SAP Minutes No. 2007-04

A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

Status of the In Utero Through Lactational Assay in the Endocrine Disruptor Screening Program (EDSP)

February 27-28, 2007
FIFRA Scientific Advisory Panel Meeting
held at the
Environmental Protection Agency Conference Center
Arlington, Virginia

Notice

These meeting minutes have been written as part of the activities of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP). The meeting minutes represent the views and recommendations of the FIFRA SAP, not the United States Environmental Protection Agency (Agency). The content of the meeting minutes does not represent information approved or disseminated by the Agency. The meeting minutes have not been reviewed for approval by the Agency and, hence, the contents of these meeting minutes do not necessarily represent the views and policies of the Agency, nor of other agencies in the Executive Branch of the Federal government, nor does mention of trade names or commercial products constitute a recommendation for use.

The FIFRA SAP is a Federal advisory committee operating in accordance with the Federal Advisory Committee Act and established under the provisions of FIFRA as amended by the Food Quality Protection Act (FQPA) of 1996. The FIFRA SAP provides advice, information, and recommendations to the Agency Administrator on pesticides and pesticide-related issues regarding the impact of regulatory actions on health and the environment. The Panel serves as the primary scientific peer review mechanism of EPA, Office of Pesticide Programs (OPP), and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. Food Quality Protection Act Science Review Board members serve the FIFRA SAP on an ad hoc basis to assist in reviews conducted by the FIFRA SAP. Further information about FIFRA SAP reports and activities can be obtained from its website at http://www.epa.gov/scipoly/sap/ or the OPP Docket at (703) 305-5805. Interested persons are invited to contact William Wooge, SAP Designated Federal Official, via e-mail at wooge.william@epa.gov .

In preparing the meeting minutes, the Panel carefully considered all information provided and presented by the Agency presenters, as well as information presented by public commenters. This document addresses the information provided and presented by the Agency within the structure of the charge.

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Steven G. Heeringa, Ph.D.
FIFRA SAP Chair
FIFRA Scientific Advisory Panel
Date: May 29, 2007

William E. Wooge
Designated Federal Official
FIFRA Scientific Advisory Panel
Date: May 29, 2007

Federal Insecticide, Fungicide, and Rodenticide Act
Scientific Advisory Panel Meeting
February 27-28, 2007

Status of the In Utero Through Lactational Assay in the Endocrine Disruptor Screening Program (EDSP)

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INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) has completed their review of the status of the *In Utero* Through Lactational Assay in the Endocrine Disruptor Screening Program (EDSP). Advance notice of the meeting was published in the *Federal Register* on December 13, 2006. The review was conducted in an open Panel meeting held in Arlington, Virginia, from February 27-28, 2007. Dr. Steven G. Heeringa chaired the meeting. William Wooge served as the Designated Federal Official.

The FIFRA SAP met to consider and review the status of the *In Utero* Through Lactational Assay in the Endocrine Disruptor Screening Program (EDSP). The Agency was seeking input from the Scientific Advisory Panel on the design and methodology employed in the assay, the power of the assay, and areas for future research.

EPA is implementing the Endocrine Disruptor Screening Program (EDSP) in response to a 1996 Congressional mandate in the Federal Food, Drug, and Cosmetic Act (FFDCA) to establish a screening program using validated assays to identify pesticides that may have estrogenic effects in humans and other endocrine effects, as designated by the EPA Administrator. The Agency also has authority to include other non-pesticide chemicals that have an effect cumulative to that of a pesticide to which a substantial human population may be exposed. In developing the EDSP, EPA considered the recommendations of the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), a panel chartered pursuant to the Federal Advisory Committee Act (FACA). The screening program was also reviewed in 1999 by a joint meeting of EPA's Science Advisory Board and FIFRA Scientific Advisory Panel (SAB/SAP), as required by the FFDCA. It was recommended that EPA address both human and ecological effects and examine effects to estrogen, androgen, and thyroid (EAT) related processes, and that a two-tiered approach be used for screening. The purpose of the Tier-1 battery is to identify substances that have the potential to interact with the endocrine system. The purpose of Tier 2 is to confirm the interaction, identify any adverse effects, and establish quantitative relationships between dose and adverse effects.

Both the EDSTAC and SAB/SAP recognized the importance of chemical exposure during development *in utero* as well as during lactation and, therefore, recommended an *in utero* through lactational animal model to detect effects that may result from prenatal and early postnatal exposure. The EDSTAC and SAB/SAP also recommended that any *in utero* through lactational bioassay should be developed in a way that would allow for replacement of one or more of the other assays proposed for the Tier-1 screening battery.

The EDSP commissioned an *In utero* through lactational Detailed Review Paper (DRP) that consisted of an extensive review of the scientific literature regarding chemicals known to disrupt the EAT hormone systems during pre- and postnatal development. The DRP presented three *in utero* through lactational bioassay protocols

for possible inclusion in the EDSP. In 2001, EPA presented the DRP and its recommendations to the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) for review and discussion. The most comprehensive of the three protocols was chosen and tested with methoxychlor, a positive compound that is known to have estrogenic, anti-estrogenic and anti-androgenic effects. In general, the EDMVS agreed with this pre-validation approach with the expectation that EPA would return to a federal advisory committee such as the SAP to review and discuss the results of the *in utero* through lactational study with methoxychlor.

The purpose of the current meeting was to allow the SAP to review and discuss the protocol and assay results of an *in utero* through lactational study with methoxychlor within the current context of the EDSP and to provide advice that will inform EPA's decision to continue, modify or suspend the development of an *in utero* through lactational bioassay as a screening assay in a Tier-1 battery. The agenda for this SAP meeting included presentations on:

- ❑ An overview of the endocrine disruptor screening program/introduction to the *in utero* through lactation assay by Gary Timm, Office of Science Coordination and Policy, EPA
- ❑ The development of the *in utero*/lactational assay for the U.S. EPA EDSP by Rochelle Tyl, Ph.D., DABT, RTI International
- ❑ The *In utero* through lactational assay from a research perspective by Earl Gray, Ph.D., Office of Research and Development, EPA
- ❑ Life-stages developmental and reproductive test enhanced to detect endocrine effects in the EDSP Tier 2 by Ralph Cooper, Ph.D., Office of Research and Development, EPA
- ❑ Overview and specific charges to the SAP by Don Bergfelt, Ph.D., Office of Science Coordination and Policy, EPA

PUBLIC COMMENTERS

Oral statements were presented by:

Richard A. Becker, Ph.D. D.A.B.T., on the behalf of the American Chemistry Council

Written statements were provided by:

B. Sachau, on her own behalf

PANEL DELIBERATIONS AND RESPONSE TO CHARGE

The specific issues addressed by the Panel are keyed to the background documents, references, and the charge questions provided by EPA.

Charge Question 1. Considering the current biology and logistics, time, cost and other resources involved in conducting an *in utero* through lactational screening assay, please comment on the need, strengths, weaknesses and practicality of developing and validating the bioassay as an EDSP Tier-1 screen.

Panel Response

There are no assays within Tier 1 of the EDSTAC-recommended screening and testing scheme that assess all three hormone systems following full life cycle developmental exposures. This should not be construed as a conclusion of the EDSTAC that no merit existed in evaluating exposure to endocrine-active substances over the broad range of life stages, but, rather, an acknowledgement that no such "screening" assay existed that could be used or modified easily for inclusion at that time.

Nonetheless, during the course of EDSTAC's deliberations, its Screening and Testing subcommittee did consider what characteristics a protocol for such an assay might contain. [See Appendix O of the EDSTAC report]. This initial discussion led to follow-up efforts resulting in the three protocols described in the DRP, one of which (Protocol C) was modified to use in the (pre)validation process. Then, as now, the EDSTAC Subcommittee wrestled with the reality that a credible study design was likely to be more complex than one ordinarily considered to be a "screen." The Subcommittee often referred to this design as one that would fall into Tier 1.5: too much for Tier 1 but not quite enough for Tier 2.

Need: Why was a study design incorporating full life cycle developmental exposure considered to be of potential value by EDSTAC? As several of the background documents note, concern had been expressed that even with the broad range of the recommended assay systems (and the alternatives) proposed for Tier 1, something important might still be missed. If the weight of evidence assessment of the results in the existing screening battery supported the conclusion that the agent of interest did not exert effects on any of the three hormone systems, then there would be no appropriate trigger to move to Tier 2 and more in-depth testing, which includes protocols that incorporate full life cycle developmental exposures. In this case, a false negative outcome would result.

So, the question before the SAP now in 2007 is whether or not we have learned enough about the validity and predictive value of the original elements of Tier 1 to answer the concern about missing something important if an assay such as the In Utero/Lactation (IUL) is not a part of Tier 1. The Panel felt that the data provided on the occasion of the present meeting were inadequate to make a final judgment on the utility of the Protocol C IUL assay, that is, where might it best fit into a screening and testing strategy.

Nonetheless, there was consensus that Protocol C, as presented to the Panel, would not be suitable as a Tier 1 screen. It is too complicated and expensive to fit the criteria as a Tier 1 screen. It is recommended that if an IUL exposure screen were to be developed, it should be simplified. One suggestion is to develop a Protocol B-like assay which would incorporate exposure only to the dams during gestation and lactation, without further exposure of the offspring post-weaning, acknowledging that even this protocol may also be too complex to qualify as a Tier I screen.

Strengths: A strength of an IUL assay would be the ability to detect endocrine disrupting chemicals (EDCs) at a sensitive life stage that would not be detected through the other proposed Tier 1 assays.

Weaknesses: A weakness of Protocol C is that it is too complex, perhaps asking too much with too few animals. In addition, it is costly, totaling more than the sums of the costs of the individual Tier 1 screens it would replace (i.e., the uterotrophic, Hershberger and male and female pubertal assays).

Practicality: While the prevalidation study showed that the protocol can be technically conducted as written, this assay is too complex for a Tier I screen.

Cost: As noted above, its cost exceeds that of the sum of the costs of the assays it would replace.

Charge Question 2. Please comment on the basis for supporting continued development and validation of an *in utero* through lactational screening assay using Protocol C in the EDSP Tier-1 battery.

Panel Response

The Panel was not confident that any of the proposed protocols (A, B, C) would find utility as a routine Tier 1 screen. If thousands or even hundreds of substances are to be subjected to a battery of Tier 1 endocrine disruptor screens, inclusion of the IUL assay would add exorbitant costs to the registration process. Initial screens should be much less costly, more specific, rapid and highly reproducible in many laboratory settings.

That said, there is no sentiment to exclude *in vivo* testing in Tier 1. Indeed, it was recalled that EDSTAC recommended in vivo screening using additional animal subphyla and classes besides mammals. A positive response in one or more of these might also identify candidates for an IUL protocol.

It was suggested that a simplified "*in utero*" or a "lactational" protocol could become a useful option to regulators and applicants. It could be used frequently, but not as a mandatory screen of every chemical (i.e., as a Tier 1.5 test). Perhaps the term "adjunct test" would be more useful. It was not entirely clear to the Panel where this sort of protocol belongs in the overall scheme of EDSTAC, nor was it certain whether a full validation of "optional" or "adjunct" tests should be necessary.

As described in the response to Question 1, a less comprehensive IUL protocol (closer to Protocol B) might be preferred over Protocol C, while still covering the critical in utero period of exposure. Exposure to chemicals during the fetal stage through the maternal route could alter the disposition of subsequent pre- and postweaning exposures after birth. For example, imprinting of enhanced or repressed biotransformation enzymes may occur in utero and could change the disposition of subsequent postnatal exposures (Lucier et al. 1979). Biotransformation in the dam could lead to a different metabolite profile in the developing fetus relative to exposure in a naïve newborn animal. Biotransformation profiles can be quite unique between fetal and newborn animals (Stevens et al. 2003; Duanmu, et al. 2006). The fetus has a unique potential for endocrine disruptor assessment which may not be observed in newborn, pubertal or adult assays.

The Panel was quite concerned about a successful validation of any of the proposed IUL protocols. The modified Protocol C, from which results of the validation trial were presented, encountered serious problems for the contracting laboratory. Validation implies that a very precise, detailed protocol can be developed and certified to be successfully run in a variety of laboratory settings. Experience presented from the methoxychlor trial suggested this would be difficult to achieve. Indeed, a minority of the Panel seemed convinced that any serious validation of these protocols will be far too complex and expensive. It could cost many millions of dollars, testing several rodent strains and several test chemicals in each strain.

However, a majority of the Panel felt that a modified Protocol B could be validated as a simpler, effective screen, or as a "Tier 1.5" test, as discussed above. Another suggestion was to return to validation of more specific critical endpoints (e.g. anatomic development, estrous cycling patterns, fecundity of each sex), or of specific phases of life, as discussed above. Simpler, limited protocols might be validated with confidence. It was agreed that sufficient statistical power needs to be a core component of validation, and that it must be determined in advance of additional validation trials.

Yet another suggested approach was for the Agency to rely more heavily on literature and creative problem-solving between the applicant and Agency scientists. Perhaps one could select "customized" versions of IUL assays, based on Tier 1 screen results and current status of published information.

The Panel heard that many Tier-2 tests include a multi-generation component, which would of course include the IUL periods, but uncertainty remained whether sufficient endpoints would be covered in a large, complex Tier-2 test design. Furthermore, reliance on Tier 2 protocols still fails to identify a solution for instances when a chemical is negative in all Tier 1 screens, and, thus would not be subject to any Tier 2 testing.

In summary, while devising a method to obtain the type of information provided by elements of Protocol C seems to be important and useful to the Agency, and to EDSTAC's original outline, enthusiasm to continue work on any specific protocol was

not strong, and particularly for Protocol C. While a large-scale, multi-factorial protocol brings numerous benefits, it also brings numerous problems, particularly when it is utilized in the regulatory process.

Charge Question 3. Please comment on the basis for supporting development and validation of a revised Protocol C or alternative Protocols A or B or some other protocol for an in utero through lactational screening assay in the EDSP Tier-1 battery. What are the strengths and weaknesses of a revised Protocol C or alternative protocols and would this revised in utero through lactational screening assay be considered an alternative assay, if so, replacing what assays, or an additional assay in the EDSP Tier-1 battery?

Panel Response

The SAP's discussions reflect the conflict that may exist between scientists belonging to two distinct experimental cultures dealing with environmental endocrine disrupting chemicals (EDCs): toxicologists and developmental biologists each would like to have their respective premises prevail. On the one hand, some toxicologists consider EDCs as toxic, poisonous chemicals. On the other, many developmental biologists consider EDCs as modulators of developmental processes. A constructive dialog should be encouraged for the benefit of all stakeholders interested in a successful resolution of the regulatory enterprise dealing with the complex medical and social issues relating to this category of substances.

Exposure to endocrine disruptors does not result in acute, blatant effects, but rather contributes to the morbidity of wildlife and humans in a slower, even "silent" way. EPA's concern is based on what these EDCs may do to us both in the short- and long-term. To identify these substances, the concept of a two tier screening and testing strategy has been devised. We are here concerned with proposing a set of reliable, quick assays for a Tier-1 stage and a more complex, *in vivo* Tier-2 stage in which the information provided by the Tier-1 assays could be confirmed and additional data gathered and evaluated using one or more suitable *in utero*/lactational assays (IUL). This combination would give the regulators a defensible position before Congress and the public.

The highly intensive human-power project competently carried out by Dr Rochelle Tyl and her colleagues at RTI provided an insight into the type of multivariable protocol required to evaluate the properties of a test substance (in this case, methoxychlor). It was the consensus of members of the panel that a) not enough information was available, and b) some of the data was contradictory and counterintuitive.

In the discussants' view, the proposed bioassay in any of its variants (A, B and C) is, at its current stage, a highly complex, worthy *research* topic that deserves funding support from EPA and/or any of the branches of the NIH that deal with the multiple aspects of EDs. However, while there may be a need for an *in utero* assay in Tier 1, Protocol C as it stands is much more like a Tier 1.5 or 2 assay. Based upon the inability of Protocol C to differentiate the potential for effects of *in utero* or postnatal exposure, a much more

simplified assay similar to Protocol B could be a more modest but highly informative assay. The DRP recommended Protocol B as the best fit for a Tier 1 assay to assess *in utero* exposure. Because *in utero* exposure is unique, it cannot replace any of the *in vivo* tests which occur after birth. The Panel was puzzled as to why Protocol B was not the choice for the pre-validation exercise, rather than Protocol C.

The development of these and alternative experimental designs will enrich substantially our understanding of these compounds and potential entry into the environment where wildlife and humans live. However, they do not adequately fulfill the needs for a Tier-1 screening strategy.

As an additional response to Charge 3's request for comment on an alternative or additional assay in the battery, one panel member mentioned a bioassay that could be a component of the EDSP Tier-1 battery (but not specifically a replacement for the IUL bioassay). This panel member noted that this bioassay, called the E-SCREEN, could fulfill the first three EDSTAC defined criteria for Tier-1 screening assays (see presentation by Don R. Bergfelt). This panel member (who acknowledged his affiliation with the E-SCREEN bioassay) believes the E-SCREEN would be quick, inexpensive and easy to perform. This panel member also noted that the E-SCREEN bioassay has been validated by at least two multi-laboratory studies (Andersen et al. 1999; Rasmussen et al 2003) and a number of labs have adopted the E-SCREEN as a Tier-1 screen, both in the US and abroad.

Charge Question 4. Please comment on the basis for supporting suspension of the present course for development and validation of the *in utero* through lactational assay as an EPA screening assay in the EDSP. What research would be useful in developing an *in utero* through lactational assay that would support reconsideration of the bioassay as a *screen* in the Tier-1 battery?

Panel Response

The decision to go forward, or suspend work, on the IUL protocol require the same types of information and the two questions are linked.

The Panel needed four critical pieces of information in order to aid EPA in its deliberations on whether the IUL protocol should be further developed or suspended.

1. What is EPA's definition of a Tier 1 screening assay?

2. What components make up the Tier 1 screening battery?

3. What is the utility of the IUL protocol and has it been successfully demonstrated?

4. What is the experimental content of the mammalian Tier 2 test(s)?

1. The definition of a screen. EPA has provided this information to the panel. A screen should discriminate between substances likely to affect the endocrine system and those not likely to affect it. It should be inexpensive, quick and easy to perform. The screens should be standardized and validated and more sensitive than specific (to avoid false negatives and minimize false positives). The assays should capture multiple end points reflecting the many modes of endocrine action and yield data that can be interpreted as positive or negative to move forward (on a weight of evidence basis) to the Tier 2 test(s).

Specific for the IUL assay is that it should have the ability to replace one or more of the other assays currently being considered for inclusion in the battery of Tier 1 screens and be evaluated for its overall cost effectiveness.

If a screen is to provide only a signal of activity, rather than hazard identification and dose response information, then the IUL seems more like a test – i.e., it does identify hazards and adverse responses (but so do the pubertal assays also in Tier 1) and potential malformations. Critically, an *in vivo* study like the IUL does provide an integration of responses from numerous potential mechanisms; it overcomes some of the issues with ADME etc. Moreover, if a large number of screening end points could be incorporated in one study, this could reduce the complexity and numbers of screening studies to be conducted in Tier 1.

2. What is the constitution of the EDSP Tier 1 screening battery? This is not yet known. Without this knowledge, the panel cannot know what the potential IUL protocol could replace and therefore how it might be developed (or suspended).

It is well accepted that some of the more profound and sensitive events induced by the types of agents for which EPA has constructed the EDSP program to evaluate (EAT's), occur after exposure during critical windows of development. Is such an exposure paradigm likely to produce a more sensitive evaluation of potential activity? Should this exposure paradigm only be undertaken during the testing portion of the program? The answers to these questions depend on the format and content of the Tier 1 screening battery. The Tier 1 battery is expected to identify agents that work through the major modes of action identified with EAT's. If the mammalian battery proposed by EDSTAC is validated and adopted (ER and AR binding/transcriptional activation, H295 steroidogenesis assay *in vitro*; uterotrophic assay, Hershberger assay, and female pubertal study), then this battery would be unlikely to detect with confidence phthalate esters (which have shown to inhibit fetal testicular testosterone production to produce reproductive tract malformations) and potentially aromatase inhibitors. The only member of the aromatase inhibitor class evaluated in the proposed screens, fenarimol, was negative in the female pubertal study, yet in a multigeneration assay produced the expected effects on parturition associated with lowered estrogen levels after aromatase inhibition.

No responsible investigator would advocate the undertaking of a major tier 2 test without some kind of dose range finding study in pregnant animals. There is a large

difference in costs between Tier 1 screens and a Tier 2 test. There is a perceived need for an intermediate stage between screening and testing, or for use as an adjunct. The IUL protocol, suitably modified, could fulfill this role and extend the end points assessed in other Tier 1 screens, but it would be low throughput. However, the concept of a single assay to replace multiple optimized assays could be dangerous and not provide the appropriate sensitivity for each of the end points that would be required to be assessed in the battery.

3. What is the utility of the IUL protocol and has it been successfully demonstrated?

The data provided are inadequate to make a judgment on the utility of the IUL assay at this time. Only one compound has been run through the assay and this did not provide the expected results. At this time the Panel cannot ascertain if this is a fault of the protocol (it is complex and lengthy and should be simplified) or if there were operational issues. It did not illustrate, in the demonstration study, the value of moving to this protocol type with its unique exposure regime, as a component of the Tier 1 battery.

4. What is the Tier 2 test?

The EDSTAC realized that the current mammalian multigeneration study would have to be improved to meet the needs of EDSP as a Tier 2 test in developing the appropriate hazard and dose response information for risk assessment. EPA is considering whether to utilize the ILSI/ACSA protocol as a substitute for an enhanced multigeneration reproduction study. In its published form (Cooper *et al.* 2006), the ILSI/ACSA protocol is not a suitable replacement for the multigenerational study as a Tier 2 test. This protocol requires significant work, effort and modification as was pointed out by Dr Cooper in his presentation of the recent EPA efforts to modify the published design. The Panel has concerns with this protocol in three areas: **a) the dosing regimens employed** in pre-breed males could divorce structure from function, which, for example, would be particularly important in classification and labeling in Europe; **b) the use of triggers**; whether these should be **"opt in"** as proposed, which has not proven to be successful in the current multigeneration study and in many instances entails a very short period of time to evaluate data and decide on the incorporation and conduct of a triggered end point; versus an **"opt out"** approach where most, or all, end points would be evaluated by default, unless specific information was available to trigger removal from the study and **c) the retention of more animals to adulthood** for evaluation (as demonstrated in Dr Gray's presentation on experimental power) including histopathology, to become the Tier 2 test of choice.

It is likely that this new protocol would require a validation exercise. This is likely to be very expensive. The protocol may also suffer from the "too many ornaments on the Christmas tree syndrome!" in trying to cover numerous end points rather than reproduction in parents and offspring together with evaluations of postnatal development as a principal outcome as in the current multigeneration design. The addition of neurotoxicity and immunotoxicity end points could decrease the animals

available for reproductive evaluations and with the limited numbers proposed for these non-reproductive end points, it may be problematic in having sufficient experimental power to detect subtle changes. However, when EPA formulates a battery of Tier 1 screens, it would be unwise not to tailor the Tier 2 test(s) to reflect the screening battery results and outcomes.

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