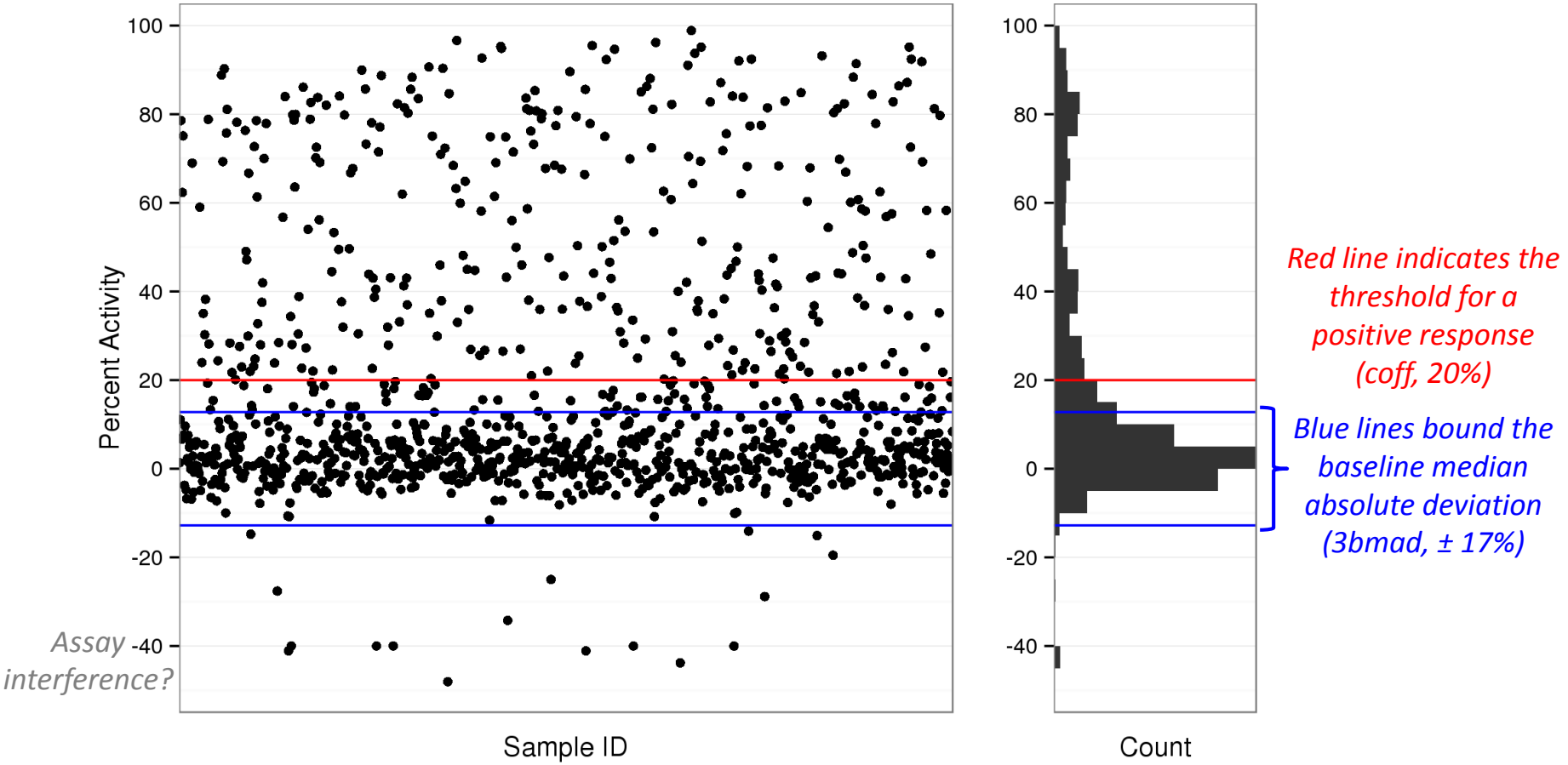
Single concentration screening

Multi-concentration screening in the AUR-TPO , CellTiter-Glo, and Luciferase Inhibition assays

Determine "selectivity" of putative TPO inhibitors

Confirmation suggests potential replacement of the guaiacol assay and perhaps a threshold for positives

Single concentration screening supported the IC_{20} cut-off and demonstrated a distribution of inhibition activities from 20-100%.



6-8 concentration-response curves were then assayed for chemicals with ≥ 20% inhibition in single concentration screening.

Figure 3,
Paul Friedman, Watt, *et al.* 2016, Toxicol Sci.

Ranking by relative potency alone gives a partial view, but does not incorporate efficacy or measures of hit-call relevance.

Chemical Name	CASRN	IC_{20}[1] (µM)	% E_{max}[2]	AUC
4-Hexylresorcinol	136-77-6	0.000052	96.9	257
Resorcinol	108-46-3	0.006	81.8	226
Methimazole	60-56-0	0.013	84.7	224
4,4'-Methylenedianiline	101-77-9	0.018	86.3	219
4-Pentylaniline	33228-44-3	0.020	83.4	218
6-Propyl-2-thiouracil	51-52-5	0.036	95.7	216
4-Methylaniline	106-49-0	0.037	73.2	201
Salicylhydroxamic acid	89-73-6	0.039	94.2	212
6-Methyl-2-thiouracil	56-04-2	0.042	90.1	195
2,2',4,4'-Tetrahydroxybenzophenone	131-55-5	0.042	87.3	209
2-Mercaptobenzothiazole	149-30-4	0.043	97.0	217
Quercetin	117-39-5	0.057	98.9	252
Sodium azide	26628-22-8	0.071	41.8	45
3-Methylaniline	108-44-1	0.079	81.9	197
4-Chloroaniline	106-47-8	0.087	78.4	187
Tannic acid	1401-55-4	0.088	87.3	140
6-Thioguanine	154-42-7	0.096	92.9	212
Catechol	120-80-9	0.100	81.5	164
Acibenzolar-S-methyl	135158-54-2	0.104	87.5	218
CI-1029	207736-05-8	0.108	95.0	196
2-Naphthylamine	91-59-8	0.115	74.1	180
4,4'-Oxydianiline	101-80-4	0.117	86.7	177
2-Anisidine	90-04-0	0.122	74.5	173
Isoproterenol hydrochloride	51-30-9	0.154	70.4	153
4,4'-Methylenebis(2-methylaniline)	838-88-0	0.171	77.0	161

[1] IC_{20} represents the concentration at which maximal activity was inhibited by 20% in the AUR-TPO assay.

[2] % E_{max} refers to the percent maximum inhibition observed in concentration-response.

314 putative TPO inhibitors were ranked by area under the curve (AUC).

This compresses potency and efficacy into a single value for comparison.

Assay is highly reproducible

- Comparison to previous training set
- rZ' scores 0.77-0.83
- rCV scores 3-4%
- Internally replicated samples

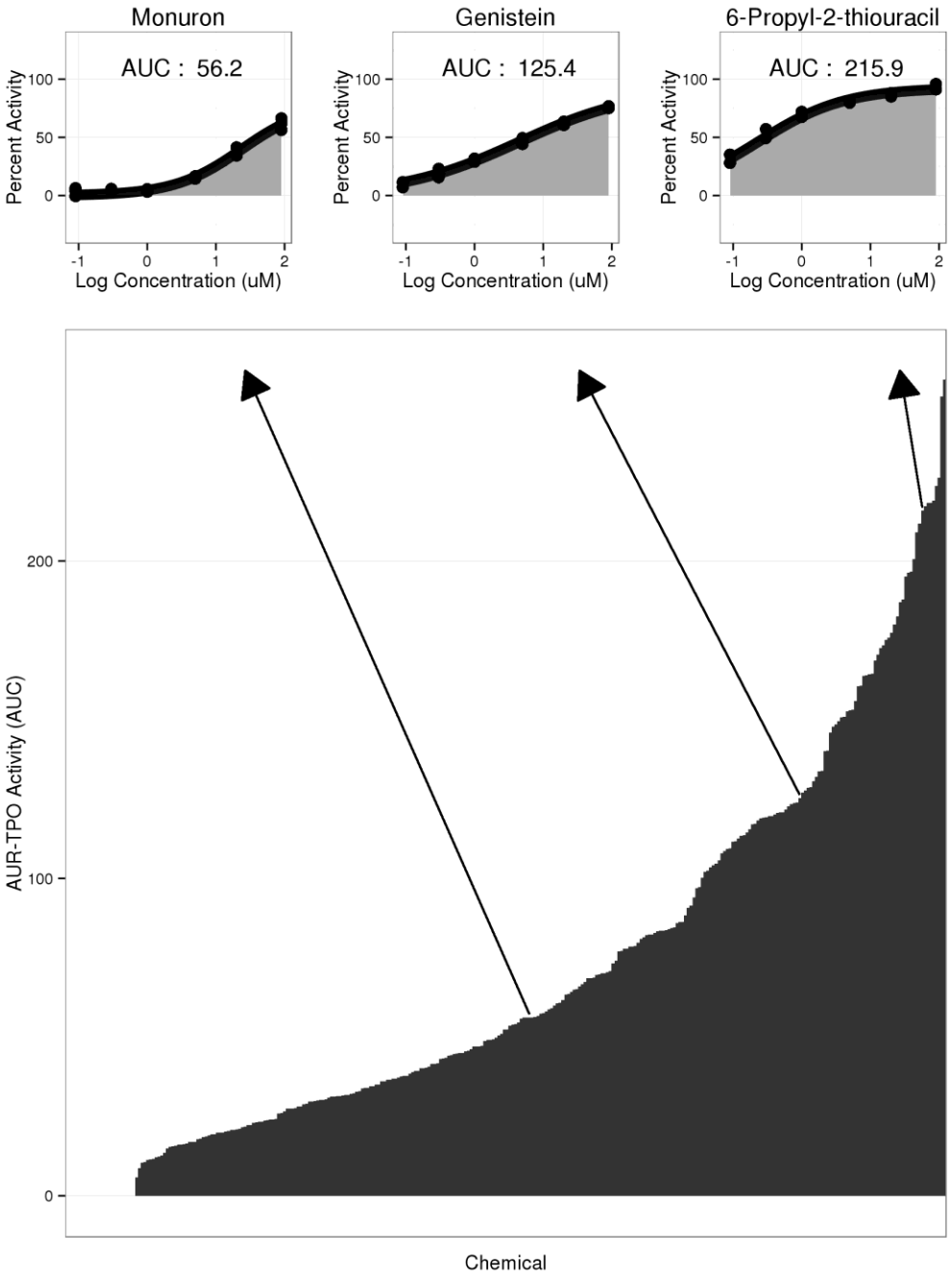
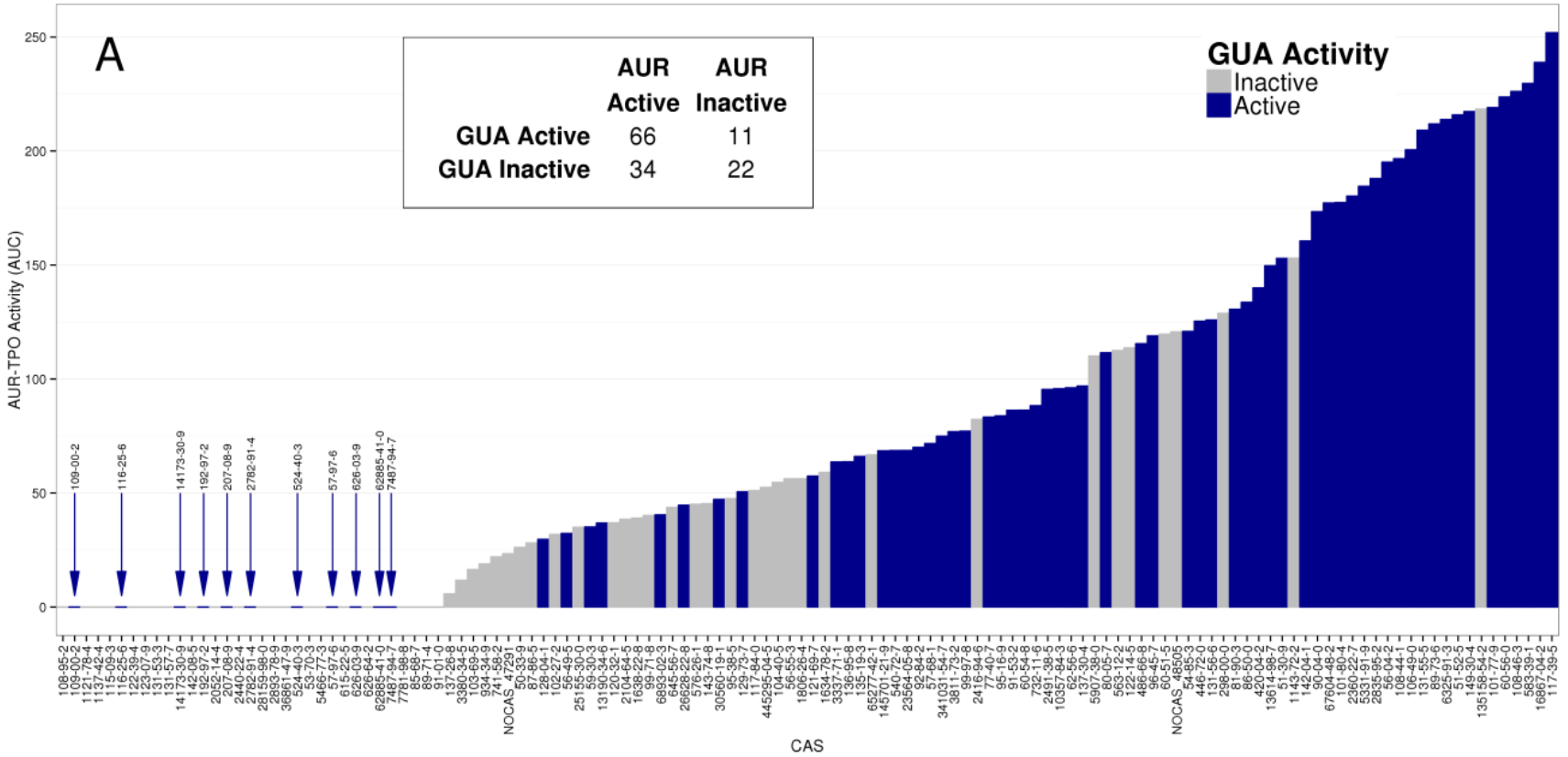


Figure 5,
Paul Friedman, Watt, *et al.* 2016, Toxicol Sci.

Considering hit-call relevance: multi-factor approach

- Comparison to the existing guaiacol oxidation assay for confirmation and determination of predictivity.
- Using selectivity, derived via comparison of log(IC_{20}) values for AUR-TPO and parallel cytotoxicity and luciferase inhibition assays, to stratify the list of putative TPO inhibitors.

150 GUA-tested chemicals ranked by AUC in the AUR-TPO assay.

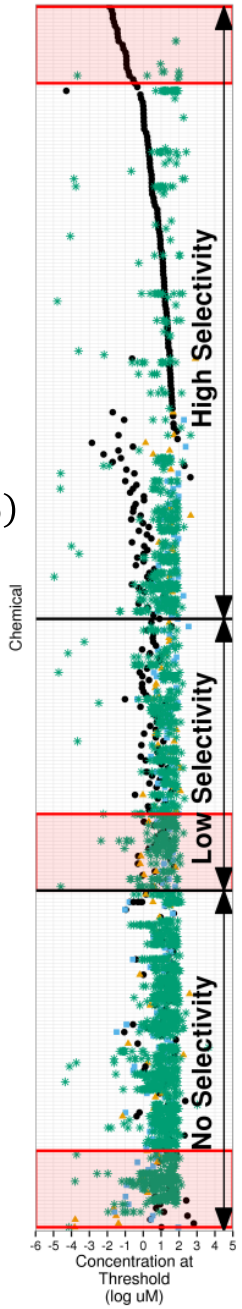


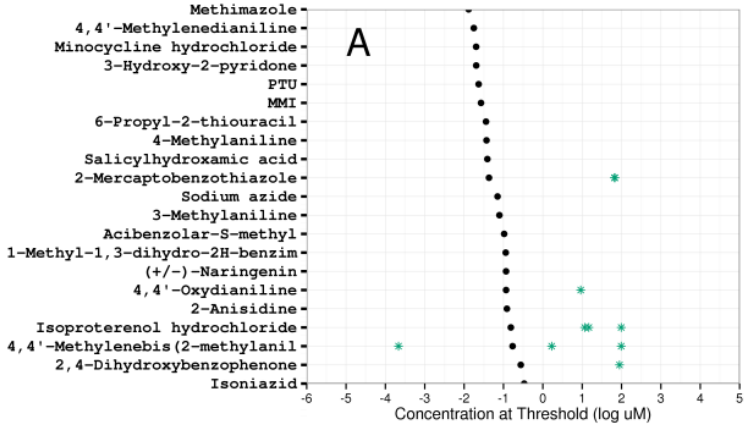
- Using GUA as a standard, AUR-TPO sensitivity = 88% and specificity = 39%.
- AUR-TPO demonstrates left-shifted potency.

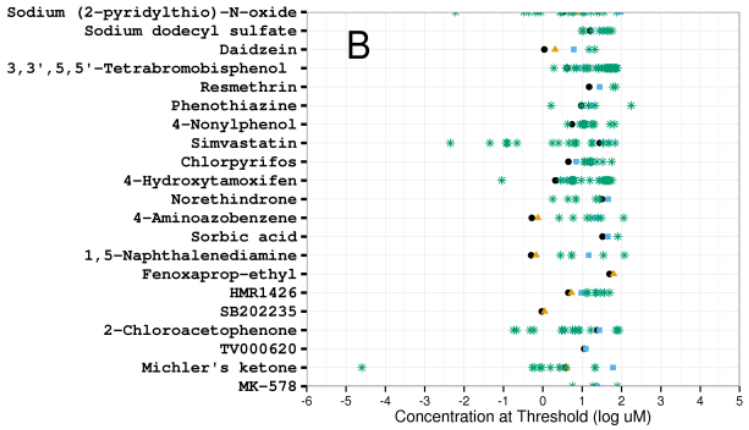
Figure 7,
Paul Friedman, Watt, *et al.* 2016, Toxicol Sci.

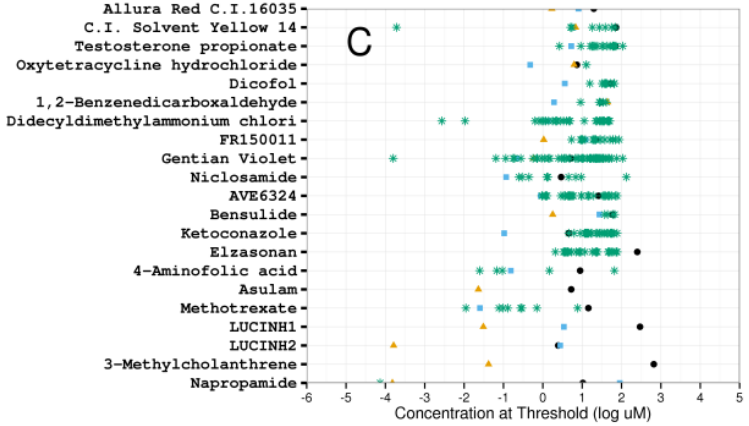
Ranking by selectivity improves interpretation and addresses the low specificity of the AUR-TPO assay on its own.

$$selectivity = min(CTG_{ACC}, QLI_{ACC}, cyto\_med) - min(AUR_{ACC}, 5)$$









Red boxes designate sections that were magnified for the insets.

Figure 6,
Paul Friedman, Watt, *et al.* 2016, Toxicol Sci.

The data have been processed using the tcpl v1.0 pipeline and are available for download and viewing.

Conclusions and future directions

- We have demonstrated a tiered approach to screening in addition to one approach to ranking putative TPO inhibitors by “selectivity.”
- In the future this could be combined with exposure prediction in order to further understand priority.
- PBPK modeling could be used to better understand the possibility of a chemical reaching a threshold of concern in the thyroid gland (see Leonard et al., 2016 Toxicol Sci).
- Future systems biology models of thyroid hormone homeostasis and its disruption could employ this assay system.