Significance of Hormone Perturbation Patterns in a High-Throughput Steroidogenesis Model

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Overview

Background: ToxCast data from a high-throughput H295R (HT-H295R) human adrenocortical carcinoma model of steroidogenesis are available for over 2000 chemicals with effects on 13 steroid hormones including progestogens, glucocorticoids, androgens, and estrogens. All chemicals were screened at a single high concentration, up to at most 30% cytotoxicity. 575 chemicals that affected at least 4 hormones (compared to DMSO controls) were retested in concentration-response (Karmaus, et al. 2016). One hundred three chemicals were tested as replicates.

Objectives:

- Apply a new statistical analysis to improve the sensitivity and ultimately the specificity of predictions of steroidogenesis disruption;
- Use patterns in the hormone response data to identify putative mechanisms of steroidogenesis disruption.

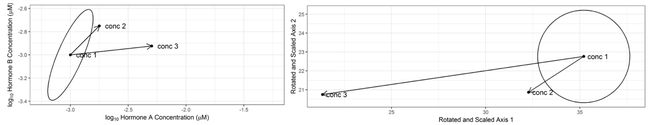
Methods: We use Mahalanobis distance to quantify disruption of steroidogenesis across 11 hormone measurements, and similarities between that and Hotelling's T^2 statistic to develop a statistically-based critical value for calling "hits". Evaluating patterns of response to treatment yields insight into modes of action.

Results:

- Hit calls for 84% (87 / 103) of replicated chemicals agree.
- 95% (533/559) of the chemicals with non-conflicting calls tested in concentration-response format were flagged as potential steroidogenesis disruptors using this methodology, as compared to about 78% (411 / 524) having hits in at least one hormone (Karmaus et al., 2016).
- Patterns are frequently consistent with affects on multiple enzymes.
- Response patterns suggest mechanisms that have not been included in current kinetic models built using a similar H295R system (Saito et al., 2016).

What is Mahalanobis Distance?

Mahalanobis distance measures differences between observations that include multiple features (like changes in multiple hormone levels after treatment). It adjusts for different levels of and correlations between measurements of the features.



The three points are the mean ($\log_{10}$) concentrations of hormones A and B at three concentrations of a test chemical. Conc 3 is twice as far from conc 1 as is conc 2 (Euclidean distance). The ellipse represents the joint error distribution for both hormones.

Variables are changed by a rotation and rescaling so that the error distribution on the new axes is uncorrelated. Now, conc3 is four times as far from conc1 as is conc2 (Euclidean distance in the transformed space is Mahalanobis distance based on the original coordinates).

The mean Mahalanobis Distance (mMD) for a given test compound between the hormone concentration at the c^{th} concentration relative to that at the lowest concentration is:

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

where $\mathbf{y}_j$ 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 $\sum$ is the estimate of the covariance matrix. It the Mahalanobis distance divided by the square root of the number of hormones evaluated.

References

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT (2016) High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. *Toxicol Sci* **150**(2) 323-32.

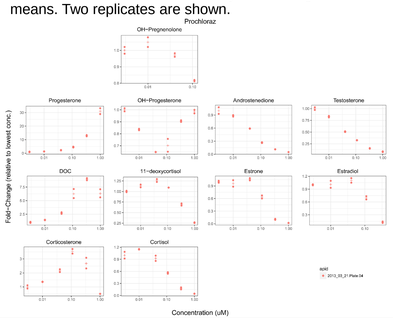
Mardia, K. V., Kent J. T., and Bibby, J. M. (1979). *Multivariate Analysis*. Academic Press, London.

Nakamura T and Imada T (2005) Multiple comparison procedure of Dunnett's type for multivariate normal means. *J. Jpn. Soc. Comp. Statist.* **18**: 21-32.

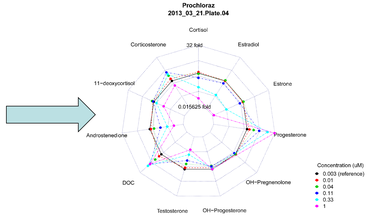
Saito R, Terasaki N, Yamazaki M, Masutomi N, Tsutsui N, and Okamoto M. (2016). Estimation of the Mechanism of Adrenal Action of Endocrine-Disrupting Compounds Using a Computational Model of Adrenal Steroidogenesis in NCI-H295R Cells. *J Toxicol* 2016, 4041827.

Computing mMD and Max mMD – an Example

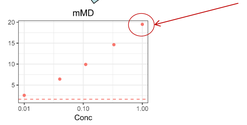
Concentration-response plot of effect of prochloraz on 11 hormones. Scaled so the geometric mean response at the lowest concentration is 1. '+' symbols are geometric means. Two replicates are shown.



Same data, using radar chart to represent the 11-dimensional nature of the data.



max mMD used for prioritization and calling 'hits'

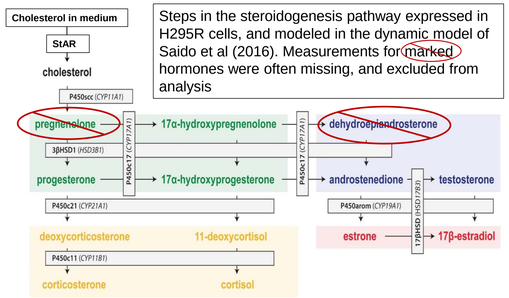


mMD of each concentration from the response at the lowest concentration of prochloraz.

Thresholding max mMD

- mMD is similar to Hotelling's T^2 statistic (Mardia, et al. 1979) for comparing two groups with multiple measurements
  - We estimate $\sum$ by pooling residuals over the whole data set and making some approximations – not quite the same setting
  - Need to adjust for multiple comparisons (like Dunnett's test).
- For thresholding the max mMD, use the results of Nakamura and Imada (2005) to compute an approximate critical value for a nominal $\alpha = 0.01$

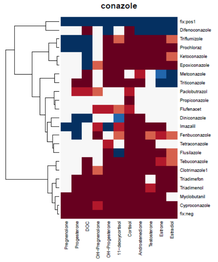
Hormone Patterns ↔ Steroidogenesis Pathway



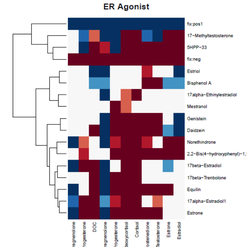
Steps in the steroidogenesis pathway expressed in H295R cells, and modeled in the dynamic model of Saido et al (2016). Measurements for marked hormones were often missing, and excluded from analysis

- Presumably, chemicals that disrupt steroidogenesis act by inhibiting, up-, or down-regulating enzymes in the pathway.
- Thus, it should be possible to map patterns of hormone concentration response back to effects at particular hormones, assuming model that captures all the relevant relationships sufficiently well. For example, inhibiting (only) CYP19A1 should increase androstenedione and testosterone, and decrease estrone and 17β-estradiol.
- A mathematical model of the pathway, *e.g.* Saito et al., 2016, should, in principle be useful to improve the specificity of the assay, by identifying hormone patterns that are inconsistent with the pathway, and thus more likely to be due to assay failures.

Exploring the H295R Steroidogenesis Pathway

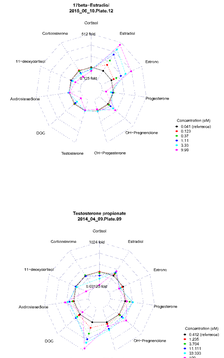


Blue is upregulation, brown is downregulation. Conazoles are a class of chemicals expected to block CYP19A1, decreasing Estrone and Estradiol. However, these chemicals tend to downregulate the entire pathway, but 3 conazoles actually upregulate Estradiol



Potent estrogens and androgens demonstrate complex but strong patterns of disruption, possibly resulting from:

- Hypothetical negative feedback mechanisms to downregulate hormone production.
- Modulation of other mechanisms present in the H295R cell, such as steroid hormone receptors.



Summary of Results

- Concordance among hit calls for replicated chemicals is good, with all the calls for 87 of 103 replicated chemicals agreeing.
- After filtering using a single-concentration screen, 95% (533 / 559) of chemicals with non-conflicting calls cross the threshold for a hit call. This is a higher rate than in the original Karmaus et al. 2016 publication (78%). Whether this represents an increase in sensitivity or decrease of specificity is currently unknown.
- Concentration-response patterns for individual hormones suggest that when chemicals disrupt steroidogenesis, multiple enzymes in the pathway are affected.
- Response patterns resulting from treatment of the HT-H295R system with steroid hormones suggest mechanisms that have not been included in current kinetic models built using a similar H295R system (Saito et al., 2016).

Future Work

- Explore and improve the thresholding to account for more of the assay noise
- To generate a model with possibly greater specificity, adapt the Saito et al. (2016) model for this specific implementation of the H295R assay, and potentially expand it to incorporate missing mechanisms to account for observed patterns of hormone changes.
- Further explore patterns of hormone perturbations using empirical methods to help map mode of action of chemicals to patterns of perturbation.
- Develop an extended list of reference compounds for use in evaluating the error rate for the assay.
- Evaluate to what extent perturbation of glucocorticoids and progestagens in the H295R model is relevant to in vivo effects.