Prioritization of Chemicals for Effects on Steroidogenesis Using an Integrated Statistical Approach to High-throughput H295R Data

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Introduction

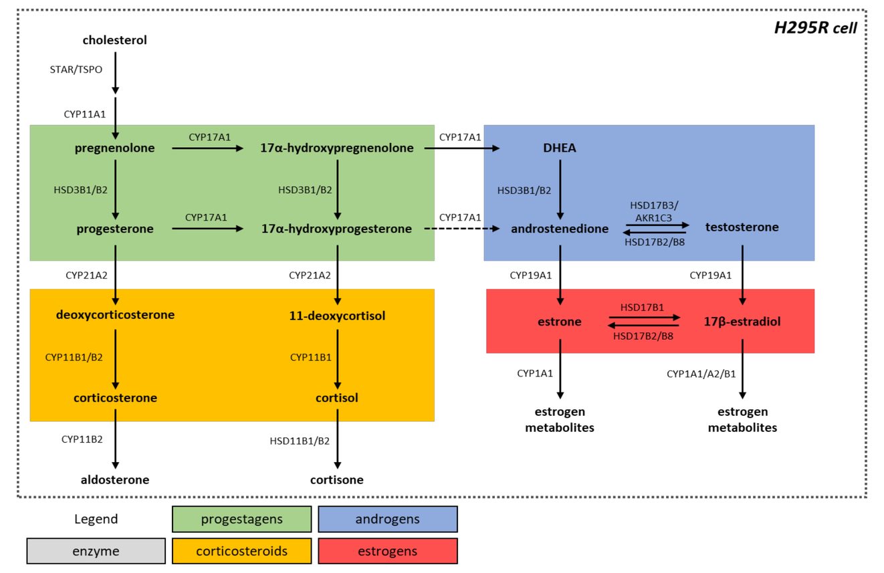
Background:

- Endocrine disruption is a toxicity of both physiological and regulatory importance; as steroid hormones regulate reproduction, development, and other biological processes, it is a priority to identify chemicals that may interact with production of these hormones.
- A high-throughput H295R assay (HT-H295R) was developed as part of the U.S. EPA's ToxCast program that includes measurement of 11 hormones across the steroid hormone biosynthesis pathway in H295R cells, including progestagens, corticosteroids, androgens, and estrogens.
- HT-H295R has been used to screen a total of 2012 chemicals in single-concentration and 656 chemicals in multi-concentration.

Mahalanobis Distance Metric:

- We previously developed a statistical metric using the mean Mahalanobis distance (mMd) to quantify the effect of a chemical on the overall steroidogenesis pathway in HT-H295R.
- Mahalanobis distance incorporates the effect size for each steroid hormone measure after adjusting for covariance between the steroid hormone measures (Equation 1).
- The maximum mMd (maxmMd) value for each chemical was selected to indicate the magnitude of steroidogenesis pathway perturbation.

Steroidogenesis in H295R Cells



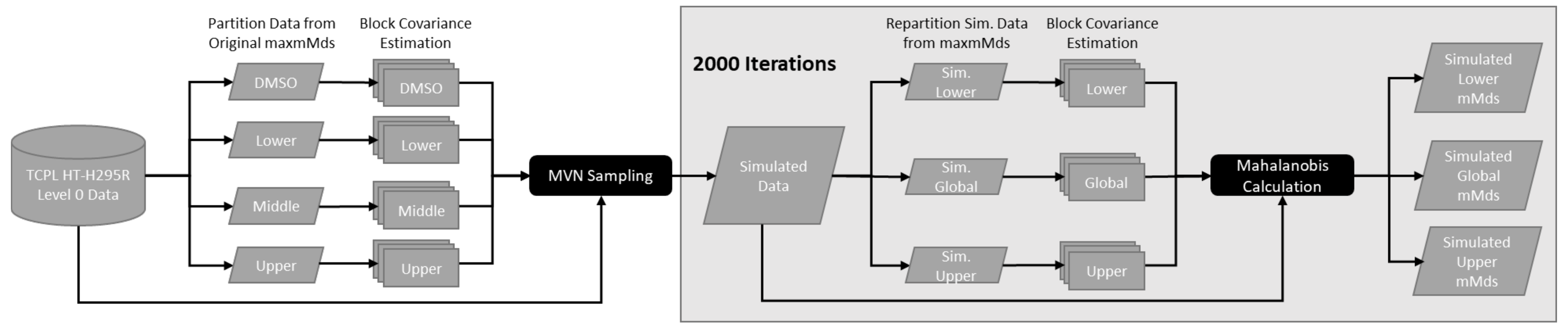
Eq. 1 $$mMD = \sqrt{(\mathbf{y_c} - \mathbf{y_1})'\mathbf{\Sigma}^{-1}(\mathbf{y_c} - \mathbf{y_1})/N_h}$$

Where y_j is the vector of log-transformed hormone concentrations for the j^{th} concentration, N_h is the number of hormones with measurements for this chemical, and ∑ is the estimate of the covariance matrix

Primary Questions:

- Is the covariance structure stable when you only use low or high responding chemicals for the mMd calculation (*i.e.* if the dataset changed), and how does this influence resulting mMd values?
- How well did we approximate the type I error rate, and what is the sensitivity of the mMd approach to detect significant perturbations with different effect sizes and steroid hormone responses?
- Do putative aromatase inhibitors have a unique steroid hormone pattern in HT-H295R?
- Can high-throughput screening markers of cytotoxicity and mitotoxicity be used to contextualize the mMd as a prioritization metric?

Data Simulation to Test Stability of the Approach



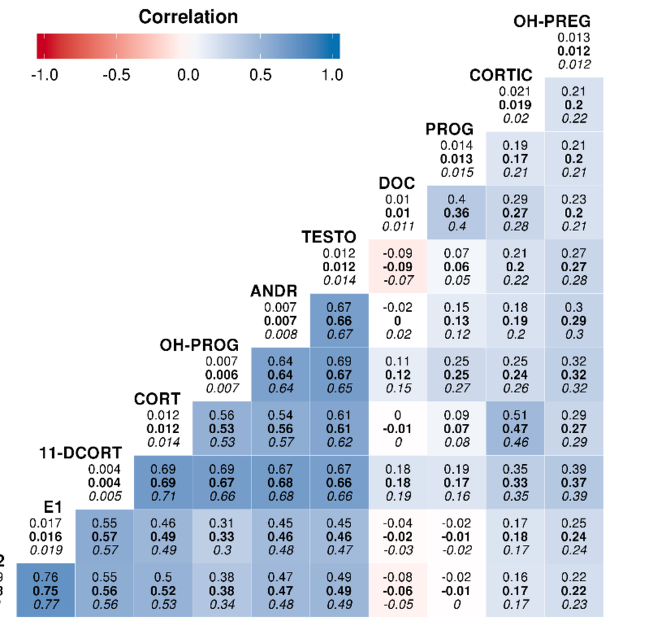
We generated 2000 simulated HT-H295R datasets using multivariate normal sampling.
For each simulated dataset:

- Estimate three different covariance matrices based on weak responders (lower), all data (global), and strong responders (upper).
- Derive new mMds using these covariance matrices for a total of three sets of mMd values to compare.

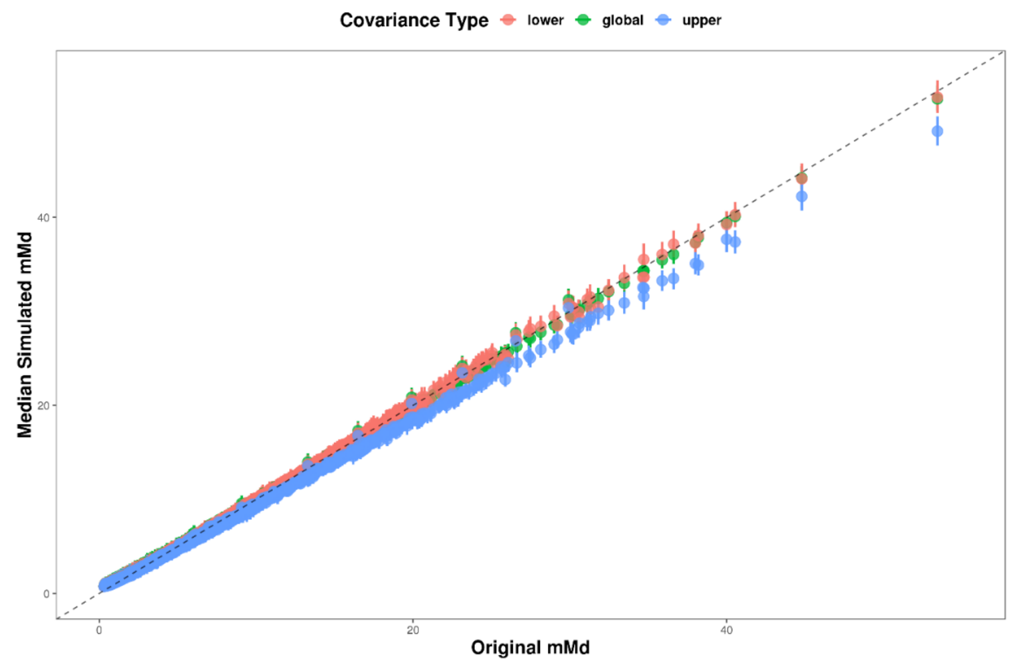
The Mean Mahalanobis Distance (mMd) Approach is Stable

Changes in covariance matrices from data simulation suggest that the covariance structure is stable, leading to reproducible mMds.

A.



B.



A. Comparison of the correlation of the three estimated covariance matrices.
   - Correlation of the covariance of steroid hormone measures are largely the same.
   - Variance of the steroid hormone measures is also similar.

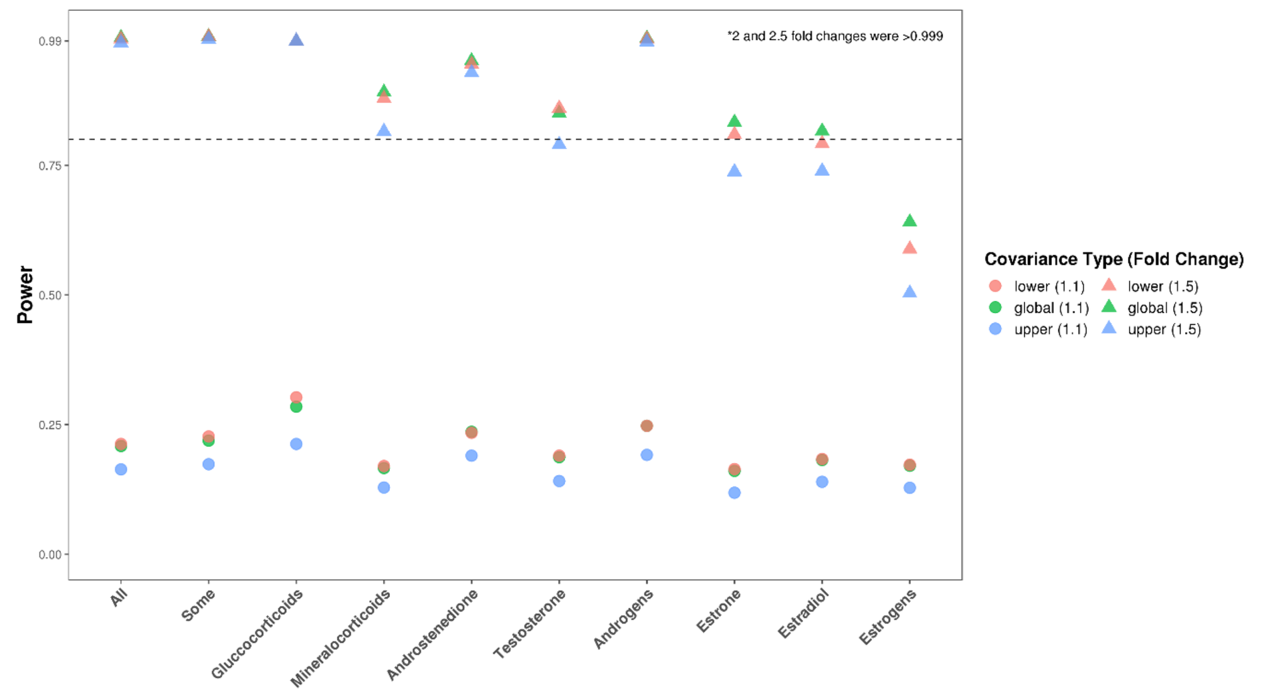
B. Scatterplot of the difference between the original mMd and the median of the simulated mMd for the three covariance types.
   - Practically no difference was observed between the simulated mMds and the original mMds (dashed identity line).

What is the true type I error rate (false positive rate) and the power to detect fold-changes in steroid hormones?

A.

	Covariance Type		
Error Rate	**Lower**	**Global**	**Upper**
0.05	0.015	0.019	0.012
0.01	0.007	0.008	0.006

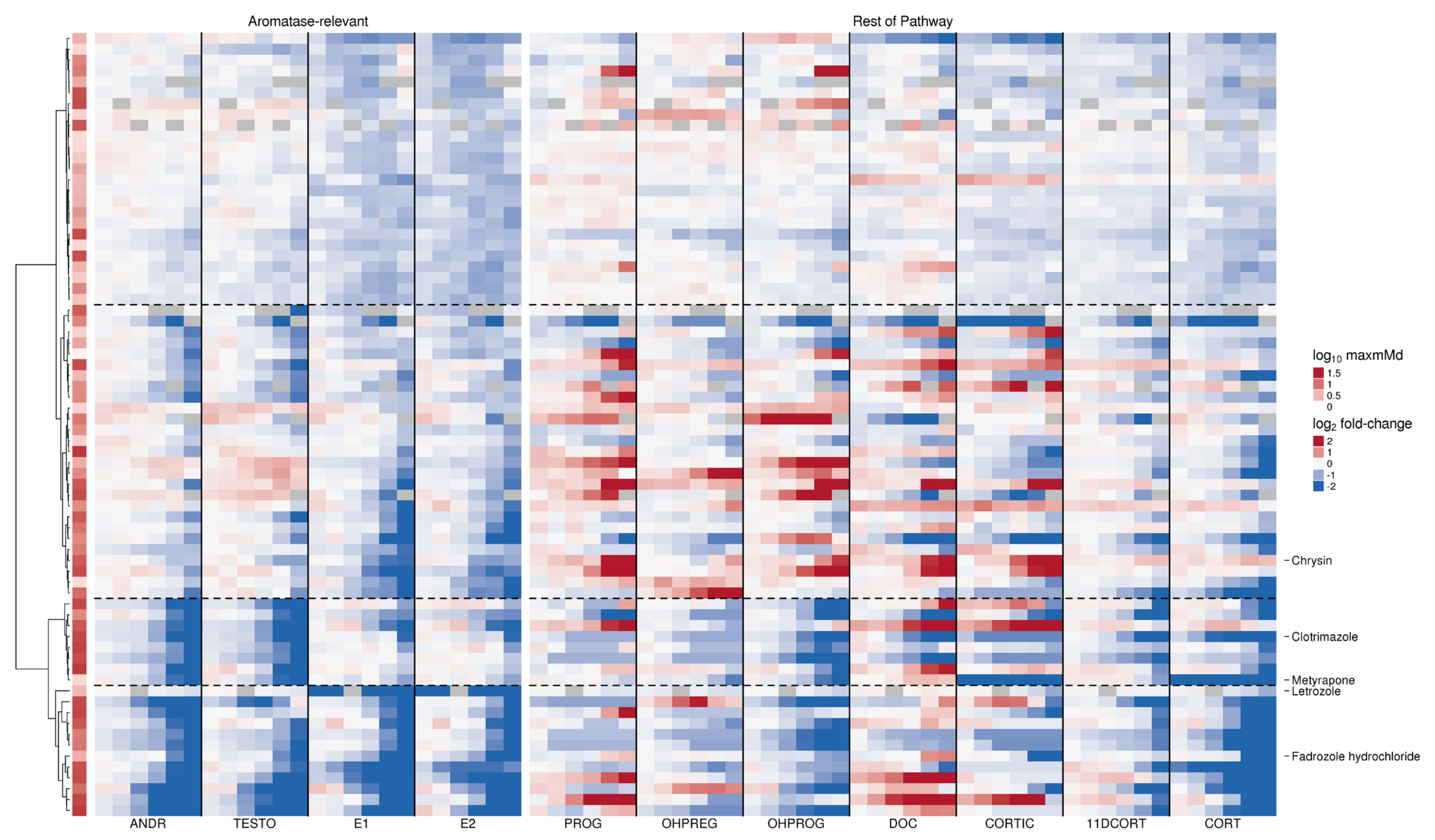
B.



A. Estimated type I error rate of a simulate true negative HT-H295R response profile at two α levels and the three covariance matrix types.

B. The power to detect experimental significance using the mMd approach.
   - Fold-change effect sizes ≥1.5-fold resulted in sufficient power to detect significant effects on steroidogenesis.
   - The lowest power was observed for steroid hormone changes in both estrone (E1) and estradiol (E2).

Steroid Hormone Profiling of Putative Aromatase Inhibitors



Steroid response heatmap for putative aromatase inhibitors.

- 72 out of 766 chemical samples had a putative aromatase inhibitory signature (1.5-fold decreases in both E1 and E2).
- Hierarchical clustering of androstenedione, testosterone, E1, and E2 responses identified four predominant clusters.
- Known aromatase inhibitors decrease estrogens *and* androgens but have myriad effects on upstream steroid hormone patterns.

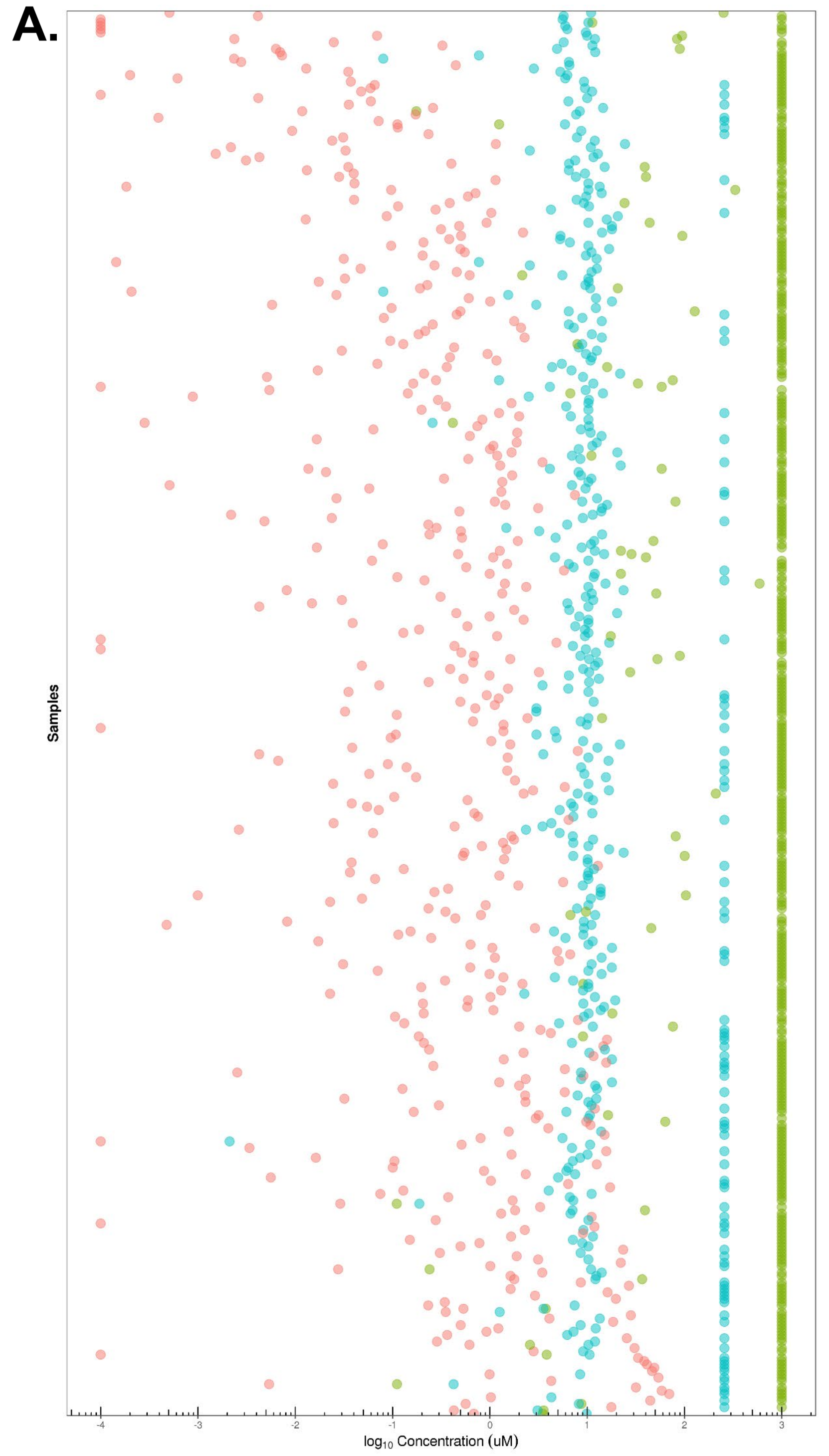
Chemical Name	Cas No.	maxmMd	HT-H295R Estrone	HT-H295R Estradiol	HT-H295R Fold Change	HT-H295R Aromatase Positive	NVS Aromatase Positive
4,4'-Oxydianiline	101-80-4	18.35	↓	-	Yes	Yes	No
Clotrimazole	23593-75-1	18.35	↓	↓	Yes	Yes	No
Cyproconazole	94361-06-5	8.58	↑	↑	Yes	No	Yes
Epoxiconazole	133855-98-8	14.68	↓	↓	Yes	Yes	Yes
Fadrozole hydrochloride	102676-31-3	16.90	↓	↓	Yes	Yes	Yes
Fenarimol	60168-88-9	16.70	↓	↓	Yes	Yes	No
Flusilazole	85509-19-9	9.27	↓	↓	Yes	Yes	Yes
Imazalil	35554-44-0	22.62	↑	↓	Yes	Yes	Yes
Letrozole	112809-51-5	14.02	↓	↓	Yes	Yes	Yes
Myclobutanil	88671-89-0	16.43	↓	↓	Yes	Yes	Yes
Prochloraz	67747-09-5	18.56	↓	↓	Yes	Yes	Yes
Propiconazole	60207-90-1	5.14	-	-	No	No	Yes
SSR150106	NOCAS_47362	9.06	↓	↓	Yes	Yes	No
Tetraconazole	112281-77-3	3.41	↓	-	No	No	Yes
Triadimenol	55219-65-3	17.25	↓	↓	Yes	Yes	Yes
Triflumizole	68694-11-1	17.19	↓	↓	Yes	Yes	Yes
Vatalanib dihydrochloride	212141-51-0	8.34	↓	↓	Yes	Yes	No

Concordance of putative aromatase inhibitors from the Tox21/ToxCast aromatase assays and HT-H295R.

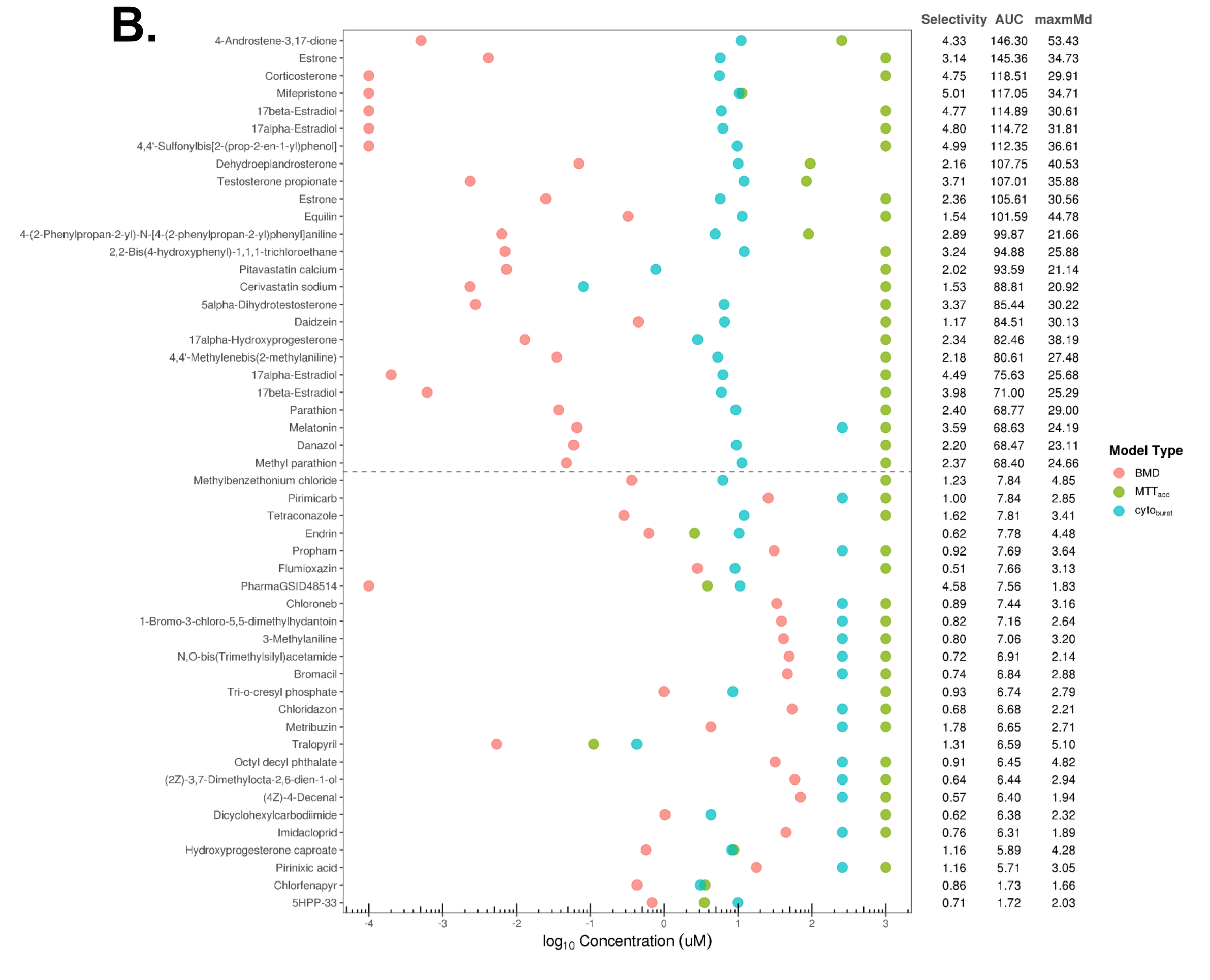
- 17 putative selective aromatase inhibitors from Tox21 confirmatory screening were tested in both the ToxCast NVS aromatase assay and HT-H295R.
- 14 of the 17 putative aromatase inhibitors were considered selective positives in HT-H295R, and 12 were considered positives in the ToxCast NVS assay.
- 9 chemicals were positive in both the ToxCast NVS assay and HT-H295R.

Development of a HT-H295R Prioritization Metric

A.



B.



We developed a rank prioritization metric using a selectivity score which accounted for cytotoxicity and mitotoxicity (as indicated by MTT).

A. 428 of 709 (60%) chemical samples with sufficient ToxCast cytotoxicity assay coverage were considered selective in HT-H295R and were ordered by efficacy in the mMd response.

B. Top and bottom 25 most selective and efficacious chemicals samples in HT-H295R.
   - Most selective chemicals included steroid hormones and known steroidogenesis disruptors.

Summary and Future Directions

- The mMd appears to be robust to changes in the covariance matrix derived from different effect size partitions of the HT-H295R chemical space.
- The observed type I error rates were conservative and similar to the approximated values used in the critical threshold calculation, suggesting that the hit call method used for the mMd approach is appropriate and robust.
- An effect size of a 1.5-fold increase in hormone levels results in a maxmMd response value greater than the critical threshold with a power of 0.8 or higher, except for changes in both estrogen steroid hormones.
- The HT-H295R assay as implemented can identify aromatase inhibitors, but the myriad responses across the majority of the steroid hormone measures for these chemicals suggests that proper mechanistic evaluation requires multiple timepoints to better model the complex and dynamic steroidogenesis pathway present in H295R cells.
- Inclusion of orthogonal aromatase assays can increased confidence in identifying aromatase bioactivity.
- Removing chemical samples that had BMDs within the cytotoxicity/mitochondrial toxicity 'burst' range using the selectivity score, and then ordering by the AUC estimates appeared to efficiently rank active chemicals with steroid hormones and known steroidogenesis disruptors ranking highest.

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