OIRA SHOULD NOT BUY A PIG IN A POKE: CRE'S COMMENTS ON EPA'S RESPONSE TO PEER REVIEW OF THE ER BINDING ASSAY

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CRE Comments on EPA's Response to Peer Review of the ER Binding Assay

The Center for Regulatory Effectiveness ("CRE") submits these comments to OMB's Office of Information and Regulatory Affairs ("OIRA"). CRE asks OIRA to consider these comments during OIRA's review of EPA's Information Collection Request for Tier 1 of EPA's Endocrine Disruptor Screening Program. EPA first made its peer review response for the ER Binding Assay publicly available on July 9, 2009. Given the complexity and length of the documents involved, CRE could not have submitted these comments on EPA's peer review response before now.

1) **There is still no final peer review for the ER Binding Assay because EPA's final assay protocol is not available to OIRA or to the public, assuming there is a final protocol.**

EPA states in the ER Binding Assay Peer Review Report that

> "The final peer review for the ER binding assay will include this peer review report consisting of the peer review comments, as well as documentation indicating how peer review comments were addressed by EPA, and the final EPA work product."[1]

EPA has changed the ER Binding Assay in response to the Peer Review comments.[2] To the best of CRE's knowledge, "the final EPA work product" (*i.e.*, the final EPA assay protocol for the ER binding Assay), is not publicly available and has not been sent to OIRA. Consequently, there is no final peer review for the ER Binding Assay, and OIRA should not approve an ICR for this assay.

2) EPA should validate the final ER Binding Assay protocol before it is used.

EPA Response to Peer Review states at page 53:

> "The Agency recognizes that the ER-RUC assay is sensitive to small changes in techniques, and that ideally a validation study would be conducted only on the final protocol. However, the Agency also recognizes that the time it takes to conduct such a validation study would delay the Screening Program significantly. In its judgment, the modifications to the protocol are not of such magnitude that a new validation study is required."

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[1] Page 1-3 of Peer Review Results for the Estrogen Receptor (ER) Binding Assay (April 13, 2009), available online at http://www.epa.gov/endo/pubs/er-binding_peer_review.pdf

[2] EPA Response to Comments on ER-RUC Assay, *e.g.* pages 19, 46, 49, 50, available online at http://www.epa.gov/endo/pubs/assayvalidation/er-ruc_response_to_comments.pdf

EPA admits that "small changes in techniques" can affect the performance of the ER Binding Assay, and that the assay has been changed in response to peer review and for other reasons. EPA further admits that the best science would be to conduct validation "only on the final product:" *i.e.,* the final assay protocol. However, EPA is not validating the final assay protocol because EPA is in a hurry to get Tier 1 test orders out, even at the sacrifice of sound science. Sound science should not be tromped by EPA's internal schedule.

EPA also thinks that the changes it's made to the assay protocol aren't sufficient to warrant further validation. How do we know that the changes in the ER Binding Assay protocol "aren't sufficient to warrant further validation"? Until EPA produces a final ER binding assay protocol for public and OIRA review, there is no way of knowing whether EPA is right.

In other words, EPA is asking OIRA and the public to buy a pig in a poke.[3] You don't know what you're paying for, or what you're going to get.

3) Where is the final assay protocol and peer review record for the H295R Assay?

The final ER Binding Assay protocol is not the only one missing in action.

The Peer Review Report for EPA's H295R EDSP assay includes the following comment, "Overall, the test guideline has the potential to be a screening tool for steroidogenesis but requires further testing and refinement."[4]

EPA's only response to this comment is "No response needed."[5]

Similarly, EPA responds to 10 other significant peer review criticisms of H295R by stating, "This is being investigated and the protocol modified, if appropriate." To the best of CRE's knowledge, EPA is still investigating these peer review criticisms of the H295R assay. EPA's responses to the many peer review criticisms of H295R acknowledge that the test needs work and is not final.

This needs-work conclusion is consistent with the following EPA response to a peer review criticism:

> "There will be a complete and separate H295R protocol after this assay undergoes peer review both by the US-EPA and OECD which will combine all of these aspects."[6]

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[3] http://en.wikipedia.org/wiki/Pig_in_a_poke

[4] *Disposition to Peer Review Panel Comments on the H295R Steroidogenesis Assay*, last page, available online at http://www.epa.gov/endo/pubs/assayvalidation/peerreview_responses.pdf

[5] Id., last page.

[6] Id., Topic 2.4.1

To the best of CRE's knowledge, H295R has not been additionally peer reviewed by EPA and the OECD. To the best of CRE's knowledge, EPA has not given OMB or the public any final H295R assay protocol that responds to the many critical peer review comments on the draft protocol.

Consequently, there is no final and complete peer review of H295R, and OIRA should not approve an ICR that includes this assay. Once again, EPA is asking OIRA and the public to buy a pig in a poke. Actually, this poke is full of pigs.

4) Where are the final Assay Protocols for all the rest of EPA's proposed Tier 1 Assays?

CRE has been unable to find final protocols for most of the rest of EPA's proposed Tier 1 assays. To the best of CRE's knowledge, EPA has not submitted final EDSP Tier 1 assay protocols to OIRA for purpose of ICR review, or for any other purpose.

Under EPA's own standards quoted above, peer review is not complete for any EDSP Tier 1 assay until and unless there is a final validated assay.

Given the current incomplete record, OMB should not approve EPA's proposed EDSP Tier 1 ICR.

5) The Tier 1 tests will likely yield inconsistent results, and EPA does not know how to weigh the test results.

In EPA's Response to Peer Review on the ER binding assay, EPA states at page 54-55:

> "The Agency recognizes that there is the potential for apparently non-concordant results between the several assays in the Tier 1 Battery that are relevant to the estrogen hormone system. Interpretation of such results will depend on the particular circumstances of the studies. For example, were each of the studies well-conducted with little variability between replicates or were there substantial differences in reliability between studies? Also, these Tier 1 assays do not give sufficient information to determine precise mechanisms of action of a test chemical, so there may be instances where apparent inconsistencies may have a plausible explanation. For example, since the transcriptional activation assay integrates the results of more intracellular processes than simple binding to the receptor, it is possible that a chemical could be positive in the binding assay but negative in the transcriptional activation assay. A complete description of how all of the details available to the Agency will be combined into a weight-of evidence determination is not feasible.
>
> See also the response to comment 1.4."

Peer Review Comment 1.4 and EPA's response to it state:

Peer Review Comment

> "What is less clear is the weight to give to the result obtained for an unknown chemical using the ER-RUC assay (interactive or not with the ER) within the battery of the Tier-1 program.
>
> It should be interesting to give, in the introduction of the integrated summary report (ISR) (page 2, under C. "The Tier1 battery of assays"), a description of the strategy that will be used to classify a chemical as negative or positive after the Tier 1 screening, that includes various in vitro and in vivo assays (ISR, page 3, table 1), and to give the weight of each assay in the final decision of the Tier 1 screening."

EPA Response

> "The 'weight of evidence' evaluation of the Tier 1 Battery will depend on the specific data and circumstances for a specific chemical, taking into consideration, for example, in vitro/in vivo discrepancies (if any), metabolism, and route of exposure. No general statement can be made about the weight to be given to the ER-RUC assay."

In summary of the above, EPA concedes that the different Tier 1 assays may give inconsistent results (positive or negative or inconclusive) for the same substances. The only purpose of Tier 1 assays is to determine which substances have to conduct Tier 2 testing. Whether a substance is subject to Tier 2 testing will be determined on a "weight of evidence" standard.

Yet EPA cannot provide any meaningful guidance on how this standard will be implemented to yield data that have utility.

OIRA should not approve EPA's proposed EDSP ICR because EPA cannot explain how the test data will be interpreted and used, and because EPA does not have a plan for using the test data. Therefore, the tests lack practical utility.

6. EPA should be using human recombinant assay instead of rat uteri.

Peer reviewers commented, and EPA agreed, that a human recombinant assay is a far superior test to using rat uteruses. A representative comment and response follow:

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Peer Review Comment

"The main weakness of the assay comes from the use of animals to prepare the binding fraction, with not only ethical, but also technical consequences (lack of reproducibility in receptor preparations). The solution is the use of recombinant ER binding assays, using both alpha and beta isoforms of the ER."

EPA Response

"The Agency agrees that it is appropriate to investigate the use of human recombinant ER as a potential replacement for the cytosol preparation used in the ER-RUC assay. It is currently participating in an international, multi-laboratory effort to validate the hrERα assay for use in screening. However, availability of recombinant ERβ for widespread screening purposes is limited due to patent considerations."

EPA has not explained how "patent considerations" preclude a human recombinant assay. Moreover, patent considerations haven't prevented the following validation processes:

- The OECD is working on 2 test methods using human recombinant ERalpha

- CeeTox is working with NEERL to validate a human recombinant ERalpha assay (Wilson, VS., Judith E. Schmid, JE, David P. Blakeman, DP, Jeffrey F. Pregenzer, JF, McKim JM, Jr, Goldman, JM, and L. Earl Gray, Jr. 2007. Evaluation of an *In Vitro* Pre-screening Strategy to Prioritize Environmental Chemicals for Further Testing: Androgen and Estrogen Receptor Mediated Activity. Presented at the 46th Annual Meeting of the Society of Toxicology, Charlotte, North Carolina).

- DiscoverRX is working with the NIH Chemical Genomics Center on a high-throughput human recombinant ERalpha assay (Olsen, K. Presentation at NTP Request for Information Meeting: High Throughput screening approaches to toxicology, September 11 – 12, 2008. NIEHS, Research Triangle Park, NC).

In other words, the rat uterus cytosol assay--the test that EPA wants to use because EPA is in a hurry--is the only assay in the world that uses rat uteruses. Purification of the receptor is the primary source of variability for this version of the assay, and that inherent variability is one of the main reasons why scientists everywhere are working to develop an alternative assay.

7. EPA is in too much of a hurry to address other peer review concerns.

Peer reviewers made several comments about problems with the ER binding assay. Some of these comments relate to the possibility of false negatives from the assay test. EPA responded to some of these comments by stating that the Agency will consider them in the future, and if necessary revise the assay sometime in the future.

For example, on pages 8 to 9 of EPA's Response to Comments, a peer reviewer commented

"A second point regading the biolgical relevance of the assay is the role of the molecular chaperone hsp90 and its role in maintaining the estrogen receptors in a ligand binding state. Previous work with hsp90 associated receptors, in particular receptors like the GR, has demonstrated an important role for the hsp90 complex. In the case of the estrogen receptor ERα experiments have demonstrates that hsp90 is important but not crucial, for ligand binding. I am concerned that during cytosol preparation, the hsp90 complex may dissociate which would negatively impact on the receptors ligand binding activity. The presence of molybdate will stabilize the complex but it may not be sufficient.

In the case of the second estrogen receptor isoform ERβ, very little is know regarding the putative role of hsp90 and regarding the stability of the complex. Again this may cause problems and in particular may explain some of the interlaboratory variation."

EPA responded by acknowledging this to be a concern that EPA may address sometime in the future**:**

"The Agency recognizes the importance of heat shock proteins in maintaining the binding activity of ERα. A saturation binding assay is required for each batch of cytosol to provide assurance that the receptor is functioning as expected. If in the future the Agency decides to further refine the ER-RUC assay, the effect of molybdate may be an appropriate topic for further study, as may be the interaction of hsp90 with ERβ."

Similarly, at page 8 of EPA's response to Peer review, one peer reviewer pointed out a problem that could cause false negatives:

" In my opinion the assay does not take into full account some issues. Recent experiments have shown that one of the estrogen receptor isoforms namely ERβ is under circadian control in the mouse. The circadian system is well conserved so it therefore likely that this is occurs also in the rat model.

Depending on the timepoint of cytosol preparation, the levels of ERβ expression may be very low.

These low ERβ levels may result in cytosol preparations that fail to detect compounds t hat preferentially interact with the Erâ isoform and thus may considered "safe".

This point should be taken into account to avoid missinterpretation of obtained results."

EPA responded as follows:

"The Agency notes that the research on circadian control of ERβ has been carried out only in male mouse lung (Cai W, Rambaud J. et al. Mol Cell Biol 28(2): 784-93 and that there is insufficient information on which to base selection of an optimal time point, if any, at which to collect uteri from rats. If further research substantiates the importance of

this effect in rat uterus and allows determination of an optimal time point for collection of tissue, future versions of this protocol may be adjusted."

Waiting until the validation of a human recombinant assay would moot these false negative concerns. It would also eliminate the need for "future versions of this protocol."

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