In Vitro Profiling of Chemical Effects on Steroidogenesis

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ABSTRACT

Steroidogenesis, including both steroid hormone biosynthesis and metabolism, is critical for proper endocrine function. Disruption of steroidogenesis by environmental chemicals results in altered hormone levels that may cause adverse reproductive and developmental effects. This study presents a high-throughput adaptation of the OECD validated H295R human adrenocortical carcinoma steroidogenesis assay. A 96-well format and hormone quantification by HPLC-MS/MS allowed for the evaluation of a diverse library of chemicals for effects on 13 major hormones in the steriodogenic pathway. Steroidogenesis was induced by pre-stimulation with 10 µM forskolin for 48 h followed by chemical exposure for 48 h. Media were removed and quantification of progestagens, glucocorticoids, androgens, and estrogens was conducted. Initially, nearly 2000 ToxCast chemicals were tested at a single non-cytotoxic concentration, of which 936 chemicals altered levels of at least one measured hormone. Based on the single concentration analysis, 395 chemicals altering the levels of ≥4 hormones were selected for six-point concentration-response evaluation (0.003–100 µM). Compared to results from OECD guideline criteria, which requires only changes in testosterone and/or estradiol, our criteria of ≥4 altered hormones identified chemicals with 91% sensitivity. Furthermore, the profiles generated by quantifying 13 hormones in concentration-response not only characterized chemical-elicited disruption in steroidogenesis, but also identified distinct putative modes of action. For example, distinct patterns of decreased glucocorticoid levels with concurrent increases in progestagen levels distinguished putative HSD3B inhibition. Additionally, increased progestagen levels along with decreased androgen and estrogen levels was observed for putative CYP17A disruptors. These data suggest that a high-throughput adapted evaluation of steroidogenesis using the OECD validated H295R platform can provide additional insight into chemical-mediated effects on steroidogenesis.

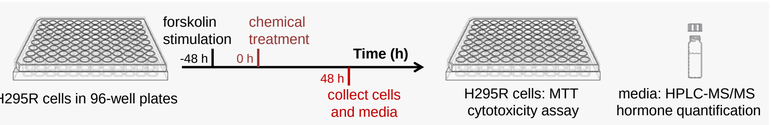
INTRODUCTION & OBJECTIVES

There are a broad spectrum of environmental chemicals that elicit adverse health effects by disrupting endocrine function. To-date endocrine disruption has been largely evaluated in the context of chemical effects on hormone receptor signaling. However, endocrine disruption comprising reproductive and developmental toxicity may also result from disruption of hormone levels. Hence, the evaluation of chemical effects on hormone biosynthesis and metabolism (steroidogenesis) is critical. Unfortunately, there are no high-throughput *in vitro* models currently amenable to the evaluation of chemical-mediated effects on steroidogenesis.

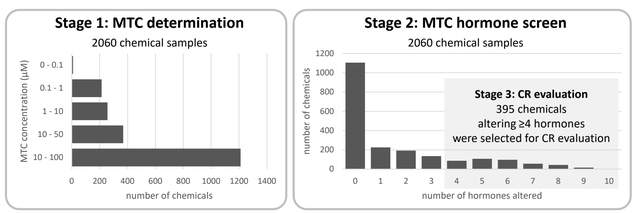
The objectives of the current study were:

- Develop a quantification method allowing detection of 13 major steroid hormones
- Establish H295R cells as a high-throughput model for the evaluation of steroidogenesis
- Screen the effects of a large library of chemicals on steroidogenesis in concentration-response

STUDY DESIGN



▲ **Figure 1. Cell culture**: H295R human adrenocortical carcinoma cells were stimulated with 10 µM forskolin for 48 h prior to chemical treatment for 48 h. Media were harvested for hormone quantification by HPLC-MC/MS while cells were tested for viability by MTT assay to ensure cytotoxicity did not confound results.

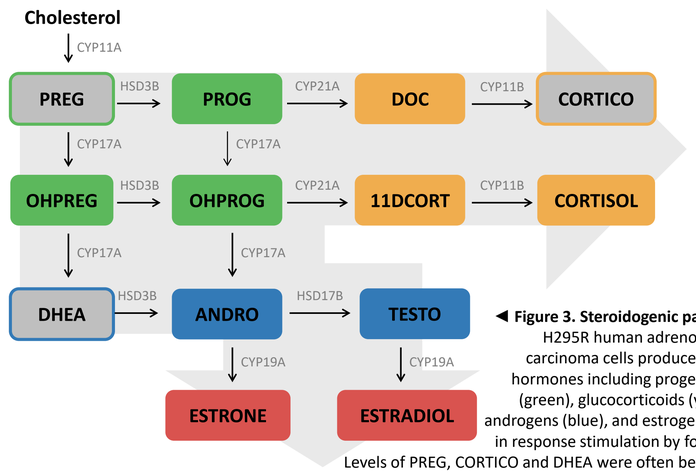


▲ **Figure 2. Study stages**: This study was conducted in three stages: 1. Determination of a maximum tolerated concentration (MTC; ≥70% cell viability); 2. Quantification of hormone levels upon MTC treatment; 3. Six-point concentration-response (CR) evaluation for selected chemicals. MTC concentrations were established for 2,060 chemical samples, with the majority of samples having an MTC ≥10 µM. All 2,060 chemical samples were evaluated for MTC effects on hormone levels. Samples altering ≥4 hormones in the MTC screen (highlighted in the shaded region in the Stage 2 graph) were included for CR evaluation.

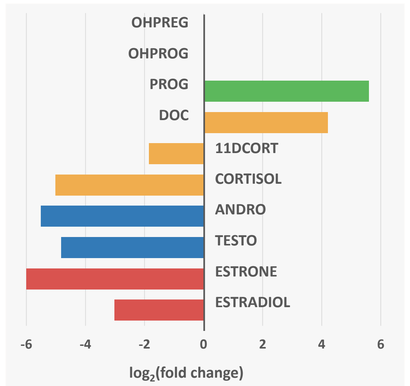
QUANTIFICATION OF HORMONES ACROSS THE STEROIDOGENESIS PATHWAY

Table 1. Hormones quantified by HPLC-MS/MS and Limits of Detection (LOD)

Hormone	Short Name	LOD (ng/ml)
Pregnenolone	**PREG**	2-400
17α-OH Pregnenolone	**OH-PREG**	5-1000
Progesterone	**PROG**	0.2-40
17α-OH Progesterone	**OH-PROG**	0.2-40
Deoxycorticosterone	**DOC**	0.5-100
Corticosterone	**CORTICO**	0.5-100
11-Deoxycortisol	**11DCORT**	5-1000
Cortisol	**CORTISOL**	0.5-100
Dehydroepiandrosterone	**DHEA**	3-600
Androstenedione	**ANDRO**	1-200
Testosterone	**TESTO**	0.1-20
Estrone	**ESTRONE**	0.03-6
Estradiol	**ESTRADIOL**	0.03-6



◄ **Figure 3. Steroidogenic pathway:** H295R human adrenocortical carcinoma cells produce steroid hormones including progestagens (green), glucocorticoids (yellow), androgens (blue), and estrogens (red) in response stimulation by forskolin. Levels of PREG, CORTICO and DHEA were often below the limit of detection and omitted from subsequent analyses.



▲ **Figure 4. Prochloraz-mediated effects on steroidogenesis:** Prochloraz is a conazole fungicide known to inhibit CYP17A1, and resulted in increased PROG and DOC levels while significantly decreasing androgen and estrogen levels, as expected. Maximum fold change achieved in CR evaluation is plotted.

PROFILING AND COMPARISON TO OECD GUIDELINE

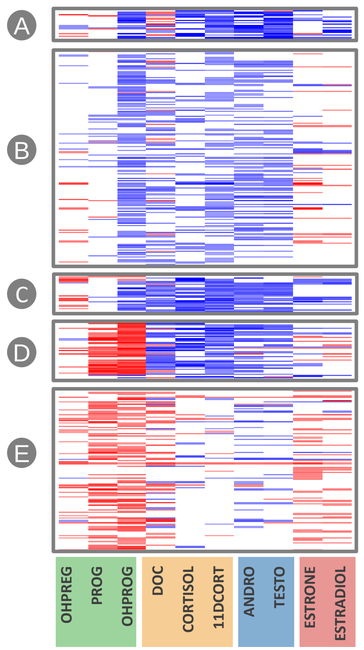


Table 2. Comparison of Criteria Between MTC Screen and OECD Guideline

		OECD Criteria		
		Positive	**Negative**	**Total**
MTC Screen Criteria	**Positive**	211	192	**403**
	Negative	21	100	**121**
	Total	**232**	**292**	**524**

In total 524 chemicals were evaluated in both MTC and CR stages (including 395 meeting MTC criteria and select borderline and negative chemicals). This table compares results for these 524 chemicals evaluating our MTC screening criteria (effect > 1.5-fold on ≥4 hormones) to the OECD criteria (concentration-dependent effect on TESTO and/or ESTRADIOL). When using OECD criteria as truth, our approach achieved 91% (211/232) sensitivity for identifying steroidogenesis disrupting chemicals.

◄ **Figure 5. Profile-driven clustering of chemical effects on steroidogenesis:** 336 chemicals elicited concentration-dependent effects on at least one hormone were evaluated using k-means clustering to identify profiles of steroidogenesis disruption. White indicates no data or no effect, increases in hormone levels are indicated in red, and decreases in hormone levels are shown in blue. Unique profiles including prochloraz-like CYP17A1 inhibition (cluster A) and putative HSD3B inhibition (cluster C) are discernable.

SUMMARY

Established H295R cells as a high-throughput model for the evaluation of steroidogenesis

- Modified the H295R OECD guideline assay into 96-well format and established a three-stage study design to allow high-throughput evaluation without cytotoxicity confounding the results.
- Addition of a pre-stimulus with forskolin allowed for the robust dynamic detection of both increases and decreases in hormone levels.
- Developed an HPLC-MS/MS method to quantify 13 major steroid hormones across the steroidogenesis pathway.

Screened the effects of 2,060 chemicals on steroidogenesis

- 2,060 chemicals were evaluated to identify an MTC, and were tested for their effects on hormone levels.
- 936 chemicals had effects on at least one hormone when evaluated in MTC screening.
- Our selection criteria using ≥4 hormones altered in MTC screening identified 91% (211/232) of the chemicals that would have been identified as disrupting steroidogenesis using the OECD guideline criteria of simply detection TESTO and/or ESTRADIOL effects.
- Concentration-dependent effects on at least one hormone were seen for 336 chemicals.
- By quantifying hormone levels across the steroidogenesis pathway, distinct profiles of steroidogenesis disruption can be distinguished by k-means clustering.

The H295R high-throughput model can evaluate large sample libraries to identify chemicals that perturb steroidogenesis. The quantification of hormones across the pathway provides the unique the ability to use a profiling approach to help propose possible mechanisms of action.