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FOR RISK ASSESSMENT

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EDUCATION



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Report on The Chromium Mode of Action Studies Peer Review Report: Mouse 90-Day Study

**Expert Review Organized by
Toxicology Excellence
For Risk Assessment (TERA)**

www.tera.org

For more information contact:

Ms. Joan Strawson,
Peer Review Coordinator

910-528-9768

Strawson@tera.org

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INTRODUCTION

In July 2009, Toxicology Excellence for Risk Assessment (TERA) convened a Science Advisory Board (SAB) that provided guidance on a protocol for a series of studies investigating the mode of action by which hexavalent chromium is carcinogenic in rats and mice following drinking water exposure. The research project was organized by ToxStrategies. The final report of the July peer review was issued in late 2009 (available at: <http://www.tera.org/Peer/Chromium/Chromium.htm>) and expressed the expert panel's recommendations on studies designed to assess chromium's mode of action following a 90-day drinking water exposure in mice and rats. Following release of the peer report, ToxStrategies incorporated the panel's recommendations and engaged several research laboratories to conduct the proposed studies. Due to laboratory limitations, the various studies were conducted in a staggered fashion. The first study to be completed is the mouse 90-day study, which includes the toxicokinetic, biochemical and pathological analyses that have been completed on tissues from that study. ToxStrategies has prepared a draft manuscript reporting the results of the mouse 90-day study that will be submitted to a peer reviewed journal following review by members of the SAB.

Toxicology Excellence for Risk Assessment (TERA) arranged for a written peer review of ToxStrategies' draft manuscript reporting the results of the mouse 90-day study. This review was conducted by four risk assessment experts who were members of the original SAB. The scientists who conducted this review are:

- Michael Dourson, Toxicology Excellence For Risk Assessment (TERA)
- Xianglin Shi, University of Kentucky
- Dave Gaylor, Gaylor and Associates
- Kirk Kitchin, U.S. EPA

The objective of the review was to provide ToxStrategies with independent scientific and technical expert opinion and comment on their draft manuscript. The experts provided their own personal opinions, and did not represent the opinions of their employers or other organizations they may be affiliated with. The information in this report does not represent the opinions of Toxicology Excellence for Risk Assessment. This work was done under contract to ToxStrategies and was sponsored by the American Chemistry Council.

The experts received the draft manuscript and the charge questions on February 14, 2011. Reviewers were asked to carefully review the document and provide written responses to the

charge questions, including a clear rationale and support for their opinions. This report is a compilation of the four reviewers' written comments, organized by the charge questions.

Review of Chromium Mode of Action Studies Peer Review Report: Mouse 90-Day Study

General Comments

Reviewer 1

Wow. Nice study. I have a few suggestions, of course, but see no need to keep this out of the hands of the journal reviewers.

Reviewer 2

Although my review raised quite a few issues, these issues are not major ones. Overall the quality of the manuscript is high and the study design is logical. The results obtained support the conclusions and the conclusions are solid. It is up to the authors whether they want to consider my suggestions or comments. The current version is up to the standard for publication.

Reviewer 3

None.

Reviewer 4

Items 1-4 in Study design, the results, the conclusions and study reliability are all generally fine. All in all you have made a lot of good positive progress and have assembled a good integrated data set from which one could eventually start to build a model .

Comments in Response to Charge Questions

1. Study Design - *Based on your knowledge of toxicological study protocols, please comment on the experimental design of the study:*

- *Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?*
- *In light of the chemical and toxicological profile for chromium (VI), comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.*

- **Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?**

Reviewer 1

I am not an expert experimentalist, but have reviewed countless studies for risk assessment purposes. This study appears to be well conducted and the results will be most useful for understanding the underlying the Mode of Action (MOA) for chromium's tumorigenicity.

Reviewer 2

The role of GSH in the mechanism of Cr(VI) can have two aspects. (a) GSH is a Cr(VI)-reducing agent. Reaction of GSH with Cr(VI) generates Cr(V) and glutathionyl radical GS• radical. Cr(V) is very reactive and can cause DNA damage and is able to react with cellular hydrogen peroxide to generate hydroxyl radical. GS• radical can also react with other GSH and molecular oxygen to generate GSS• and oxygen centered radical. (b) GSH also functions as an antioxidant to remove reactive oxygen species. It is unclear whether the authors use the change of GSH level as an indicator of Cr(VI) reduction or an as indicator of antioxidant status. In the discussion section, the results of GSH measurements should be clearly discussed.

Several different antioxidants should be used as an indicator of antioxidant status during the Cr(VI) 90 day study. These antioxidants are superoxide dismutase (SOD) against superoxide radical and catalase or glutathione peroxidase (GPx) against hydrogen peroxide. The use of GSH alone as an antioxidant indicator is too weak. However, as an independent manuscript, it is sufficient for publication.

Reviewer 3

Appropriate statistical analyses were correctly conducted indicating statistically significant dose response trends and doses with statistically significant differences from controls. These analyses identified which biological effects were statistically significantly associated with exposure to chromium VI.

Reviewer 4

It seems a bit odd at first to have ICP-MS Cr determinations of tissue Cr in the same paper as colorimetric determinations of Cr in drinking water for the dosing solution. Would an AAS Cr assay be worth the effort? How much money are you saving by using colorimetry versus a more sophisticated assay? Is Cr/water so easy that colorimetry is all that is needed?

- **In light of the chemical and toxicological profile for chromium (VI), comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.**

Reviewer 1

My only additional thought would be to determine the pH values of the gastric contents in rodents and compare them with humans. I suspect an increase in reductive capacity in humans based on known pH differences. The authors might be able to address this idea in their discussion section.

Reviewer 2

8-OHdG was chosen as the only indicator for Cr(VI)-induced oxidative DNA damage. The kit assay was used. An additional assay measuring 8-OHdG levels should be used.

Reviewer 3

This question is outside my area of expertise.

Reviewer 4

None.

2. Study Results - Please comment on the strength, credibility, and relevance of the toxicological results of the study under review:

- *Were the individual animal data correctly summarized?*
 - *Are there nomenclature issues that need clarification?*
 - *Was adequate statistical information provided for quantitative dose-response analyses?*
 - *For each lesion or finding presented in the study, please comment on the strength of the evidence supporting the authors' conclusions that the lesion or finding is treatment-related.*
-
- **Were the individual animal data correctly summarized?**

Reviewer 1

I suggest a few changes in the text based on the figures and tables. These changes do not change the conclusions. Please see the annotated text.

Reviewer 2

The data were summarized properly.

Reviewer 3

Yes

Reviewer 4

Yes

- **Are there nomenclature issues that need clarification?**

Reviewer 1

None of which I can think, although if the authors are going to use a 75¢ word that is not commonly encountered (e.g., syncytia---multinucleated masses of protoplasm), they may want to explain it in the text.

Reviewer 2

No.

Reviewer 3

No.

Reviewer 4

The abbreviation SDD does not mean CrVI to me and probably not to some others as well. So consider not naming it SDD but some other thing such as NaCrVI or CrVI with an asterisk explaining what was really administered. You are trying to administer CrVI so why not say that more directly and clearly.

- **Was adequate statistical information provided for quantitative dose-response analyses?**

Reviewer 1

This is not my area of expertise, but I found the information to be valuable.

Reviewer 2

In Figure 3A, the error bars are missing for the oral mucosa group. The labeling of “duodenum” and “oral mucosa” is also missing.

Reviewer 3

Yes

Reviewer 4

Yes

- **For each lesion or finding presented in the study, please comment on the strength of the evidence supporting the authors’ conclusions that the lesion or finding is treatment-related.**

Reviewer 1

The descriptions of the lesions, with few exceptions, seemed appropriate and relevant. Please see the annotated text for the few exceptions, none of which affect the conclusions of the authors. It would be extremely valuable for the authors to expand the idea of “a clear progression,” however. Ideally this would be in the discussion section, where this idea comes up, specifically, page 24 starting at line 10.

Reviewer 2

(a) Gross and microscopic findings: These results reflect the routine and standard analyses. This reviewer did not have any question or concern.

(b) GSH/GSSG measurements. As discussed above, the role of GSH in the mechanism of Cr(VI) can have two aspects. (i) GSH is a Cr(VI)-reducing agent. Reaction of GSH with Cr(VI) generates Cr(V) and glutathionyl radical GS• radical. Cr(V) is very reactive and can cause DNA damage and is able to react with cellular hydrogen peroxide to generate hydroxyl radical. GS• radical can also react with other GSH and molecular oxygen to generate GSS• and oxygen centered radical. (ii) GSH also functions as an antioxidant to remove reactive oxygen species. It is unclear whether the authors use the change of GSH level as an indicator for Cr(VI) reduction or as an indicator of antioxidant status. In the discussion section, the results of GSH measurements should be clearly discussed and should discuss how the results of GSH concentration will support the MOA.

(c) 8-Isoprostane. The observation of 8-isoprostane increase is important to support the role of oxidative stress in the mechanism of Cr(VI)-induced carcinogenesis. Since 8-isoprostane is an indicator of oxidative stress occurring in the lipid phase, measuring additional indicators of oxidative stress in aqueous solution will be helpful.

(d) Cytokine and chemokine measurements. Various cytokines were analyzed and the results were obtained. The authors did not discuss the results nor use them to support their conclusions.

(e) The iron status. The iron status was measured. The authors did not provide an adequate rationale why the iron status should be measured. The results obtained were not discussed either.

Reviewer 3

Adequate evidence was supplied.

Reviewer 4

(a) Isoprostanes. The assay you are using is determining the “free” isoprostanes and missing the unesterifiable isoprostanes that could be liberated with a treatment with base, heat and some time (maybe 1 N NaOH, 60 degrees C and 1 hour for example, recipes vary on these points). It would be safer and preferable to measure the total unesterifiable isoprostanes rather than just the free isoprostanes.

I am delighted that some increases in isoprostanes have shown up. So far no increase in 8-OHdG have shown up. 8-OHdG is a difficult assay to do well. Not finding an increase does not mean that 8-OHdG is not increased. It can easily be that it means your baseline is high and lots of dG to 8-OHdG oxidation is going on. People get very enthusiastic about antioxidants/ chelators such as EDTA, desferal, TEMPO etc. in protecting dG from oxidation. Whole articles have been written in this area. This is a difficult area with substantial quicksand about. You may want to contract with a group that has substantial 8-OHdG expertise, experience and quality control and gets low basal oxidation of dG to 8-OHdG and have them do the assay rather than attempt it yourselves.

While significantly better than nothing, ELISA based assays for isoprostanes and 8-OHdG are not state-of-the-art. For example a good LC-MS method for multiple isoprostanes in human urine is that of Yan, Byrd and Ogden (J of Lipid Research 48(7) 1607-1617 2007). Morrow's group in Vanderbilt usually uses GC-MS methods for isoprostanes. For 8-OHdG, either LC-electrochemical detection or LC-MS/MS methods are much better than ELISAs. Since heavy deuterium forms dG and 8-OHdG are commercially available, it makes LC-MS/MS methods quite attractive.

I recommended isoprostane and 8-OHdG determinations in the earlier peer review and probably the Senft GSSG assay as well. I had no idea if they would be done or how they would be done. Now that some data is in, it is clear they are all three of these parameters are important to the overall MOA case the group is trying to argue for and so the best available assays should be used. Thus, LC-MS or LC-MS/MS rather than ELISA assays should be used in any future work for isoprostanes (could be GC-MS also for isoprostanes) and 8-OHdG. For GSSG LC-MS/MS is much better than the Senft method. I did not specifically state that

LC-MS/MS should be used for these parameters in earlier reviews. My view is they are "critical". For the group to convince EPA to use their Cr model, the best available analytical biochemistry needs to be used.

(b)GSH/GSSG. As GSH/GSSG is an important low dose responding variable in your study, you might want to think about increasing the quality of the GSSG measurement. The best way I can think of to do this is switch to a LC/MS based method where the GSH sample is exposed to either NEM or iodoacetate. This inactivates the GSH by derivatizing it. The GSSG is not affected. Then in positive ion mode and electrospray, both GSH-NEM for example and GSSG show up as positive ions. There are several papers published on this approach including a long review article on GSH type methods (Monostori, Wittmann, Karg and Turi, J of Chromatography B 877 p3331-3346 2009). The major advantage is that GSH to GSSG oxidation is minimized. The GSSG amount is very important to you as it is coming either from CrVI exposure or during the time course of how you do your assays.

With a generally less than 40% decrease in GSH why will this greatly reduce CrVI reduction by GSH? There is still mM GSH to reduce uMolar CrVI. At a >1000 to 1 molar ratio, I expect the reduction of CrVI should still be going fairly well.

3. Study Conclusions

- *Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made?*
- *Do you agree with the authors' conclusions of the study?*

- **Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made?**

Reviewer 1

In the results section, I apparently missed the statistically significant trend stated on page 23 line 20 regarding "decreased 8-OHdG levels in the duodenum." Please make this trend more obvious, or change the text in the discussion accordingly. Whether or not the trend is statistically significant is not critical to the study's overall conclusions.

Reviewer 2

Did not provide a response to this question.

Reviewer 3

None.

Reviewer 4

Did not provide a response to this question.

- **Do you agree with the authors' conclusions of the study?**

Reviewer 1

Yes, I am quite happy to see this work done and look forward to the next set of results. This overall body of work will be of utmost importance to our society as we grapple with the appropriate low dose response assessment for chromium VI.

Reviewer 2

Did not provide a response to this question.

Reviewer 3

Generally, yes. However, focusing only on statistically significant doses fails to indicate biologically significant doses, e.g., benchmark doses, that generally are lower.

Reviewer 4

Did not provide a response to this question.

4. Study Reliability – Describe the reliability of the study for consideration in the derivation of EPA IRIS quantitative health benchmarks and the qualitative characterization of cancer risk. Describe any major strengths or uncertainties with this study that might preclude it from being used as consideration for:

- *derivation of a noncancer reference dose,*
- *determination of the mode-of-action and weight-of evidence for chromium (VI)'s cancer risk*
- *derivation of a cancer slope factor*

- **derivation of a noncancer reference dose,**

Reviewer 1

I am not sure what the existing critical effect is for chromium VI as the basis of the Reference Dose (RfD), but judge that this study will be highly relevant in the overall determination of the critical effect when compared with other data, since the No Observed Adverse Effect Level (NOAEL) or expected benchmark dose (BMD) is in the range of other chosen points of departure, which have been considered for determination of the critical effect (International Toxicity Estimates for Risk (ITER). 2011. <http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?./temp/~vRu03z:1>).

Reviewer 2

From the present study one cannot derive a noncancer reference dose due to the limited number of doses used.

Reviewer 3

The data are amenable for benchmark dose analyses in order to calculate points of departure for setting a noncancer reference dose.

Reviewer 4

Did not provide a response to this question.

- **Determination of the mode-of-action and weight-of evidence for chromium (VI)'s cancer risk**

Reviewer 1

More importantly, the results of this study appear sound and highly relevant for understanding the MOA for chromium's tumor response in rodents. Additional data from this study on kinetics and genomics will also be highly relevant. It is gratifying to see that the authors are following EPA (2005) guidelines on investigating relevant MOAs in a series of hypothesis-driven research studies. The authors have also picked up on numerous suggestions of the previous external review panel. After some additional work as suggested in this review, the results will be highly credible and very relevant.

Reviewer 2

The results of 8-OHdG measurement provides a high degree of uncertainty for the Cr(VI) MOA. The measurements of GSH/GSSG measurements did not help very much due to the reasons discussed above mainly because GSH functions both as a Cr(VI) reducing agent and as an antioxidant.

Reviewer 3

This question is outside my area of expertise.

Reviewer 4

Did not provide a response to this question.

- **Derivation of a cancer slope factor**

Reviewer 1

In part this depends on the expected MOA in humans. I suggest that the authors follow the EPA/ILSI/IPCS guidelines for evaluating MOA and human relevance.

Reviewer 2

One cannot derive a cancer slope factor due to the limited number of doses used.

Reviewer 3

The data are amenable for benchmark dose analyses in order to calculate points of departure for deriving a cancer slope factor.

Reviewer 4

Did not provide a response to this question.

5. Please identify and discuss any other relevant scientific issues or comments not addressed by the above questions.

Reviewer 1

I am not sure how Figure 5 was relevant to the MOA argument being proposed in part. Rather than showcase this figure, I suggest that the authors replace it with one based on how the NTP

intestinal tumors relate to the sequence of atrophy, apoptosis and/or regenerative crypt cell hyperplasia found in different parts of the GI tract. My initial impression is that the tumor percentages in different parts of the GI tract would map quite nicely to the regenerative crypt cell hyperplasia in different parts of the GI tract as seen in this study.

Reviewer 2

None.

Reviewer 3

Page 19, line 22. Transferrin should be changed to ferritin.

Page 30. The symbol (*) needs to be added to the Table 2 footnotes.

Page 32, Table 3 footnotes. For *** should p be less than 0.001, not 0.01?

Page 38, Figure 2A and 2B. Equal spacing of the dose groups distorts the dose response and makes it appear linear, whereas there are some relatively large changes at low doses which are compatible with a power curve.

Reviewer 4

It is not clear to me what delta E is and how it is measured. (Table 3)

Table 5 is a good summary for the duodenum results. Instead of a check mark for a positive result at $P < 0.05$, I suggest putting an up or down arrow showing the direction under the dose and not just at the left in the Table where it is easier to miss. The degree of statistical significance can be shown by the number of arrows e. g. three arrows for $P < 0.001$

There should be a results summary - Table 5 for the jejunum, ileum, oral cavity and plasma as well. When all five anatomical sites results are spread out in front of you, then you can visualize the whole study results very easily. Right now it is not easy to keep track of multiple parameters.

Figure 5 Somewhere on this figure you should put the mg/L drinking water levels (0.3520 mg/L numbers). The Log x axis does not easily give you this information which is how all the other concentrations are referred to.

In Figure 3 it is better to normalize to mg tissue or mg protein instead of ml of tissue homogenate.

It somewhat surprises me that nothing in the area of inflammation has shown up positive so far.

Appendix A: Instructions to Reviewers

Dear Reviewers,

Thank you again for your willingness to provide a peer review of the Mouse 90-day study, which has been conducted as part of the series of studies investigating the mode of action by which hexavalent chromium is carcinogenic in rats and mice following drinking water exposure. This research project was organized by ToxStrategies. This email provides you with the review materials and instructions. The study and charge questions are attached.

The subject of this review is a draft manuscript that has been prepared by ToxStrategies describing the mouse 90-day study. The final report of mouse 90-day study prepared by the authors, Southern Research Institute, is also available for your consideration at the following link: <http://dl.dropbox.com/u/6893750/13026.01.01%20Amended%20Report.pdf>. Following your peer review, ToxStrategies will be submitting the draft manuscript for publication.

For this peer review, the reviewers are asked to carefully review the study and provide written responses to the charge questions for each assessment. Please address each charge question (as appropriate given your expertise), and provide clear rationales and support for your opinions. Please identify the page number and line number of the text that you are commenting on to allow ToxStrategies easy reference to the specific text. We will need an electronic copy of your comments preferably in MS Word. We prefer that you use the attached charge file as a template and add your answers to it. If you would like a copy of any cited references, please send your request to me (Strawson@tera.org or 910-528-9768).

Your written review should be sent by email to me by Monday, February 28, 2011. After the reviews are submitted, we may schedule a follow-up conference call with ToxStrategies to resolve any issues or to answer any clarifying questions that ToxStrategies may have for the reviewers. The need for follow up will be determined upon receipt of the reviews. A draft compiled report with the other experts' comments on this study will be forwarded to you and you will be provided the opportunity (albeit brief) to revise your comments if you feel that is needed. At the completion of this review, please destroy any copies of the review materials, as they are draft and are not for distribution outside of the review panel.

If you have questions regarding the review, please contact me. Thank you again for being willing to do this review in such a short time frame. ToxStrategies very much appreciates your assistance.

Joan Strawson
Review Coordinator
910-528-9768

Appendix B: Reviewer 1's Annotated Draft

Available upon request.