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UNIVERSITY
SCHOOL of MEDICINE
THE BOWMAN GRAY CAMPUS

January 8, 2004

Dr. Joseph J. Merenda
Director, Office Science Coordination and Policy
US Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington, DC 20460

Dear Dr. Merenda:

RE: **COMMENTS AND/OR RESPONSE TO QUESTIONS ON THE PUBERTAL FEMALE RAT, PUBERTAL MALE RAT, INTACT ADULT MALE AND AROMATASE SCREENS, LIST OF NEGATIVE COMPOUNDS FOR EDSTAC ASSAYS AND VAGINAL OPENING DIFFERENCES BETWEEN THE US AND THE UNITED KINGDOM.**

Pubertal Rat Female and Male Assays

EPA Questions:

1. *Does the EDMVS agree that the pubertal assays show adequate sensitivity over a range of chemicals for use as a Tier 1 assay? That is, are the pubertal assays ready for an interlaboratory validation study? If not, what specific areas need further prevalidation work?*

   Response:

The key parameter measured in the female pubertal assay is vaginal opening (VO), and in the male, preputial separation (PPS). There is clearly low sensitivity in the female pubertal and the inherent high variability of vaginal opening measurements, making the assay of somewhat questionable value. For example, the Research Triangle Institute's studies were unable to pick up any effect on VO by the weak estrogen Bisphenol A, (Sharpe et al., 2003), at doses where body weight effects achieved or exceeded the default MTD. In addition, both the EPA (Laws *et al.*, 2000) and Ashby (Ashby *et al.,* 2002) found significant delays in VO with doses of atrazine below the 75 mg/kg/day level in the RTI study which speaks further to the lack of sensitivity of female pubertal assay.

Further, there is an apparent lack of specificity for these endpoints for both the male and female pubertal assays since puberty it appears to be influenced by body weight gain

(Trentacost *et al.* 2001), the phytoestrogen content of the diet (Lewis *et al*., 2003) and/or strain of rat used (Ashby *et al*., 2002).

Therefore, we recommend that EPA complete further pre-validation studies of the male and female pubertal assays. These should include the use of non-specific systemic toxicants which do not specifically alter body weight or have the capacity to elicit endocrine manifestations, to test whether the assays are specific for hormonal effects. It will be important to further establish that observed alterations in VO or PPS are produced by a hormonally mediated mechanism rather than just from delayed onset of puberty due to significantly reduced body weight gain and delayed development.

In light of the sort of comments made during the December meeting that "everything may be an endocrine disruptor", it bears restating that definitions of "endocrine disruptor" should be restricted to substances that mimic or inhibit a hormonal action or secretion *by a specific mechanism that can be elucidated*, and that generically toxic substances, or chemicals that only induce general toxicity at MTD levels, should not be considered as true endocrine disruptors. Although many physiologic functions, particularly those involving reproduction (e.g. puberty, ovulation, fertilization, pregnancy) require support of numerous hormones, they also rely upon proper function of every other body system. Tier 1 screens must have the ability to distinguish specific endocrine disruptors from other kinds of toxicity (i.e., minimize the incidence of false positives), because follow-up Tier 2 testing will be much more difficult and expensive for registrants to conduct.

2. *Based on the currently available data, are there endpoints that should be added or dropped from the assay?*

Response:

CLA advocates weight-of-evidence criteria to trigger the necessity of Tier 2 studies based on the results of Tier I screens. Decisions to move to Tier 2 studies should not be triggered on the results of a single positive study, especially those from the pubertal assays where changes in apical endpoints (onset of puberty, organ weight changes) may have been obtained only at doses above the MTD.

Comments on the Intact Male Assay.

From the information presented and/or published by Dr O'Connor, it would appear that the intact adult male assay should continue to be included in the validation process of the Tier I screening battery. The Agency should perform a side-by-side evaluation of the pubertals and intact male for all the prevalidation work rather than a partial review as presented by Dr. Gray. This could be invaluable in determining whether or not the intact male is a viable alternative to either or both female and male pubertal assays.

It is clear that the intact male is capable of providing mechanism of action data for a broad array of EAT hormonally mediated endpoints with comparable or better sensitivity than the pubertals (O'Connor et al., 2002). Further, the intact male assay is shorter in duration (15 days vs. 40+ days in the pubertal assays) and provides a screening platform of a sexually mature less variable male than either of he pubertals assays.

In addition, and perhaps more importantly, inclusion of the intact male in the Tier 1 screen may have the potential to reduce and refine animal use, and reduce resource commitment by replacing the Steriodogenesis (and alternative aromatase assay), Hershberger assay and Female (or male) pubertal assays.

Aromatase Questions

1. *Does the EDMVS agree that the prevalidation studies demonstrate that both the placental and recombinant assays are effective in identifying chemicals that inhibit aromatase?*

Response: Yes.

2. *EPA is planning a series of studies to better define and control the sources of variability observed in the work discussed today. Pending the successful resolution of this, does EDMVS believe that prevalidation has been successfully completed for both assays? In not, what additional studies should be performed before beginning interlaboratory studies?*

Response: Yes.

3. *Should EPA continue to validate both the placental and recombinant assays?*

Response:

Although there might be some merit to validate both assays, the recombinant assay would appear to provide a more stable and standard assay and avoid the potential confronting of the variability associated with human placental materials derived from a variety sources which might contain polymorphic forms of aromatase.

4. *If the answer to question 3 is to continue the validation of the placental assay, should the preparation of placental microsomes be included as a parameter in the interlaboratory studies?*

Outline of Validation Study Plan

- *Assumptions:*

– *Need to validate both assays–Select 5 laboratories (RTI +4 labs)*
– *Need to include validation of the preparation of placental microsomes*
– *Conduct studies in triplicate–Use 6-8 chemicals in coded study including one negative chemical*

- *Approach:*

1. *Demonstrate estrone production and inhibition of aromatase activity using various concentrations of positive control (4-OH ASDN) with recombinant and centrally supplied placental microsomes*
2. *Run coded chemical studies with centrally supplied placental microsomes and recombinant microsomes*
3. *Labs 2 and 4 will prepare placental microsomes and supply to labs 3 and 5.*

The four labs will repeat the coded chemical study with these microsomal preparations.

Response: Yes.

List of Negative Chemicals:

A sample list, only substances beginning with the letter A, that should be devoid of inherent EAT endocrine activity is attached. This was found in the "Generally Recognized as Safe", or GRAS Food Additives List. The full list from A to Z can be found at: http://vm.cfsan.fda.gov/~dms/eafus.html. In addition, we are providing a link to the FDA's slide presentation on GRAS Food Additives for a better understanding; http://www.fda.gov/oia/embslides/additives. A similar list of pharmaceuticals and pesticides could be developed, on a more selective basis, but the generalized GRAS list avoids the potential of goring someone's sacred cow. There are certainly thousands of substances, many extremely toxic, that do not interact directly with any hormonal system.

Vaginal Opening Differences Between US And United Kingdom:

We have contacted Dr. John Ashby regarding the later age of vaginal opening observed for control Sprague-Dawley and AP Wistar pubertal female rats in the study [Ashby J, Tinwell H, Stevens J, Pastoor T, Breckenridge CB. The effects of atrazine on the sexual maturation of female rats. *Regul Toxicol Pharmacol.* 2002; 35(3):468-73.] that was called into question during our December EDMVS meeting. John has agreed to provide the Subcommittee with a concise position paper that will explain the reasons for these differences. In the meantime, we offer this linked abstract from *Experientia.*1991; 47(10):1027-38 (attached).

VO paper abstract.doc

This paper suggests a dichotomy exists, for undefined reasons, in the timing of preferred vaginal opening in certain rat strains: an early period, at 31-35 days, and a late period, at 36-40 days.

Thank you for the opportunity to comment.

Cordially yours,

J. Charles Eldridge, Ph.D.
Professor of Physiology and Pharmacology

James T. Stevens, Ph.D.
Professor of Physiology and Pharmacology

References:

Ashby J, Tinwell H, Stevens J, Pastoor T, Breckenridge CB. The effects of atrazine on the sexual maturation of female rats. *Regul Toxicol. Pharmacol*. 2002; 35(3):468-73.

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