Screening the 10K Tox21 Chemical Library for Thyroid Hormone Receptor Modulators

Keith Houck[1], Audrey Bone[1], Katie Paul Friedman[1], Chia-Wen Hsu[2], Srilatha Sakamuru[2], Menghang Xia[2], Ruili Huang[2], Vikas Soni[3], Diana Stavreva[3], Kevin Crofton[1]

[1] NCCT/ORD, U.S. EPA, RTP, NC; [2] NCATS/NIH, Rockville, MD; [3] NCI/NIH, Bethesda, MD

Contact: houck.keith@epa.gov

Orchid iD: 0000-0002-0055-2249

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Background

Disruption of the hypothalamic–pituitary–thyroid axis by environmental chemicals is of high concern for developmental and other toxicities. Key components of this axis are the thyroid hormone receptors, TRα and TRβ. Several environmental chemicals, in particular PCBs and hydroxylated PDBEs, have been reported as modulators of TR. However, relatively few other chemical structures beyond pharmaceutical derivatives of the endogenous hormones, T3 and T4, are known to directly interact with TR. Here we screen the Tox21 chemical library containing diverse environmental and industrial chemicals and drugs for activity against TR to determine the frequency of interaction with chemicals having potential human and environmental exposure.

Objectives

- Screen the Tox21 chemical library for activity against the thyroid hormone receptors (TR)
- Evaluate the pharmacology of the active compounds identified as candidate ligands

Outline of Methods

Primary qHTS

- TR transactivation assay (TRTA)
  - Rat pituitary cell line (GH3.TRE-Luc)
  - Firefly luciferase reporter gene assay
  - Endogenous TRα/β
  - Agonist and antagonist mode
  - Cell viability run in parallel
- Chemical library
  - Tox21 library (10,496 samples/8,306 unique structures)
  - 15 concentrations each/triplicate

Confirmation Assays

- Agonist
  - GH3.TRE-Luc assay
  - hTRβ mammalian one-hybrid assay (HEK293)
  - RXR mammalian one-hybrid assay (HEK293)
  - Coactivator recruitment assay
- Antagonist
  - GH3.TRE-Luc + T3 assay
  - hTRβ mammalian one hybrid assay (HEK293)
  - Increased agonist competition in GH3.TRE-Luc + T3
  - Coactivator recruitment assay
  - Nuclear translocation assay

qHTS Results

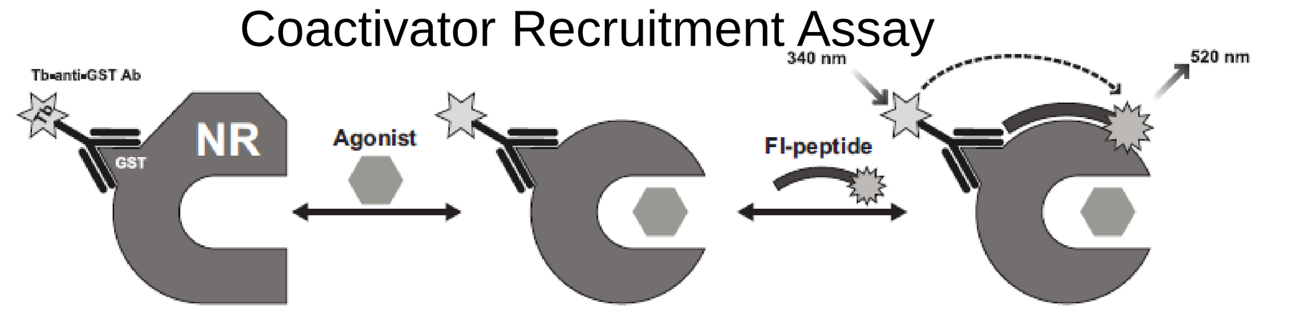
qHTS and Confirmation Assays Summary

Category	Agonist	Antagonist	Comment
Actives	31	2357	Original screen actives after QC review of curve fits
# IC50cytotox/IC50TRTA > 10	31	747	# actives with cytotoxicity greater than 10-fold less potent than transactivation assay
Retested	73	491	Selection criteria: availability; cytotoxicty; assay promiscuity; near hit
# Confirmed	26	448	# actives in GH3 confirmation assay

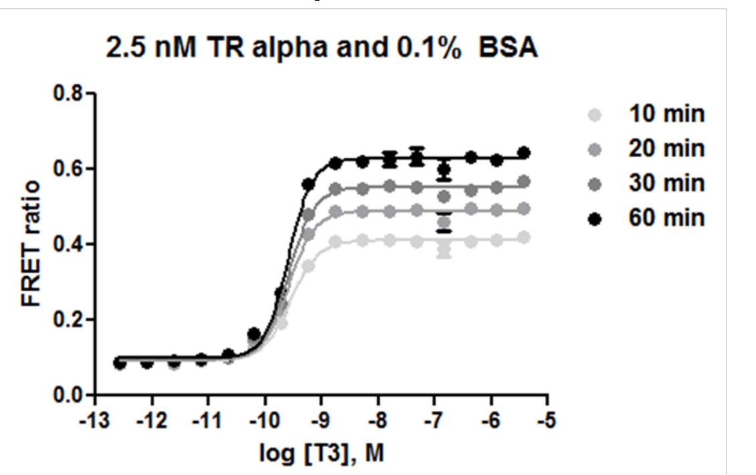
Primary qHTS data were normalized and fit to concentration-response curves using the EPA's tcpl R package (ToxCast Data Analysis Pipeline). Active hit calls with tcpl warning flags were manually examined and adjusted for obvious artifacts where appropriate. All active agonists as well as some marginally active compounds but negative hit calls were retested in agonist mode. A subset of active antagonists were selected using criteria in the table above for confirmation in antagonist mode.

Agonist Characterization

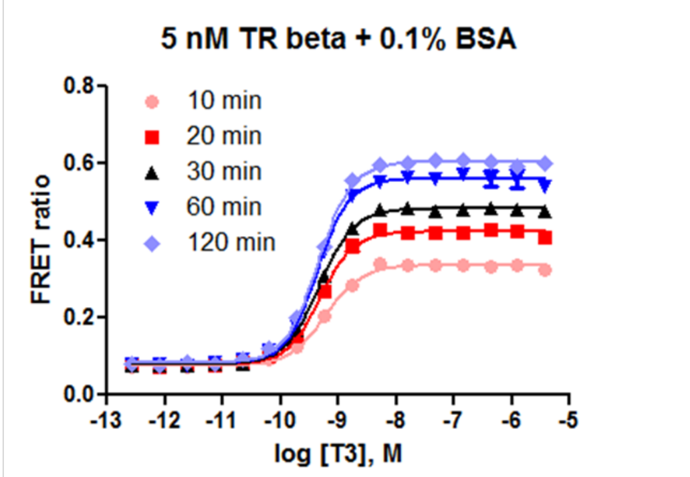
Coactivator Recruitment Assay



Confirmed agonists from the qHTS were characterized in a coactivator recruitment assay. Agonist binding to the ligand-biding domain stimulates conformational change favoring binding of coactivator to the receptor. The Invitrogen LanthaScreen assay uses either TRα or TRβ ligand-binding domains expressed as fusion proteins with GST. The receptor is then labeled with a Tb-anti-GST antibody and the coactivator with a fluorescent tag. Recruitment of coactivator to ligand-bound receptor results in a TR-FRET signal.



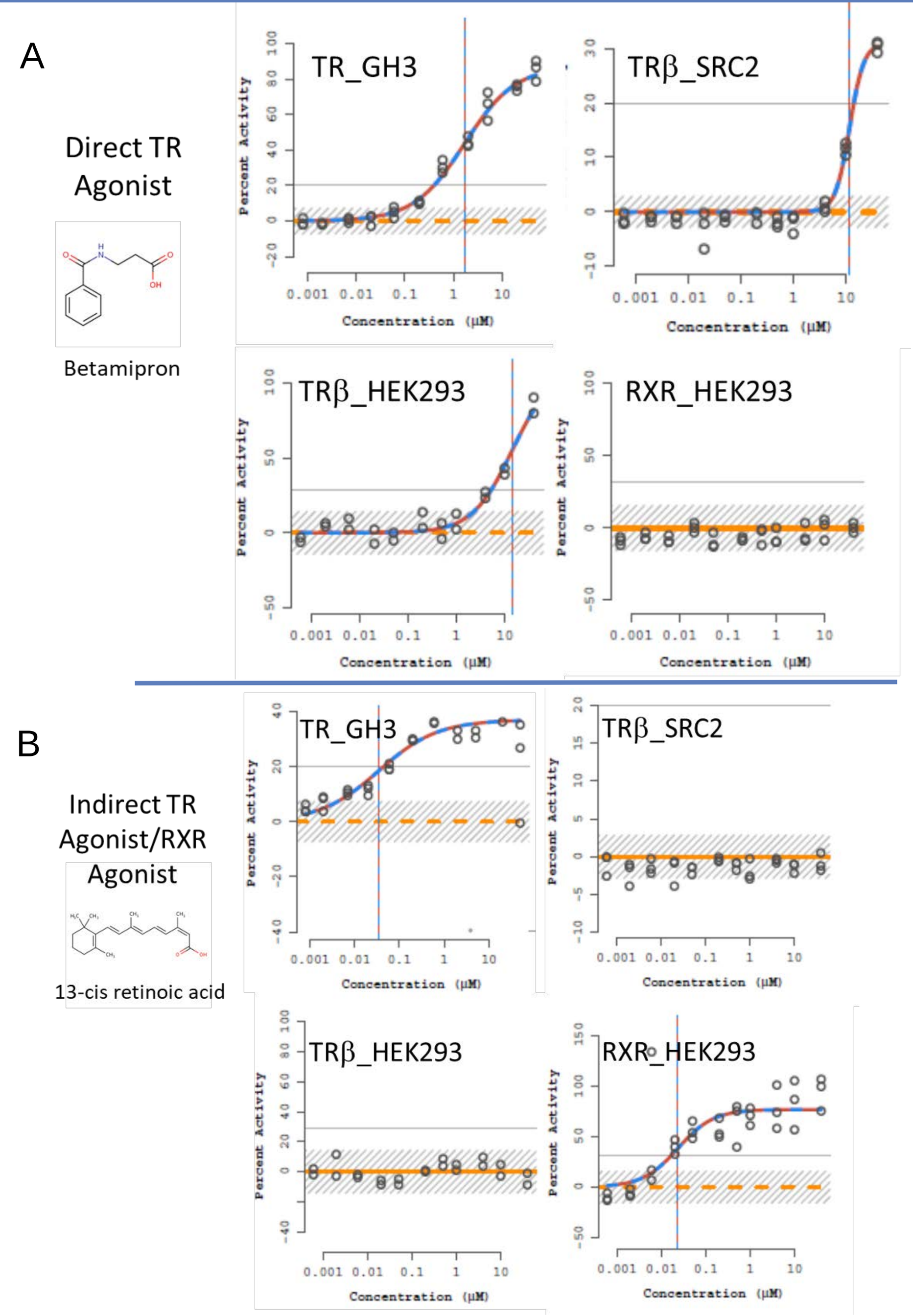
2.5 nM TRα-LBD	S/B	CV%	EC50 (M)
10 min	5.0	3.6	2.786E-10
20 min	5.9	3.3	2.605E-10
30 min	6.7	4.2	2.629E-10
60 min	7.0	4.6	2.571E-10



TRβ-LBD	S/B	CV%	EC50 (M)
10 min	4.4	3.9	5.911E-10
20 min	5.3	4.7	4.786E-10
30 min	6.1	4.6	4.661E-10
60 min	6.8	4.8	4.380E-10
120 min	7.4	5.7	4.507E-10

The assays were optimized for both human TRα and TRβ LBDs using the endogenous ligand T3. All agonist compounds confirmed in the GH3 assay were tested in concentration-response mode against both receptors.

Agonist Characterization



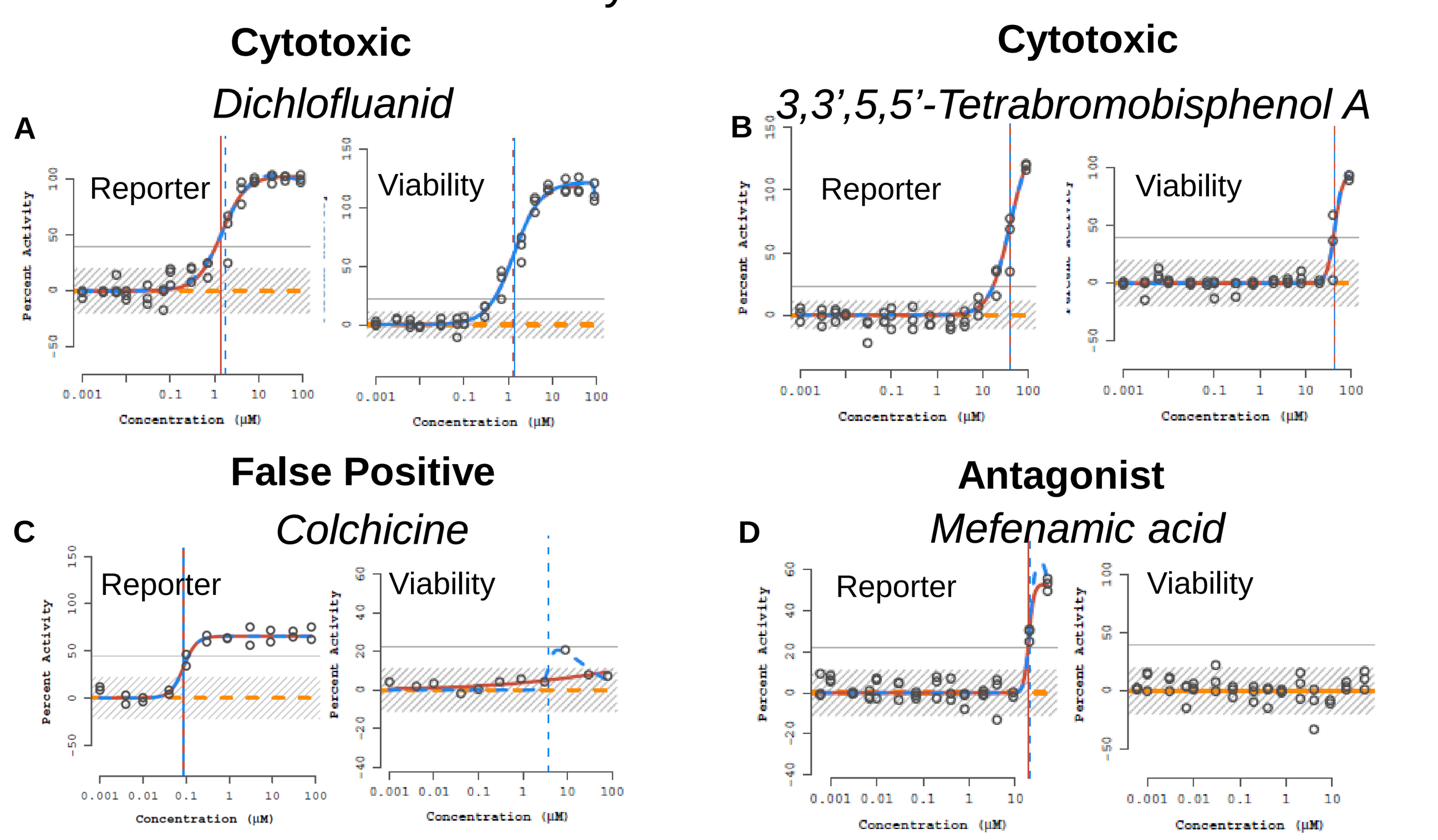
Confirmed agonist actives were tested in the coactivator recruitment assay, a TR one-hybrid assay in HEK293 cells and an RXRα one-hybrid assay in HEK293 cells. Results for two compounds illustrate the two classes of actives identified: A) direct TR agonists and B) indirect TR activators working through the TR heterodimer partner RXR.

Final Agonists Identified

Samples			AC50													Emax (% Control)						Category
			GH3				TR-Coactivator				TR one-hybrid		RXR		Viability	GH3		TR-Coactivator		TR one-hybrid	RXR	
Sample Name	CASRN	Sample QC	hitc	TR_LUC_GH3_Agonist (uM)	hitc_GH3_Agonist_Followup	TR_LUC_GH3-followup_agonist (uM)	hitc_TRa_Coa_Agonist	TRa_Coa_Agonist (uM)	hitc_TRb_Coa_Agonist	TRb_Coa_Agonist (uM)	hitc_TRb-bla	TRb-bla (uM)	hitc_RXR-bla	RXR-bla (uM)	TR_LUC_GH3_viability (uM)	EMAX_TR_LUC_GH3_Agonist (% Con)	EMAX_TR_LUC_GH3-Followup (% Con)	EMAX_TRa_Coa_Agonist	EMAX_TRb_Coa_Agonist	Emax_TRb-bla	Emax_RXR-bla	Categorization Code
CP-634384	290352-28-2	A	1	0.0015	1	0.018	1	0.003	1	0.002	1	0.087	1	44.299	>92	129.5	103.7	158.4	137.4	189.2	54.9	TR-Ag
3,5,3'-Triiodothyronine	6893-02-3	A/C	1	0.0011	1	0.0007	1	0.003	1	0.003	1	0.001	0	1000	41.687	121.6	107.7	170.0	144.9	104.1	6.8	TR-Ag
Levothyroxine	51-48-9	A	1	0.006	1	0.008	1	0.034	1	0.139	1	0.036	0	1.362	>92	117.2	100.3	170.8	138.9	115.9	21.2	TR-Ag
3,3',5,5'-Tetraiodothyroacetic acid	67-30-1	ND	1	0.0009	1	0.006	1	0.248	1	0.227	1	0.021	1	23.551	51.286	113.5	123.6	153.3	121.6	204.2	61.6	TR-Ag
3,3',5'-Triiodo-L-thyronine	5817-39-0	A	1	0.218	1	0.537	1	0.707	1	1.000	1	0.641	0	1000	>92	111.6	87.2	152.6	117.6	93.8	17.4	TR-Ag
Tiratricol	51-24-1	ND	1	0.00001	1	0.0002	1	0.006	1	0.005	1	0.001	1	7.872	>92	110.9	105.1	161.7	143.8	110.4	77.6	TR-Ag
3,3',5-Triiodo-l-thyronine sodium salt	55-06-1	A	1	0.0002	1	0.002	1	0.013	1	0.013	1	0.004	0	1000	>92	110.9	96.5	169.7	146.3	111.1	15.3	TR-Ag
Betamipron	3440-28-6	A	1	11.841	1	1.679	1	10.403	1	11.765	1	14.030	0	1000	>92	72.8	90.7	120.3	30.8	85.5	2.9	TR-Ag
trans-Retinoic acid	302-79-4	B/C	1	0.234	1	1.159	0	1000	0	1000	1	1.480	1	0.788	66.737	29.3	97.2	-0.3	0.0	31.1	81.6	RXR-Ag
Acitretin	55079-83-9	I	1	4.327	1	0.940	0	1000	0	9.701	0	1000	1	0.564	>92	24.4	79.4	3.4	4.8	4.1	104.9	RXR-Ag
13-cis Retinoic acid	4759-48-2	I	1	0.133	1	0.035	0	1000	0	1000	0	1000	1	0.024	81.283	29.3	46.3	-0.3	-0.1	6.8	99.4	RXR-Ag
Bexarotene	153559-49-0	A	0	0.008	1	0.017	0	1000	0	1000	0	1000	1	0.005	10.351	26.0	37.1	-0.4	-0.4	11.8	86.7	RXR-Ag
9-cis Retinoic acid	5300-03-8	Ac	1	4.180	1	2.068	0	1000	0	1000	0	1000	1	1.848	>92	31.8	76.0	-0.1	-0.3	5.7	83.4	RXR-Ag
Sincalide	25126-32-3	A	1	12.376	0	8.588	0	1000	0	1000	0	1000	1	10.496	>92	80.1	19.9	-0.4	-0.1	30.9	36.1	RXR-Ag
Thiostrepton	1393-48-2	A	0	6.374	1	5.472	0	1000	0	1000	0	1000	0	1000	>92	15.2	28.2	-0.6	-0.2	4.1	17.2	RXR-Ag
Clofoctol	37693-01-9	A	0	1.374	1	3.004	1	8.920	1	8.928	0	1000	1	6.796	8.035	23.6	34.4	55.5	64.9	100.7	36.1	other

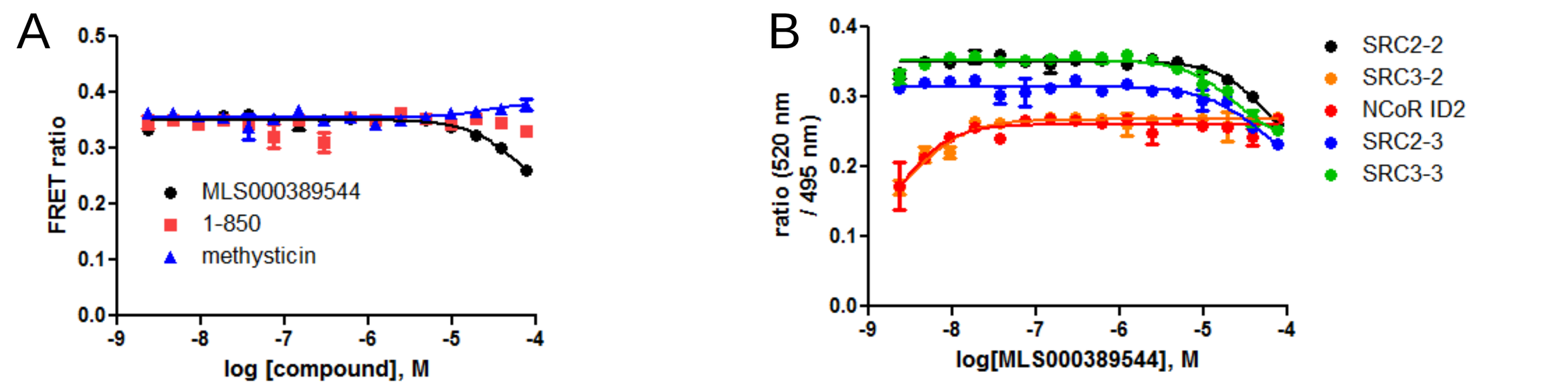
Antagonist Characterization

Cell Viability Counterscreen



Comparison of TR antagonist assay and cell viability assay run in parallel. A and B are examples of actives classified as cytotoxic. Note TBBPA is a reported TR antagonist. C is an example of a likely false positive as colchicine is an inhibitor of microtubule polymerization and typically cytostatic in cell cultures. However, the viability assay does not show this. D represents an apparent true TR antagonist. Using a 10-fold minimum ratio of viability to antagonist AC50's, 747 compounds were considered potential TR antagonists.

Coactivator Recruitment Assay



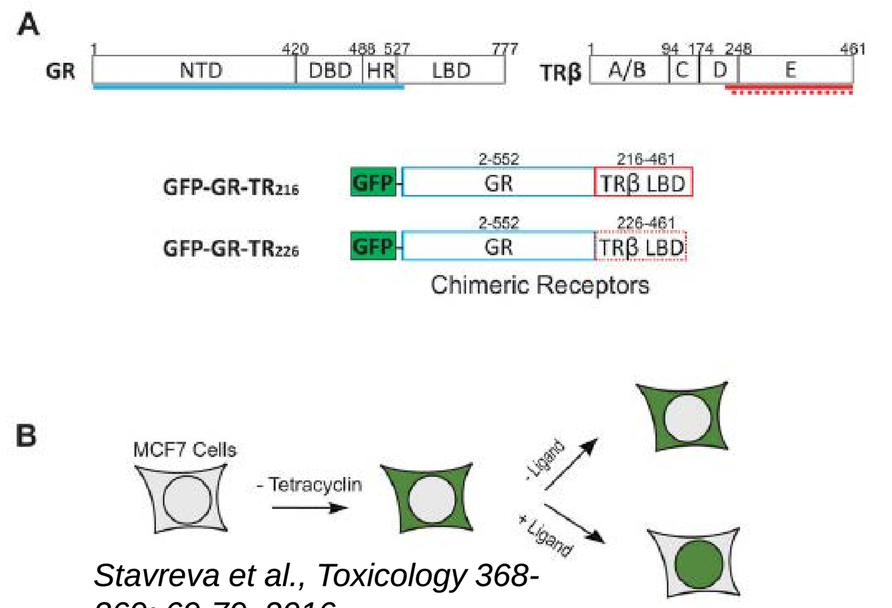
A. Three known TR antagonists tested with the SRC2-2 peptide and TRβ in the presence of 1.5 nM T3. B. MLS000389544 tested on various coregulator peptides in the presence of 1.5 nM T3. Decision made not to use for antagonist characterization.

Agonist Competition Potency Shift Assay

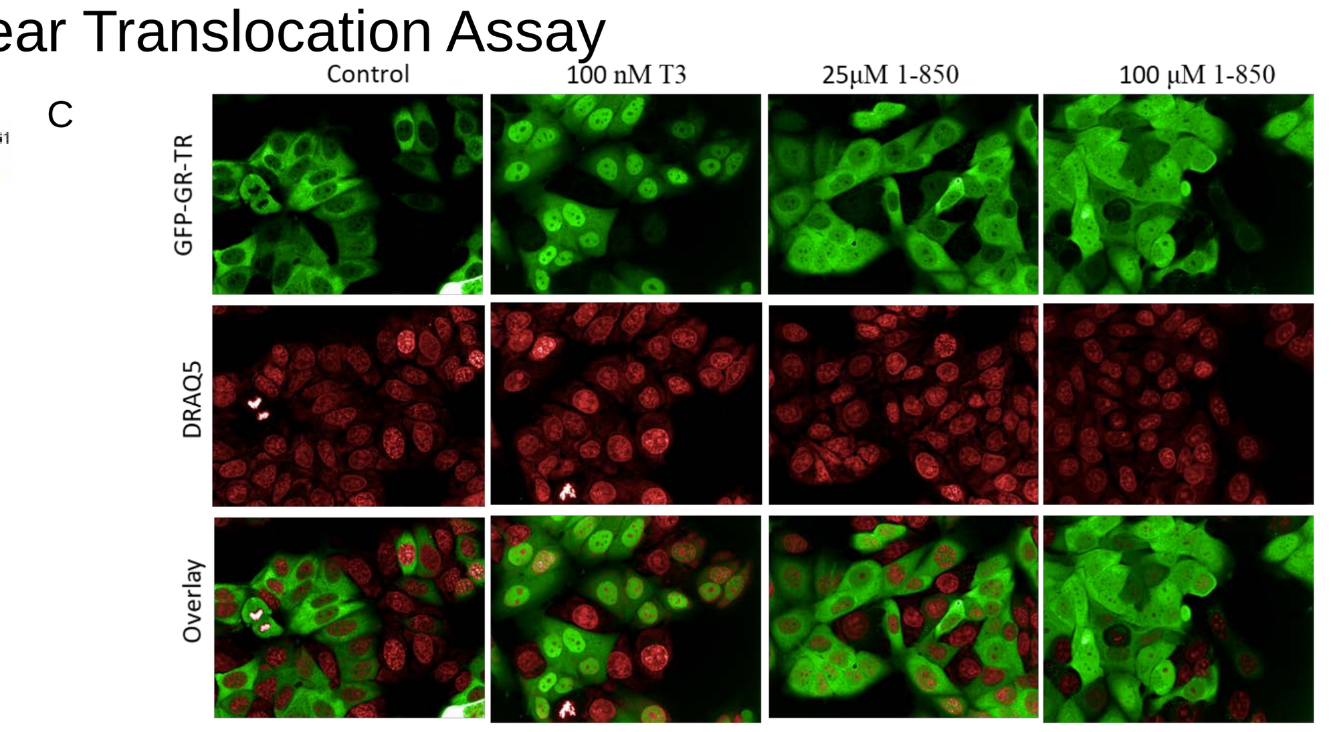
Candidate TR antagonists were retested in the GH3 assay in the presence of 1, 5, 10, 100 nM T3. Increasing agonist concentration should cause an increase in the AC50 for true competitive antagonists. No shifts with a concentration dependence were found. These competition results were not used in further characterization of potential antagonists (*data not shown*).

Antagonist Characterization

Receptor Nuclear Translocation Assay



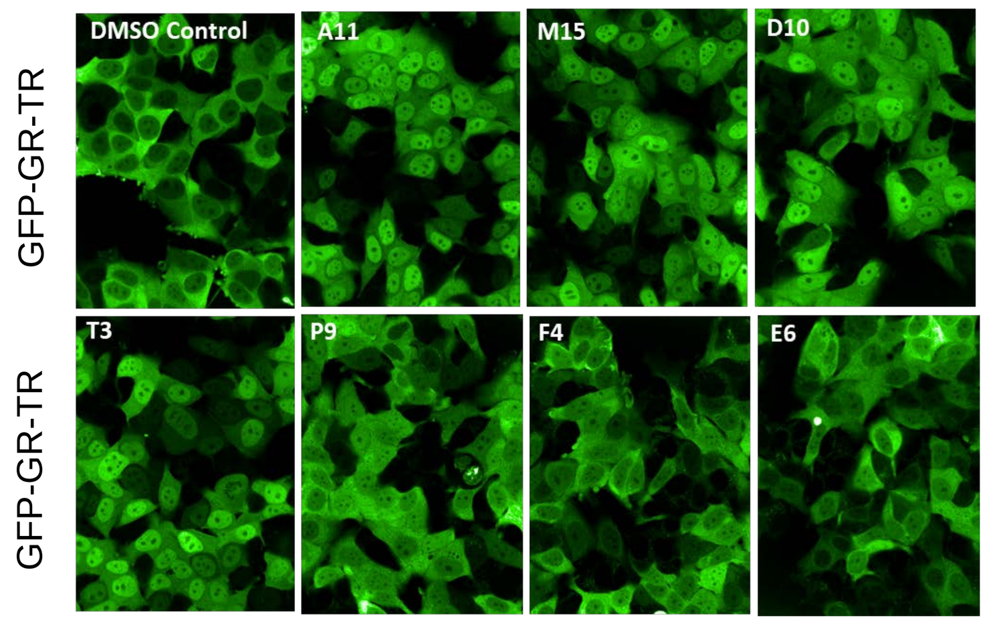
Stavreva et al., Toxicology 368-369: 69-79, 2016



A) A GFP-GR-TRβ chimeric receptor was constructed by fusing the human GR N-terminus, DNA-binding domain and hinge region to the human TRβ ligand-binding domain. B) The chimeric receptor was stably expressed in MCF 7 cells using a tetracyclin-off system. C) Cells were treated with the indicated compound for 3 hr, fixed with paraformaldehyde, the nuclei stained with DRAQ5 and cells imaged on the Perkin Elmer Opera Image Screening System. Translocation was calculated as a ratio of the mean GFP-GR- TRβ intensity in nucleus and cytoplasm and each value was further normalized to the value for the control (DMSO) sample on the same plate. Results for a positive control agonist, T3, and a positive control antagonist, 1-850, are shown.

Images of cells treated with the indicated compound, all at 100 µM (except T3 at 100 nM).

A11: Mefenamic acid
M15: Risarestat
D10: Diclazuril
P9: Tomelukast
F4: Guggulsterone E
E6: Vatalanib



Final Antagonists Identified

Samples			AC50			Nuclear/Cytoplasm Ratio			
			GH3		Viability	TR Nuclear Translocation			
chnm	casn	Sample QC	TR_LUC_GH3_Agonist (uM)	TR_LUC_GH3_Antagonist (uM)	TR_LUC_GH3_Antagonist_viability (uM)	Normalized Nuclear/Cytoplasm Ratio @ 12.5 uM	Normalized Nuclear/Cytoplasm Ratio @ 25 uM	Normalized Nuclear/Cytoplasm Ratio @ 50 uM	Normalized Nuclear/Cytoplasm Ratio @ 100 uM
Mefenamic acid	61-68-7	A	1000	19.505	>92	1.48	2.15	2.63	3.38
Risarestat	79714-31-1	B	1000	5.630	>92	1.77	1.96	2.51	3.31
Diclazuril	101831-37-2	A	1000	4.700	>92	1.41	1.62	2.03	2.69
Tomelukast	88107-10-2	A	1000	25.919	>92	1.03	1.04	1.23	1.64
Vatalanib	212141-54-3	A	1000	19.856	>92	1.01	1.08	1.26	1.47
Guggulsterone E	39025-24-6	A	1000	18.010	>92	1.01	1.11	1.29	1.43
Pazopanib	444731-52-6	A	1000	13.956	>92	1.09	1.23	1.42	1.40
Gestrinone	16320-04-0	A	1000	24.281	>92	1.04	1.11	1.28	1.39
Quetiapine fumarate	111974-72-2	A	1000	23.491	>92	0.98	1.01	1.11	1.32

Summary

- Extensive characterization of primary screening actives is necessary to confirm activity against the target
- Few agonists or antagonists were found in a large library of environmental chemicals
- Other modes of toxicity should be the focus of understanding potential disruption of the HPT axis by environmental chemicals

U.S. Environmental Protection Agency
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