H295R Steroidogenesis assay (OECD TG456): a CropLife Europe initiative to review S-modality data contributing to the ED WoE evaluation of active ingredients.

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INTRODUCTION

The H295R steroidogenesis assay is one of several Level 2 (in vitro) assays identified in the OECD Conceptual Framework for Testing and Assessment of Endocrine Disruptors (ED) as providing information on endocrine activity. Since the entry into force of the ED criteria, this assay is regularly requested as part of the ED assessment of active ingredients to determine any potential interference with the steroidogenesis (S)-modality. The assay is designed to provide information on testosterone (T) and estradiol (E) secretion following exposure of the H295R cells to a test item. Concerns have been raised by industry stakeholders about the high incidence of low but statistically significant changes in T and/or E secretion in this assay and the impact such findings have on further testing requirements. Specifically, the S-modality is the only modality with no available follow-up short-term in vivo (Level 3) assay. Moreover, Level 4 pubertal assays are not considered robust enough to clearly identify adversity due to altered steroidogenesis. According to the EFSA/ECHA ED guidance document, a Level 5 reproductive toxicity assay is required in order to contextualize any in vitro steroidogenic activity.

METHODOLOGY

To determine the biological relevance of weak positive H295R steroidogenesis assay outcomes, CropLife Europe (CLE) commissioned a project to take a deep dive into data generated in this assay. Blinded data were compiled for test items, negative controls and the two quality control (QC) reference compounds (forskolin and prochloraz) used in the guideline studies. Anonymised datasets for 60 test items, generated by several contract labs or in house, from a total of 11 companies within CLE, were provided to the service provider (Integrated Laboratory Systems, USA) and used to create an integrated database of all raw H295R data (E and T concentrations and cytotoxicity). This was then interrogated to determine overall assay performance based on the supplied QC data and to identify outcome consistency from various statistical analyses across test items in the database.

RESULTS

A database was compiled with ~16000 data points from the H295R assay for which QC plates accounted for nearly half the data in addition to 60 test items. No outlier or single source of variability in outcome could be attributed to a particular testing site. Guideline criteria were achieved for cell viability based on the QC data used to determine assay acceptability. In contrast, the criteria were not reliably achieved for prochloraz inhibition of E nor for forskolin induction of T. Initial evaluation of the test item data with low fold changes in hormone levels and at least one equivocal conclusion revealed that different statistical analyses could affect interpretation. For example, the guideline requires pairwise p-values from ANOVA with Dunnett's post hoc test; conversely, linear trend testing for concentration-response can yield different outcomes. Thus, pairwise significance testing per concentration may be overly sensitive and result in more equivocal outcomes. Further examination of these data will be presented to determine which of the hormones and the direction of the changes are most impacted by low fold changes and statistical analysis approaches.

DISCUSSION

The H295R steroidogenesis assay (OECD TG456) is one of the Level 2 assays used to provide information on endocrine mechanisms. A compendium of data for reference (QC) compounds and test items was reviewed, to understand the low but statistically significant changes observed in this assay, given the impact that such findings have on further testing requirements. Our analyses reveal that acceptance criteria are not robustly achieved for the reference compounds on QC plates, which suggests that this assay is difficult to conduct within the outlined requirements for induction/inhibition dynamic ranges. Furthermore, the statistical method suggested in the guideline (pairwise testing to controls) may be overly sensitive compared to linear trend methods and may contribute to the number of positive outcomes in this assay, thus leading to a potential increase in the number of Level 5 reproductive toxicity assays to be performed, and consequently increased animal usage. Overall, the unique dataset compiled herein allowed for a comprehensive analysis of the performance in H295R steroidogenesis testing, revealing that limited dynamic range and statistical analyses contribute to the sensitivity and interpretation of the assay.