Screening the Tox21 10K library for thyroid stimulating hormone receptor agonist and antagonist activity

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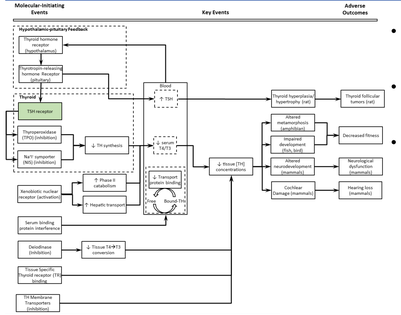
Abstract and Background

Thyroid-stimulating hormone (TSH) regulates thyroid hormone (TH) production via binding to its receptor (TSHR). The roles of TSHR in human pathologies including hyper/hypothyroidism, Grave's disease, and thyroid cancer are known, but it is currently unknown whether TSHR is an important screening target to identify chemicals that may perturb THs. The Tox21 collaboration enabled testing of the null hypothesis that TSHR is not targeted by environmentally-relevant chemicals. The TSHR is a G protein-coupled receptor that, when agonist-bound, activates adenylate cyclase with a resultant increase in cyclic adenosine 3',5' monophosphate (cAMP) that upregulates TH production. Commercially-available HEK293-TSHR cells were used in a 1536-well assay format to demonstrate agonism or antagonism of the TSHR, using cAMP as a marker of TSHR activation. A wildtype control was used to identify confounders, e.g. nonspecific modulators of cAMP. Homogeneous time-resolved fluorescence technology was used to quantify cAMP using a competitive immunoassay between native cAMP and dye-labeled cAMP. In agonist and antagonist mode optimization, TSH demonstrated signal-to-background ratios of 3.8 and 2.6, Z-factors of 0.75 and 0.53, and CVs of 3.6 and 5.7%, respectively, and an EC50 = 0.28 ng/mL. The Tox21 library (9667 chemicals) was tested in concentration-response in triplicate, and data were normalized by plate and plotted as %control, with the greater of a 20% change or 6*baseline median absolute deviation as the activity cutoff. Candidate agonists (493) and antagonists (340) were identified, with >70 chemical hits flagged as potentially nonspecific based on activity in wildtype cells, including cytotoxic organometallic compounds, autofluorescent or dye compounds, and cAMP upregulators active through other receptors or mechanisms. Potency (EC50/IC50) values ranged from 1e-5 to 150 µM for agonists and 1e-3 to 170 µM for antagonists. These chemicals have been prioritized for further screening as TSHR modulators using orthogonal assay technologies. *This abstract does not necessarily reflect the policy of the US EPA.*

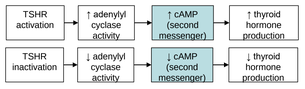
Question: Do environmentally-relevant chemicals interact with the TSHR?



- Multiple molecular-initiating events may be important for screening for thyroid hormone perturbation.
- Currently unknown if TSHR activity is modulated by environmentally-relevant chemicals.
- Though the Tox21 library contains many chemical use types, screening this library for TSHR activity represents the broadest effort to date to determine if environmentally-relevant chemicals might modulate its activity.

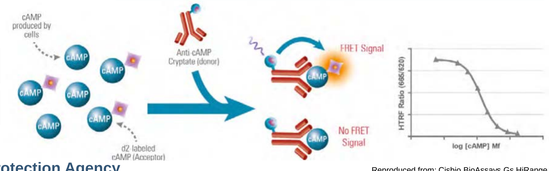
Assay principle

Putative mode-of-action



cAMP is the signal measured in this assay platform

Cisbio homogenous time-resolved fluorescence (HTRF)



Reproduced from: Cisbio BioAssays Gs HiRange Kit
http://www.cisbio.com/sites/default/files/ressources/cisbio_dd_pi_62AM6PEB-62AM6PEC-62AM6PEJ.pdf

Data Analysis: Positives, preliminary ranking of activity, and potential confounders to enable screening deprioritization

- The ToxCast data analysis pipeline (tcpl) was used for analysis of these assay data.
- Area under the curve (AUC) (toxboot, R package v0.1.1) was used to compress efficacy and potency into a single value for preliminary ranking.
- Using data-based hypotheses on potential assay confounders, chemicals will be prioritized for orthogonal screening.

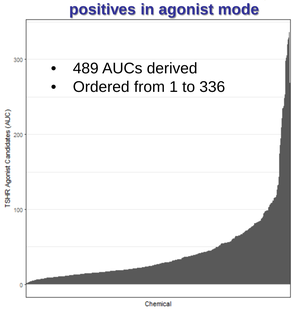
Assay metrics

Assay endpoint name	# chem samples tested	# chemical samples positive	# unique chemicals positive	Hit rate	Robust Z' factor, mean ± SD (calculated by plate)
TOX21_TSHR_Agonist_ratio	9668	588	493	6%	0.701 ±0.122
TOX21_TSHR_Antagonist_ratio	9667	392	340	4%	0.549 ±0.092
TOX21_TSHR_wt_ratio	9668	76	64	0.8%	0.836 ±0.065

Ranking by AUC and example positive responses

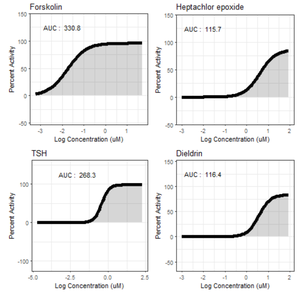
TOX21_TSHR_Agonist_ratio

Ranking of area under the curve (AUC): positives in agonist mode



- 489 AUCs derived
- Ordered from 1 to 336

Example positives in agonist mode



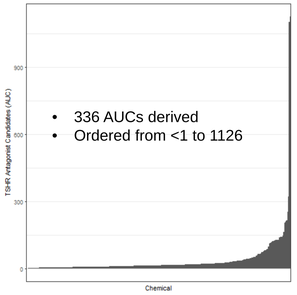
Top 20 'Agonists' by AUC

	CASRN	Chemical Name	AUC (unitless)
1	54239-37-1	Cimaterol	336.4482
2	51-30-9	Isoproterenol hydrochloride	328.8974
3	66575-29-9	Forskolin	326.7781
4	598-55-0	Methyl carbamate	319.8661
5	94749-08-3	Salmeterol xinafoate	318.3742
6	37148-27-9	Clenbuterol	305.6025
7	75-05-8	Acetonitrile	302.2813
8	7683-59-2	Isoproterenol	297.7042
9	NA	TSH	268.3225
10	54240-36-7	Mabuterol hydrochloride	253.1438
11	72332-33-3	Procaterol	248.5352
12	9066-59-5	Lysozyme hydrochloride	237.6576
13	78416-81-6	Trequinsin hydrochloride	235.4024
14	60-92-4	cAMP	234.5791
15	30392-40-6	Bitolterol	231.2025
16	18559-94-9	Albuterol	221.5552
17	363-24-6	Dinoprostone	208.8234
18	134865-33-1	Meludrine	194.8564
19	387867-13-2	Tandutinib	185.2481
20	51-42-3	Epinephrine bitartrate	174.1244

B-adrenergic agonists are indicated by purple shaded rows.

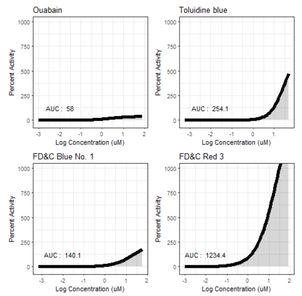
TOX21_TSHR_Antagonist_ratio

Ranking of area under the curve (AUC): positives in antagonist mode



- 336 AUCs derived
- Ordered from <1 to 1126

Example positives in antagonist mode



Top 20 'Antagonists' by AUC

	CASRN	Chemical Name	AUC (unitless)
1	15905-32-5	Erythrosin B	1126.431
2	16423-68-0	FD&C Red 3	1101.648
3	35189-28-7	Norgestimate	320.2482
4	92-31-9	Toluidine blue	254.126
5	2353-45-9	FD&C Green No. 3	214.1366
6	7220-79-3	Methylene Blue trihydrate	213.4273
7	61-73-4	Methylene blue	205.6179
8	11024-24-1	Digitonin	204.2618
9	76-87-9	Triphenyltin hydroxide	164.3488
10	115-39-9	Bromophenol blue	146.9767
11	605-91-4	Carbocyanine	141.2232
12	379-52-2	Triphenyltin fluoride	140.4712
13	3844-45-9	FD&C Blue No. 1	140.1462
14	20562-02-1	alpha-Solanine	140.0264
15	637-03-6	Phenylarsine oxide	137.4048
16	900-95-8	Triphenyltin acetate	129.1063
17	115-09-3	Methylmercuric(II) chloride	128.6814
18	15086-94-9	Tetrabromofluorescein	127.7025
19	1461-22-9	Tributyltin chloride	126.9051
20	596-03-2	4',5'-Dibromofluorescein	125.3338

Dyes are indicated by purple shaded rows.

Positive responses: confounder or putative TSHR modulator?

Hits in all three assay modes

- TOX21_TSHR_wt_ratio (wildtype) lacked the TSHR.
- 10 chemicals generated positive responses (hitc = 1) in the wildtype (wt), agonist, and antagonist modes of this assay.
- Many of these chemicals appear to be cytotoxic organometallic compounds.
- Many organometallic compounds appeared positive in TOX21_TSHR_Antagonist_ratio.

Chemicals with positive hitcalls in all three assay modes

	wt	agonist	antagonist	CASRN	Chemical name
1	1	1	1	62-38-4	Phenylmercuric acetate
2	1	1	1	3520-42-1	C.I. Acid Red 52
3	1	1	1	1461-22-9	Tributyltin chloride
4	1	1	1	7487-94-7	Mercuric chloride
5	1	1	1	2155-70-6	Tributyltin methacrylate
6	1	1	1	56-72-4	Coumaphos
7	1	1	1	100-56-1	Phenylmercuric chloride
8	1	1	1	13331-52-7	(Acryloyloxy)(tributyl)stannane
9	1	1	1	122-64-5	Phenylmercuric lactate
10	1	1	1	20018-09-1	Diiodomethyl 4-methylphenyl sulfone

Autofluorescent or dye compounds

- Autofluorescent and dye compounds appeared to generate some of the higher AUC values in TOX21_TSHR_Antagonist_ratio.
- 15 unique chemicals were positive in at least one TOX21_AutoFluor_ assay endpoint and TOX21_TSHR_Antagonist_ratio.
- Additionally, dyes (particularly blue) appeared prominently on the putative antagonist list.

Chemicals with positive hitcalls in ≥ 1 TOX21_AutoFluor_ assay endpoint and TOX21_TSHR_Antagonist_ratio

	Antagonist AUC (unitless)	CASRN	Chemical name
1	1126.431	15905-32-5	Erythrosin B
2	1101.648	16423-68-0	FD&C Red 3
3	127.7025	15086-94-9	2',4',5',7'-Tetrabromofluorescein
4	68.08341	17372-87-1	Eosin
5	36.38343	6317-18-6	Methylene bis(thiocyanate)
6	35.83095	952-23-8	Proflavin hydrochloride
7	29.16467	3520-42-1	C.I. Acid Red 52
8	20.94445	1811-28-5	Proflavin hemisulfate
9	16.81901	786-19-6	Carbophenothion
10	16.36241	69235-50-3	Acriflavine hydrochloride
11	15.30084	517-28-2	Hematoxylin
12	15.25632	905-97-5	3,3'-Diethylthiacarbocyanine iodide
13	8.188388	1641-17-4	Mexenone
14	8.17692	481-49-2	Cepharanthine
15	2.737559	52417-22-8	9-Aminoacridine, monohydrochloride, monohydrate

cAMP upregulators

- The TOX21_TSHR assays detect cAMP and non-TSHR-mediated effects include:
  - Agonists of other adenylyl cyclase-linked GPCRs expressed by HEK293 cells (e.g. β-adrenergic receptors, prostaglandin receptors); acting directly or indirectly
  - Other activators of adenylyl cyclase
- Distinguishing true TSHR agonist activity may require orthogonal screening.

Do environmentally-relevant chemicals modulate TSHR activity? More investigation needed.

- Organochlorine pesticides appeared to have activity in both the agonist and antagonist modes, with greater potency in agonist mode. Inverse agonism of TSHR has been reported previously for DDT and Aroclor 1254 (Rossi et al. 2007), but this activity may be downstream of TSHR as these compounds also inhibited forskolin-stimulated cAMP induction.
- Several dinitroaniline herbicides were positive in agonist mode.

Preliminary conclusions and ongoing work

- Additional filtering of confounders to help resolve chemicals that may modulate TSHR specifically.
- Additional analysis of chemical features, and comparison to TSHR-targeted small molecule drugs (Gershengorn and Neumann, 2012), may provide useful information for further screening prioritization of putative TSHR activity.
- Orthogonal screening, e.g receptor-binding or screening in a TSHR-responsive cell line for regulation of TSHR-responsive genes, may confirm TSHR activity, thereby addressing whether TSHR-screening identified environmentally-relevant thyroid-disrupting chemicals.

Eric D. Watt (2016). toxboot: Bootstrap Methods for 'ToxCast' High Throughput Screening Data. R package version 0.1.1. https://CRAN.R-project.org/package=toxboot

Gershengorn, M. C., and Neumann, S. (2012). Update in TSH receptor agonists and antagonists. *J Clin Endocrinol Metab* **97**(12), 4287-92.

Rossi, M., Dimida, A., Delfanno, M. T., Tincavelli, M. L., Agretti, P., Giorgi, F., Corsini, G. U., Pinchera, A., Vitti, P., Tonacchera, M., *et al.* (2007). The thyroid disruptor 1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane appears to be an uncompetitive inverse agonist for the thyrotropin receptor. *J Pharmacol Exp Ther* **320**(1), 465-74.

Titus, S., Neumann, S., Zheng, W., Southall, N., Michael, S., Klumpp, C., Yasgar, A., Shinn, P., Thomas, C. J., Inglese, J., *et al.* (2008). Quantitative high-throughput screening using a live-cell cAMP assay identifies small-molecule agonists of the TSH receptor. *J Biomol Screen* **13**(2), 120-7.