A high-throughput approach to identify and prioritize putative thyroid-stimulating hormone receptor agonists and antagonists

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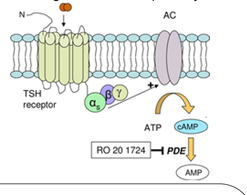
Abstract and Background

The thyroid-stimulating hormone receptor (TSHR) is a G protein-coupled receptor that signals through adenylate cyclase to increase intracellular 3',5'- cyclic adenosine monophosphate (cAMP), resulting in increased thyroid hormone (TH) production in thyroid follicular cells. Due to human health effects resulting from altered TH levels, it is important to evaluate whether environmental chemicals can disrupt thyroid function via TSHR-mediated signaling pathways. As part of the Tox21 collaboration, 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. Homogeneous time-resolved fluorescence technology was used to quantify cAMP using a competitive immunoassay between native and dye-labeled cAMP. Out of the 7,872 tested chemicals, 6% agonist, 4% antagonist, and 0.6% agonist-antagonist hits were identified, for a total of ~10% putative active chemicals. Because receptor binding is highly specific, we hypothesized that many of the hits were false positives. Thus, we developed a novel prioritization scheme to select chemicals for screening in biologically-relevant follow-up assays. Chemicals (558/778 active chemicals) were clustered by structural similarity using ChemoTyper ToxPrint fingerprints. The priority score (within cluster and for non-clustered chemicals) was penalized for: i) activity in other ToxCast cAMP enzymatic assays, ii) promiscuity according to ToxCast total assay hit rate, iii) signal interference by autofluorescence, and iv) cytotoxicity. Highly-ranked agonist clusters contain phenols, organochlorine insecticides, and retinoids. Cytotoxicity contributed significantly to the antagonist priority rank, with as many as 68% of antagonists suspected to be cytotoxic in the active concentration ranges. The prioritization scheme has identified 69/778 active chemicals that are structurally diverse for additional testing. Using this scheme, secondary screening of identified priority chemicals will be combined with structural prioritization to create an integrated predictive tool for TSHR activity. *This abstract does not necessarily reflect U.S. EPA policy.*

Background: In vitro screening of Tox21 library for TSHR activity

1. Tox21_TSHR assay principle

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



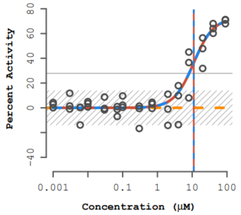
The assay is permissive, as it indirectly measures TSHR activity leading to the sub-hypothesis:

The screening assay may not be very specific for TSHR agonists, and antagonists.

Objective: Create a custom workflow to select candidates for follow-up screening in an orthogonal biological assay.

2. Curve-fitting

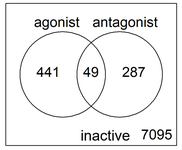
- 7872 substances were screened in the Tox21_TSHR assay in agonist and antagonist modes.
- The ToxCast analysis pipeline (tcpl) to obtain curve fits and hitcalls.



Area under the curve (AUC) was calculated to combine efficacy and potency, as an activity metric.

AUC values from agonist, and antagonist curve fits were used to rank actives in the assay.

3. Hits from primary screen



To select substances for further screening, the hits from the Tox21_TSHR assay will be grouped according to the features:

1) activity-ranking by AUC

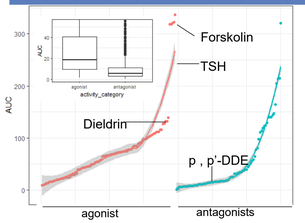
2) cytotoxicity

3) Structural diversity

Data-driven strategy: categorical and quantitative features

Chemicals selected for follow-up screening should include: **1) Highest activity; 2) Greatest specificity (based on cytotoxicity); and, 3) Highest diversity**. Additional negatives should be included for a balanced orthogonal screening set.

Activity ranking by AUC



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.

Figure 1. AUC vs Chemical for agonist and antagonist Modes.
The 181 substances cherry-picked for orthogonal screening in the large panel; inset contains boxplots for the entire library.

AUC is an informative metric for the selection of highly-active candidate hits for follow-up screening.

Prioritizing hits with highest selectivity using difference between activity and cytotoxicity

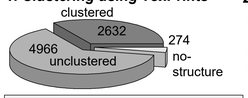
Figure 2. Ranking agonists(left), and antagonists(right) by selectivity to TSHR activity.
Positive hits in TSHR assay in agonist and antagonist modes were prioritized when activity occurred at lower concentration than cytotoxicity.

Cytotoxicity cutoff was calculated using the AC50 values from the Tox21 viability assays.**Selectivity distance** was required to be positive for prioritized selectives [log(AC50(burst))-log (AC50(TSHR)) > 0].

315/490 agonists, and 103/336 antagonists were prioritized for screening. Chemicals with antagonistic activity exhibit more cytotoxic profile than the agonists.

Identifying structure-activity relationships using clustering, and diversity maximization

1. Clustering using ToxPrints



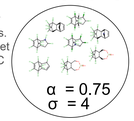
Chemicals with available structures (SMILES) are clustered according to the pairwise Tanimoto similarity using the ToxPrint fingerprints (ChemminerR). Cutoff for clustering is 0.85, and minimum cluster size is 3. Per cluster, the chemical with the highest AUC is selected as a representative of the structural class. **34** of cherrypicked substances came from **23** clusters.

2. Prioritization of clusters

Clusters are prioritized by two quantitative metrics that indicate if the cluster was highly active (active density) and if the cluster was enriched in cytotoxic substances (selective density):

- Enrichment of actives as described by **active_density (α)** = # actives/ cluster-size
- Enrichment of cytotoxic-selective hits **selectivity_density (σ)** = cluster-size/ # cytotoxic-selective chemicals, ranging **[1,5]**

Example prioritized cluster of organochlorine insecticides. Two substances have a subset of high-AUC actives with AUC values : 318 and 297.



3. Prioritization of unclustered chemicals

- Diversity of structure was scored for cytotoxicity-filtered agonist and antagonist hits (total of 381 substances). This was completed using MaxMin algorithm (RDkit).
- Unclustered substances for orthogonal screening were selected when they demonstrated the greatest diversity.

Selected chemicals were prioritized by AUC-rank

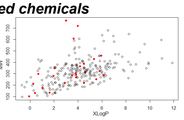
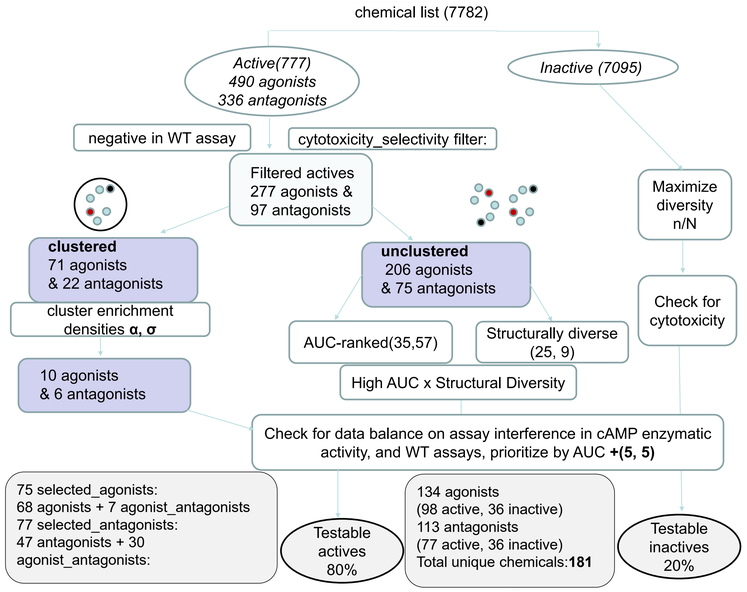


Figure 3. MaxMin algorithm selects chemicals with highest diversity.
Most diverse 25 (red) of 315 non-cytotoxic agonists were prioritized for orthogonal screening. The diverse set covers the majority of the physico-chemical space described by molecular-weight (MW), and partition coefficient(XLogP).

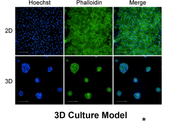
Candidates identified using prioritization workflow



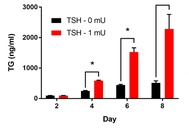
Future Direction: Develop orthogonal screening approach

Goal: Distinguish false positives from positive hits to build a model for TSHR activity.

Model: thyroid microtissues

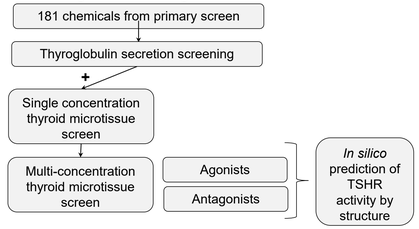


Primary human thyrocytes self-assemble into microtissues in a 3D culture model



Thyroglobulin production is the endpoint for TSHR activity in this model

Orthogonal screening workflow



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