Charge Questions

The Environmental Protection Agency (EPA) is committed to the use of validated high-throughput (HT) assays and computational models to: i) prioritize chemicals for further Endocrine Disrupter Screening Program (EDSP) screening and testing based on predicted bioactivity; ii) use as alternatives to EDSP Tier 1 assays; and iii) contribute to the weight-of-evidence evaluation of the potential endocrine bioactivity of a chemical.

Androgen Receptor (AR) Pathway Model

In December 2014, EPA and NICEATM introduced an AR pathway model during the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) where it was well received. At the time, the model integrated 9 assays and was evaluated using 23 reference chemicals. In accordance with the SAP's suggestions, the model was expanded and now includes 11 assays and has been evaluated using 65 reference chemicals of varying potencies. The SAP also asked that cytotoxicity and cell stress be monitored and confirmatory tests be employed. In the current model, cell stress and cytotoxicity are assessed using a statistical measure called a Z-score and a second confirmatory assay for AR antagonists was performed and integrated into the model. For a summary of the SAP's comments and the Agency's responses, please see Section 2.5.2 of the White Paper. For a full description of the AR model, see Section 2.

> **Question 1:** Please comment on the Agency's efforts to address the suggestions of the previous SAP, thus confirming the suitability of the current HT AR pathway model to be used as an alternative to the low-throughput (LT) Tier 1 AR binding assay (OCSPP 890.1150).

Steroidogenesis

A number of environmental chemicals have been shown to interfere with the biosynthesis of estrogens (*e.g.*, estradiol) and androgens (*e.g.*, testosterone), and the EDSP Tier 1 screening battery includes several *in vitro* and *in vivo* assays designed to detect compounds that may affect steroid synthesis. One *in vitro* assay in the Tier 1 EDSP battery, the Steroidogenesis Assay (H295R cell-based steroidogenesis assay, OCSPP 890.1550/ OECD TG 456) utilizes human adrenocortical carcinoma cells as a model of adrenal, ovarian, and testicular steroidogenic function and is used currently to screen for potential perturbations in the steroid synthesis of estrogens and androgens. Testosterone (T) and 17β-estradiol (E2) levels are measured in the cell culture medium of chemically-exposed H295R cells, and hormone concentrations in the medium serve as indicators of steroidogenesis disruption.

The EPA has developed HT H295R cell-based assay (Karmaus, *et al.*, 2016) that uses high-performance liquid chromatography followed by tandem mass spectrometry. A comparison of the LT and HT H295R assays for detecting the disruption of synthesis of T and E2 is presented. This comparison enabled evaluation of the utility of the HT H295R assay as an alternative to the LT Tier 1 H295R assay.

As an expanded component of the HT H295R assay, data from 9 additional steroid hormones (including progestagens, glucocorticoids, androgens, and estrogens) were collected (see Section 3 of the White Paper). The data for all 11 hormones were integrated using a novel statistical approach to quantify the overall impact of the chemical on the steroidogenesis pathway. In consideration of both the comparison of the LT and HT H295R assays and the new statistical approach to assess the impact on the steroidogenesis pathway, please address the following charge questions:

> **Question 2:** Based on the comparison of the performance of the HT H295R assay with the LT H295R assay, and the effects of reference chemicals on the synthesis of T and E2 levels only, please comment on the suitability of the HT H295R assay as an alternative to the LT H295R assay. See Sections 3.3 and 3.4.

Question 3: Please comment on the strengths and limitations of integrating multiple hormone responses beyond T and E2 (*i.e.* 11 hormones vs 2 hormones) in a pathway-based analysis of the HT H295R assay. Please comment on the suitability of this HT H295R pathway model (using 11 hormones) to serve as an alternative to the LT H295R assay. See Section 3.7.2.

Question 4: The work herein presents a novel statistical integration of multiple hormone responses indicative of steroid biosynthesis in the HT H295R assay. A summary statistical metric, the maximum mean Mahalanobis distance (maxmMd), has been suggested as a tool for use in prioritization of chemicals. In addition to the use of the maxmMd to indicate the magnitude of potential effects on the steroid biosynthesis pathway expressed in H295R cells, an examination of the hormone responses that contribute to the maxmMd may provide valuable biological information to inform the weight-of-evidence evaluations performed for chemicals subjected to EDSP Tier 1 evaluation. Please comment on the strengths and limitations of using the maxmMd and the pattern of steroid hormone responses in the HT H295R assay for chemical prioritization and weight-of-evidence applications. See Sections 3.2.4, 3.3.2, and 3.7.2.

Thyroid Framework

Over the last several years, EPA has significantly expanded research efforts on thyroid related HT assays, and the design of EDSP's framework for screening of potential thyroid hormone disruptors is in its early stages. Unlike screening for modulators of estrogen and androgen receptors, which captures much of the estrogenic and androgenic bioactivities of xenobiotics; chemicals that perturb thyroid homeostasis may act via one or more heterogeneous targets in the thyroid adverse outcome pathway (AOP) network (see Figure 4-1 in the White Paper). Thus, a larger set of assay targets, beyond just hormone receptors/signaling, should be considered to screen for potential disruption of thyroid hormone-related bioactivity. Currently, a number of assays are available, with several more in development; however, assays do not yet exist to interrogate every molecular initiating event (MIE) in the thyroid AOP network. Also, in contrast to the estrogen and androgen receptor pathway models, it is unlikely that multiple

orthogonal assays for each target (*i.e.*, MIE or key event [KE]) will be available in the near future.

Section 4 outlines a thyroid AOP network (Section 4.2) and presents the current status for high-throughput assays (Section 4.3). The thyroid AOP network aims to serve as a foundation for a future EDSP strategy or framework to identify and prioritize potential thyroid-disrupting chemicals. EPA seeks insights from the SAP on the direction of its proposed approach.

Question 5: Please refer to White Paper Section 4.2. EPA has identified AOPs for thyroid hormone disruption related to potential xenobiotic-induced alterations of thyroid homeostasis. Please comment on the completeness of the MIEs (Table 4-1), KEs, and adverse outcomes within the thyroid AOP network (Figure 4-1). Also, please provide information on any missing pathways, adverse outcomes, or other AOP-related information (*e.g.* MIEs or KEs) critical for capturing the complexity of systems biology controlled by thyroid hormones.

Question 6: Please refer to White Paper Section 4.3. EPA has summarized currently available assays and test guidelines informative of thyroid AOPs and is developing HT assays for a number of MIEs. Please comment on the ranked importance of MIEs (Table 4-3) and on whether assays for environmentally important MIEs are missing, and include information on both the biological and environmental relevance of these MIEs. In addition, please comment on other assays that would supplement or be orthogonal to the assays currently identified in Table 4.3 or for other KEs or AOs in the thyroid AOP framework (Figure 4-2).