Report of the Chromium Science Advisory Board Review of *Research Framework for Evaluating the Potential Mode(s) of Action Underlying the Carcinogenicity of Hexavalent Chromium Following Exposure in Drinking Water*

Prepared by The Hamner Institute

Volume I

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NOTE

This report was prepared by scientists of Toxicology Excellence for Risk Assessment (*TERA*) and reviewed by the panel members. The members of the panel served as individuals, representing their own personal scientific opinions. They did not represent their companies, agencies, funding organizations, or other entities with which they are associated. Their opinions should not be construed to represent the opinions of their employers or those with whom they are affiliated.

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Executive Summary: Key Recommendations

Overall, the panel appreciated the cutting edge nature of the research strategy as a basis to integrate information from both evolving and traditional toxicity testing for both the chromium assessment and risk assessment in general. The panel considered that the author/research team is highly qualified and expressed appreciation for the quality of their work on both the written material and presentations. The panel noted that the presentations by the research team were important for helping the panel understand purpose of research and recommended that additional information be included in the proposal so that it is self-explanatory.

Mode of Action (MOA)

The research proposal should more clearly lay out the key events for the hypothesized and alternative MOAs, identify measurable endpoints for each key event, and describe how the various endpoints being measured in the various assays help to evaluate those key events in the context of the modified Hill criteria/MOA framework.

The panel noted that the following are likely key events, although the relationship among these key events is not known.:

- Cr(VI) is taken up by the cell and causes oxidative stress as it is reduced to Cr(III).
- once Cr(VI) is in the cell and reduced to Cr(III) inside the cell, Cr(III) forms DNA adducts, complexes, and cross-links.
- cytotoxicity and inflammation, and direct DNA reactivity can all result from oxidative stress.
- gene mutation

The panel agreed that cellular uptake of chromium, oxidative stress, DNA adducts, DNA inter- and intrastrand crosslinks, mutation, cytotoxicity, and cell proliferation are all important key events and should be examined simultaneously to understand dose-response and temporal relationships. Oxidative stress could lead to any of those events.

The proposal should determine which key events are rate limiting and driving the response, recognizing that key events need to be measurable, repeatable, and essential to the outcome (although not necessarily sufficient). These rate-limiting (and dose-limiting) key events define the MOA. The proposal should also describe the inter-related key events, noting that individual key events could lie on multiple pathways to tumorigenesis. The authors should evaluate whether the proposed studies are the most effective way to evaluate those key events, how to anchor the key events to known toxicology information, and whether there are data gaps. The rationale for the choice of key events to investigate needs to be laid out.

The panel unanimously agreed that oxidative stress and mutation are proposed key events in the carcinogenicity of chromium and that the lack of biochemical testing for both of these key events in the current research protocol was a major concern. Therefore, the panel recommended that the research should include measurement of oxidative-stress related endpoints, oxidized

DNA bases (e.g., 8-OHdG), lipid oxidation measures such as malondialdehydeisoprostanes, or 4 hydroxy-nonenal, protein oxidation, anti-oxidant status in animal (e.g., GSH peroxidase, catalase, superoxide dismutase), and/or key oxidant response proteins (e.g., AP1, p53, or N-kappaB). The panel also recommended measurement of DNA interaction, such as DNA-chromium adducts (e.g., measured via ICP-MS---inductively coupled mass spectroscopy) and DNA inter- or intrastrand crosslinks. Some panel members also recommended measuring circulating inflammatory markers (e.g., IL6), although it was noted that the microarray study would identify inflammatory changes in the target tissue if those changes were regulated transcriptionally. Evaluating the temporal and dose-related patterns for these various proposed key events will help in identifying the MOA.

For each of the proposed studies, the research strategy should describe how the proposed research would be envisaged to address the weight of evidence for the hypothesized MOA including delineated key events. In order for risk assessors to fully appreciate how the research will be used in risk assessment the framework needs to present any additional information that will help risk assessors understand the usefulness of any evolving technology. For novel technologies, the research strategy needs to provide additional information on how the results of the technique are likely to support and amplify that provided by traditional technologies.

90-day Study and Microarray Analysis

The panel recommended adding 5 animals/dose (i.e., n=10, at least for the histopathology study). The panel noted that the proposed number of animals was probably adequate, from a statistical perspective, for defining the shape of the dose-response curve for continuous data, but it is marginal for pairwise comparisons of quantal data. The panel noted that the purpose of doing histopathology is to identify phenotypically anchor the genomics data. However, with only 5 animals per dose, there were concerns both about the study sensitivity to identify an effect, and the approach of starting at the high dose and conducting histopathology evaluation until a NOAEL is identified. Therefore, the panel recommended an increased number of animals to improve the phenotypic anchoring. Several panel members also recommended that the histopathology approach be modified to evaluate the controls and dose groups (except perhaps not the two low-dose groups), unless no histopathology at all is seen at the high dose. The increased number of experimental animals may also allow some of the suggested testing for oxidative stress endpoints, but the panel recognizes that some of these requested tests may lead to technical challenges.

The panel agreed with the authors to run the 90-day study using 6 dose groups and a control. Instead of using three doses lower than the lowest dose in the NTP bioassay, however, the panel recommended two unspecified low doses plus the top three doses in the NTP bioassay. Therefore, the dosing regimen should have the following approximate mg/L doses: 520, 170, 60, 14, two unspecified lower doses and a control.

Panel members concurred with the authors' suggestion to add a 1-day interim sacrifice in addition to the 7 and 90-day sacrifices for the genomics analyses.

The panel recommended the consideration of recovery time points for some key events, if experimental findings suggest that such analyses will provide data that are critical to risk assessment. In addition, stop/recovery and reversibility studies are often helpful in addressing specificity in weight of evidence analyses for hypothesized MOA(s).

The panel agreed with the authors that the MOA would not likely be different between males and females and, as such, concurred that using only females in the research was sufficient.

No specific panel recommendations were made for the microarray analysis, separate from what was recommended for the 90-day study.

HCA Analysis

The panel recommended adding measures of oxidative stress in the *in vitro* system in addition to the *in vivo* endpoints mentioned above. Such measures would include an antioxidant to promote the conversion of Cr(VI) to Cr(III), and thus limit chromium entry into the cell, and an evaluation of one or more of the same parameters as discussed above for oxidative stress. In addition, the panel recommended a literature search to locate data on use of reductants such as GSH and ascorbate in this system.

PBPK Modeling

The panel noted that it would be valuable to generate additional kinetic data in the mouse, particularly clearance data. The panel also suggested that the NTP be contacted regarding the availability of data on total chromium in urine and feces from its two-year study, as well as the data for tissues collected during the 21-day drinking water kinetics study.

MRI Analysis

The results of the pilot studies suggest to the panel that measuring accumulation of Cr(III) in intestine is not feasible following administration of Cr(VI) in drinking water at the dose levels likely to be used in the 90-day study. The panel suggested that if the research team plans additional effort to develop the MRI methods, that the control animals from the pilot studies could be used for *ex vivo* imaging of the intestine, and that gavage dosing could be done with the exposed animals, followed by relaxation time mapping to measure the rate of reduction of Cr(VI) to Cr(III). These data would be useful for refining the kinetic model.

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1. Participants

Sponsor

ToxStrategies, Inc.

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[1] Affiliations listed for identification purposes only. Panel members served as individuals on this panel, representing their own personal scientific opinions. They did not represent their companies, agencies, funding organizations, or other entities with which they are associated. Their opinions should not be construed to represent the opinions of their employers or those with whom they are affiliated.

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2. Background

Toxicology Excellence for Risk Assessment (*TERA*) was been engaged as an independent third party to convene a science advisory board (SAB) panel to provide review and guidance on the investigation of the potential mode(s) of action of hexavalent chromium underlying the carcinogenic response observed in rats and mice following exposure in drinking water. The purpose of the panel review was to evaluate whether the proposed research strategy will provide the information that is necessary to adequately determine the mode(s) of action based on the US EPA Guidelines for Carcinogen Risk Assessment (2005). ToxStrategies is coordinating the research program and the studies are being conducted at The Hamner Institutes and Charles River.

The National Toxicology Program recently completed a two-year cancer bioassay for sodium dichromate dihydrate in drinking water (NTP, 2008) and reported finding intestinal tumors in mice and oral mucosal tumors in rats following lifetime exposure to hexavalent chromium. While the NTP study provides useful information for hazard identification, it is difficult to evaluate the relevance of these findings for humans. Therefore, a research program is being initiated to investigate the mode(s) of action underlying these tumorigenic responses in rodents in order to determine the shape of the dose response curve and the human relevance of these responses prior to the development of an oral cancer slope factor for hexavalent chromium. The overall goal of these studies is to develop an understanding of the contribution of different modes of action for hexavalent chromium across a broad range of doses in order to more accurately extrapolate the results of the NTP drinking water study to humans. The observed tumors could be due to genotoxicity, or to the presence of other cellular responses, including cytotoxicity, inflammation, and high levels of oxidative stress. These secondary responses may lead to dose-dependent transitions where Cr(III) and Cr(VI) DNA reactivity lead to DNA damage, mutation, and cellular transformation. The contributions of these various pathways over the range of doses and concentrations will be determined by a combination of genome-wide microarray analyses in intact animals, high content imaging of activation of key DNA damage response or repair pathways, and consideration of pharmacokinetics in determining the relevant tissue dose.

The primary tasks for the SAB at this meeting were to ensure that the correct questions regarding MOA are being asked, that all relevant modes of action are being considered, that the individual studies are being designed to adequately address the questions being asked, and that the studies will provide sufficient information to satisfy the US EPA (2005) cancer guidelines regarding establishment of a MOA and determination of the appropriate approach for low-dose extrapolation.

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3. Panel Introductions, Conflict of Interest, and Meeting Process

The meeting opened with a welcome by Ms. Joan Strawson of *TERA*. She described the background and purpose of the peer consultation and the agenda for the meeting. Dr. Lynne Haber of *TERA* followed by noting that copies of panel members' biographical sketches and conflict of interest (COI) and bias disclosure statements were provided to all attendees (see Appendix A). The panel members then introduced themselves and noted whether they had additions or changes in their disclosure statements. Dr. Gaylor indicated that he unintentionally left off his conflict of interest questionnaire information on prior consulting work with the Chemical Industry Institutes of Technology (CIIT). This consulting work was not related to chromium and was completed in 2005. No other panel members had any substantive changes to their statements.

Dr. Dourson, the panel chair, then described how the meeting would be conducted. He explained that discussions would be organized around the charge questions and would follow the order in the agenda (see Appendix B). He noted that all panelists would have the opportunity to state their own positions on the charge items and panel members are encouraged to question one another to make sure that all the panel members and the authors understand the scientific basis for the panel's opinion. The panel would seek agreement, but if agreement is not reached, areas where panelists' disagree would be noted. Authors made brief presentations and answer clarifying questions from the panel members. The authors were also permitted to ask clarifying questions of the panelists so that they fully understood what was being suggested or said.

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4. Author Presentations

4.1 Mode of Action

Ms. Deborah Proctor, ToxStrategies, presented an overview of the current understanding of the potential modes of action (MOA) underlying the oral carcinogenicity of hexavalent chromium and the objectives for the MOA research. The slides for this presentation are located in Appendix C, page C-3. In particular, the presentation stressed the importance of inflammation in the response to hexavalent chromium. The presentation presented the following as possible key events for hexavalent chromium carcinogenicity: extracellular reduction resulting in detoxification and competing with absorption of Cr(VI) into cells of target tissue; intracellular reduction of Cr(VI); generation of oxygen radicals; generation of chromium intermediates; disruption of normal cellular homeostasis for Cr(III); DNA damage; apoptosis; inflammation; hyperplasia (small intestine); and tumors.

4.1.1 Clarifying Questions

Panel members asked how the project was initiated and about the funding sources. Dr. Mark Harris, ToxStrategies, indicated that ToxStrategies developed the idea for the project and approached several companies with a proposal. The project has been funded by a consortium of companies, but the organizations providing the funding do not have a role in determining the research approach, and are leaving that to the investigators. Another panel member asked if there was a dose response pattern for genotoxicity. Ms. Proctor responded that there is evidence that Cr(VI)-induced lesions are related to dose, but lesions occur at relatively high exposure levels. Panel members responded that it was important to look at the dose-response patterns both *in vivo* and *in vitro*. It is critically important to consider the nature and pattern of genotoxicity not only in relation to dose-response (is genotoxicity observed only at high dose?) but nature of the lesions induced (mutation or other?) and whether or not there has been any evidence of interaction with DNA in the target organ of interest for carcinogenicity. The pattern of these results may be entirely consistent with on the hypothesized mode of action (e.g., Is there genotoxicity only at cytotoxic doses?).

One panel member asked about Cr(III) essentiality and how Cr(III) gets into the cell. Ms. Proctor responded that Cr(III) can enter the cell, though it is more slowly absorbed than Cr(VI). More specifically, Cr(III) is absorbed by passive absorption, while Cr(VI) is actively transported via anion channels. She noted that not all trivalent chromium is genotoxic in the cell, which is why the research framework has proposed disruption of homeostasis as a key event. Cr(III)-DNA adducts may also represent a key event in the MOA, but the presence of Cr(III) in a cell does not necessarily mean that a mutation will occur. The same panel member asked about the equilibrium between Cr(VI) and Cr(III) in the body and asked whether Cr(III) could be converted back to Cr(VI). Ms. Proctor indicated that when Cr(VI) enters the body, it is rapidly

reduced to Cr(III), which is not as readily taken up by the cell as is Cr(VI). Additionally, she indicated that she is not aware of any data demonstrating the oxidation of Cr(III) back to Cr(VI) in the body.

Another panel member asked how Cr(III) is detoxified once it is in the cell, since Cr(III) is generally considered to be less carcinogenic because it cannot enter the cell. Ms. Proctor responded that conversion of extracellular Cr(VI) to Cr(III) is a detoxification pathway because Cr(III) does not readily pass the cell membrane. Intranuclear reduction of Cr(VI) to Cr(III) may lead to DNA damage. Reaction with glutathione in the cell is also a pathway for detoxification of intracellular Cr(III).

One panel member noted that the research framework listed oxidative stress and gene mutation by Cr(III) as possible key events in the MOA, and asked whether the framework had considered mutation by Cr(VI) or other MOAs. Ms. Proctor responded that the research team is are looking for guidance from the panel to complete the MOA framework. The team would welcome input from the panel regarding other possible MOAs or information on conflicting data that would suggest that the hypothesized MOA does not make sense. Ms. Proctor noted that one hypothesis for the oral cavity tumors is that they may be mediated via iron deficiency, as suggested by some published studies (for example, Prime et al. 1983).[2] She noted that oral cancers are associated with anemia in the literature; however, this is not addressed in the current research plan. NTP (2008) reported microcytic anemia, with rats being more sensitive than mice. The response was statistically significant only in the rat high-dose group, and measures of anemia in the mice recovered earlier than in rats. Therefore, anemia may play a key role in the development of oral cavity cancer. ToxStrategies is looking to the panel for suggestions on other possible MOAs or key events.

One panel member cautioned that the authors should be judicious in using the terms “key event” and “MOA”. Interaction with DNA could be a key event in the proposed MOA. Events being evaluated are likely to be key events in more than one MOA. In addition, there may be competing key events that converge to one MOA. Therefore, the authors need to choose key events carefully, since these are the endpoints evaluated in the research studies.

Another panel member noted that ternary complexes of Cr(III) with DNA and a ligand (e.g., GSH or ascorbic acid) were not included in the research strategy and asked if the authors considered this to have a low probability of occurring. Ms. Proctor responded that they are considering tertiary adducts to be a potential preneoplastic lesion; consistent with the first figure

[2] Prime SS, MacDonald DG, and Rennie JS. 1983. The effect of iron deficiency on experimental oral carcinogenesis in rat. Br. J. Cancer 47:413-418.

(Slide 6) in the presentation. She did not discuss this pathway in detail due to time constraints, but agreed that the Cr(III)-tertiary adducts are potentially quite important. This panel member noted that there are 5 major classes of DNA damage that result from chromium and there appears to be a better connection between DNA damage and cancer than inflammation and cancer. Ms. Proctor agreed that mutation is likely a key event and that damage of DNA is critical to the overall MOA. However, she sees inflammation as promoting cancer. Therefore, Ms. Proctor hypothesized that both processes are likely to be necessary key events fitting into the overall MOA, recognizing that the aim of the research proposal to test such hypotheses.

4.2 Introduction to Research Framework

Dr. Melvin Andersen, The Hamner Institutes, gave an overview of the research strategy program; see Appendix C, page C-5 for the slides from his presentation. Dr. Andersen explained that the emphasis of the program was on developing a research strategy for dose-response modeling methods. While The Hamner Institutes considered aspects of the IPCS Framework for Mode of Action Analysis, they did not design the studies in the research framework around the IPCS framework. The presentation summarized the hypothesis for the dose-dependent transitions in the MOA for Cr(VI) and discussed the available data to support this hypothesis. The presentation provided an overview of the various components of the research program, as well as a summary of the expected outcomes for each of the studies.

4.2.1 Clarifying Questions

One reviewer noted that the dose levels to be used in the 90-day study were not presented (Slide 13) and asked if the authors were planning on matching the four dose levels used in the NTP bioassay plus adding two environmentally-relevant doses. Dr. Andersen replied that they were planning on using a high dose that would have an adverse effect, as in the NTP assay. In addition, they plan to use two doses that are no-effect levels in order to obtain good statistics on gene expression changes and to define the inflection points and how they differ from one gene family to another. These two doses may be at the low doses of the NTP assay. The same panel member noted that there were different high dose groups in NTP's 90-day and 2-year studies and asked which LOAEL the authors were planning to use. The reviewer suggested using the higher dose, noting that if the study produces negative result at 90 days, people will ask whether that dose level would have been negative at 2 years. Dr. Andersen responded that the authors are open to discussion, since they have not specified doses for the study yet. However, they are considering using the high dose from the 2-year study.

The panel noted that this research program is different from traditional risk assessment because it is mechanism-based and asked Dr. Andersen to explain in more detail the objectives of the research. For example, in relation to Slide 6, what is the objective of the proposed study? One reviewer suggested that the research strategy should articulate the study objectives more clearly. Dr. Andersen indicated that the research program is not looking to better characterize

progression of tumors in the NTP bioassay; rather it is using shorter times *in vivo,* and potentially *in vitro,* to understand the dose-response behaviors compared to the outcomes in histology and carcinogenicity. The goal is to increase understanding of key events in the low-dose range. In addition, the research will help to describe chromium in terms of its behavior at low doses and dose-dependent transitions. Another reviewer indicated that the risk assessment *default* constructs (i.e., dichotomy between linear and nonlinear approaches) are artificial; the goal is to describe a chemical's behavior at low concentrations. Dr. Andersen agreed, but also noted that there is a need to bring more biology into the risk assessment process. It is important to look at the sequential activation of pathways and understand what happens in different dose regions. The panel agreed that generating more data at low doses is important to understand these effects. However, the proposal should state this goal more clearly.

4.3 Genomics and High Content Analysis (HCA)

Dr. Russell Thomas, The Hamner Institutes, gave a presentation summarizing the genomics studies proposed for the research program; see Appendix C, page C-8 for the slides from the presentation. He discussed the utility of genomics studies and stated that the ability to look at the entire transcriptome provides an advantage for risk assessment. Following chemical exposure, the transcriptome is changed directly due to the chemical exposure, or indirectly as the result of an adverse effect. Therefore, the purpose of these studies is to look at the dose-response for perturbations in the transcriptome. Benchmark dose methods are applied to the data to identify the points-of-departure, and bioinformatic methods are used to group genes by function. Summary statistics (e.g., mean, median, etc.) are calculated for the points-of-departure of all the genes associated with each function. The presentation gave examples of how genomics data can be used both when the MOA is known and when there is no defined MOA, by helping to identify the most sensitive pathway. Genomics data can be used to estimate both noncancer and cancer risk values. Dr. Thomas indicated that for chromium, the genomics studies will provide an understanding which genes have threshold-like behavior and which genes have linear behavior. Finally, the presentation described the HCA studies that will be conducted in cell lines.

4.3.1 Clarifying Questions

One panel member asked how a noncancer RfD value was derived from genomics data. Dr. Thomas indicated that it would be the transcriptional BMDL divided by uncertainty factors. However, other panel members cautioned that the RfD is not a gold standard because it is still extrapolating from animals. Dr Thomas replied that the goal is to provide regulatory agencies comfort that using genomics data is not dramatically different from current approaches. Another panel member asked what data are actually used when evaluating potency and dose response. Dr. Thomas indicated that they use signal values from the microarrays that are normalized based on

standard, generally accepted practices. The signal for the control and all dose groups are modeled with BMD methods. In particular, they model where gene expression deviates from the variability observed in controls – that is the BMD. The signal could go up or down as a function of dose. However, a large number of genes don't change with dose, and so those are ignored. One issue is how many genes in a pathway need to have altered expression in order to consider that pathway active. The number may vary with the pathway, so the current approach is to use the median BMD for all the altered genes in the pathway in order to describe the aggregate behavior of the pathway.

One panel member stated that with a genomics-based RfD it was important to retain the concept of critical effect, because risk assessors will eventually need to tie the RfD to the concept of clinical significance. This person also noted that using genomics affects the choice of uncertainty factors. For example, since the use of genomics addresses data gaps, we may be able to eliminate the database uncertainty factor. Dr. Thomas responded that it may be difficult to replace the database uncertainty factor, because genomics is looking only at target tissues, and is not evaluating other potential targets. The panel member also suggested that genomics data may make it possible to replace default uncertainty factors for interspecies and intraspecies differences with data-derived uncertainty factors. For example, the panel observed that the data could be used to address dynamic differences between species when the response is expressed in terms of internal dose.

4.4 PBPK Modeling

Dr. Havey Clewell, The Hamner Institutes, presented information on the PBPK model being developed for Cr(VI) and Cr(III); see Appendix C, page C-13 for the slides from this presentation. Dr. Clewell discussed the model development and model structure, noting that it is based on the original rat model developed by O'Flaherty at the University of Cincinnati to compare oral and inhalation exposure to chromium. The parameters are either physiological values available from the literature or were estimated from other models. Dr. Clewell described changes made in the model, which included adding compartments to account for the NTP target tissues, incorporating allometric scaling of rate constants and physiological parameters, and incorporating non-linear uptake from the intestinal compartment. He noted that for the buccal cavity, the model predicts that about half of total chromium is Cr(VI); however, this is probably not correct, because the Cr(VI) kinetics in the model is solely based on red cell total chromium. Therefore, The Hamner Institutes is hoping that the MRI studies will provide data to more accurately establish what proportion of total chromium is Cr(III) versus Cr(VI).

4.4.1. Clarifying Questions

One panel member asked how long it takes chromium to reach steady state in the kidney. Dr. Clewell replied that the model has acute and 24 day data, but the kidney does not reach steady state in this timeframe. Another panel member noted that it is critical to have an anchor to differentiate Cr(VI) and Cr(III). Dr. Clewell agreed and suggested that either reduction rates or an exposure marker such as adducts or cross-links could be used. However, if these approaches do not work, the modeling may not be of significant value. One reviewer asked what studies served as the basis of the modeling parameters. Dr. Clewell replied that the rat model is based on a number of different studies using oral, i.v., and inhalation exposure. The Hamner Institutes has added the target tissue compartments; they also added saturability of uptake based on the NTP studies. Dr. Clewell noted that the primary goal of the modeling is to address the Cr(III) vs Cr(VI) speciation issue.

A reviewer noted that the high dose in the NTP study is 516 mg sodium dichromate/L and asked, based on NTP data, what dose range is required for saturation of Cr(VI) reduction. Dr. Clewell replied that factors of 3-fold separated the doses in the NTP study, but the corresponding kidney concentration only goes up fractionally. Therefore, it appears that saturation occurs between the 5 and 20 mg chromium/L doses. Doses of 60 and 180 mg chromium/L are above saturation[3]. In response to a question, Dr. Clewell indicated that a mouse model does exist, although some of the parameters were based on allometric scaling from rats. Mouse data for the kidney were used to calibrate the model, and blood data were used to validate the model. Dr. Clewell also noted that NTP conducted a kinetic study in mice, and The Hamner Institutes is planning to measure total chromium in mouse target tissues. A reviewer indicated that a critical question for future improvements to the model is measuring chromium valence states, As previously discussed, it is important to be able to distinguish Cr(III) (and other valence states) vs. Cr(VI) in order to address critical MOA issues. One panel member asked if the Hamner Institutes planned to measure total chromium in buccal cells. Dr. Clewell replied that they are doing the study in the intestine and can consider doing the same in buccal cells.

One panel member asked about how to use studies to estimate the rate of reduction of Cr(VI) to Cr(III). He noted that if the MRI studies can generate data on the rates of reduction and tissue levels, then the PBPK model can be improved. If one knows the amount of Cr(VI) coming into an animal at steady state, the amount of excretion, and the amount of reductants, would it be possible to calculate the rate of reduction? Dr. Clewell responded that they had considered that question, and concluded that understanding residence times in the gut and lumen is the biggest issue. He noted that if the MRI studies can generate data on the rates of reduction, they can be used in the model to estimate Cr(VI) kinetics.

[3] The doses listed in the report are not the same as the doses shown in the presentation materials because they were revised by The Hamner Institutes after the meeting.

4.5 MRI Studies

Dr. Vinrod Kaimal, Charles River, presented information on the MRI pilot studies being conducted to address the issue of speciation between Cr(VI) and Cr(III); see Appendix C, page C-15 for the presentation slides. Dr. Kaimal explained that the premise for using MRI to understand chromium speciation is that Cr(III) is paramagnetic and causes T1-shortening in magnetic resonance (MR) images but Cr(VI) is non-paramagnetic; therefore it does not affect contrast in MR images. Results from pilot studies conducted at the University of North Carolina and Charles River Labs were presented. The goal of the pilot studies was to look for evidence of Cr(III) accumulation in tissues. The sensitivity of the system is on the order of about 5 mg/kg. Therefore, based on the information provided by the PBPK modeling, the tissue concentrations may not reach a level needed to see a signal; the expected concentration is ~5 mg/kg in kidney, which accumulates chromium, and may be lower in intestine. Dr. Clewell stated that data collected by NTP on day 13 suggests that the only tissues with chromium concentrations above 5 mg/kg are liver and glandular stomach at the high dose.

Dr. Kaimal explained that they were still dosing animals and asked the panel for suggestions to the refine imaging and/or the dosing protocol. The following suggestions were proposed by the research team, with feedback requested from the panel: imaging the abdomen and ignoring the oral cavity, focusing on the target organs; using a surface coil placed under animals; increasing the sample sizes to enhance ability to see statistically significant differences; conducting the imaging at the end of the rat's dark cycle in order to reduce the time for chromium elimination from the gut (since rats feed at night during this dark cycle); measure chromium ex vivo in small intestine tissue and compare the results with controls; and/or homogenize the tissue and measure its relaxation properties.

4.5.1 Clarifying questions

One panel member noted that, based on the reference tubes, the authors calculated a limit of detection of 0.1-2 mM. Since the tube references are a best-case scenario, what would be the correction factor to *in vivo* sensitivity? Dr. Kaimal replied that the pilot study conducted by UNC suggests that limit of detection derived from the reference tubes seems consistent with the *in vivo* limit of detection. Dr. Kaimal noted that they do have tissue homogenate data that may provide additional information. The reviewer then asked about the capabilities for T1 relaxation time mapping and asked if this was a feasible alternative. Dr. Kaimal indicated that they have the capability to look at this, agreeing that although the approach is more complex, it is more quantitative and therefore it may give a higher sensitivity. A panel member then suggested that dosing by oral gavage might change this situation, because the rapid peak exposure may create a

need for more dynamic information. T1 mapping may not have temporal resolution to capture conversion of Cr(VI) to Cr(III) after gavage dosing.

Another reviewer agreed that ex-vivo imaging would be a good way to generate the data. However, this reviewer noted that the organs will have a mixture of Cr(VI) and Cr(III) and asked if Cr(VI) will be degraded or converted to Cr(III) if the organs are removed or tissue homogenized. Other panel members agreed that the conversion of Cr(VI) to Cr(III) is relatively fast, so that even if tissues are fixed quickly, the conversion processes is ongoing. Dr. Kaimal replied that it might be possible to try to image over time to address that question, but this approach would not address the changes that occurred before the first image.

Regarding the ex-vivo imaging, one panel member asked if pooling the GI tract organs from all 5 rats would help improve the sensitivity. Dr. Kaimal responded that the assay would likely have enough sensitivity for single animals if they are doing homogenates, so there is not benefit to pooling organs from different animals. Other panel members agreed, noting that the concentration in the entire sample would need to be higher in order to increase the sensitivity. Pooling animals would decrease variation but would not increase sensitivity. Another reviewer asked if it was possible to evaluate *in vitro* metabolism using organ homogenates, which would allow evaluation of the rate of conversion in the tissue. Another panel member suggested additional methods that could be useful, such as NMR to measure intracellular and extracellular markers or using perfused organs. However, these methods are not widely used and would need to be further developed before it determining if they are worth pursuing.

A reviewer asked how the authors expect that the MRI results will help in the evaluation of MOA. Dr. Clewell replied that they hope to measure steady state chromium at three concentrations in the intestine to compare with total chromium, so they have a metric of Cr(III) and Cr(VI) to examine if the ratio changes with dose. Alternatively, they will measure rate of reduction in small intestine, lumen, and enterocytes, and incorporate the data in the PBPK model to predict the ratio of Cr(III)/ Cr(VI). The panel member asked how the ratio of Cr(III) to Cr(VI) will relate to MOA and assist with the risk assessment. Dr. Clewell responded that the goal is to determine whether Cr(VI) or Cr(III) concentrations track best with markers of significant cellular responses. Cr(VI) appears to be more related to oxidative stress markers. If Cr(III) concentrations appear to track with DNA damage or repair, this will suggest that the DNA damage is due to Cr(III), not oxidative stress. The reviewer replied that Cr(VI) itself does not cause DNA damage, so there is no need to measure it and evaluate its effect. In addition, this reviewer stated that all Cr(VI) eventually becomes Cr(III), so the only issue is how fast it is removed from body. Dr. Clewell disagreed, indicating that the authors believe effects of Cr(VI) and Cr(III) are different, and their goal is to obtain data to show that Cr(VI) tracks with oxidative stress, Cr(III) with DNA damage.

One panel member asked about the method used to conduct the imaging experiments. Dr. Kaimal explained that they conducted imaging between 9 am and noon. In addition, Dr. Clewell suggested that by using gavage dosing rather than drinking water, imaging could be conducted at the time of peak tissue concentrations, improving the signal and addressing the sensitivity issue. In contrast, the drinking water study will evaluate Cr(III) accumulated in tissue at that time point; it will be possible to estimate Cr(VI) by measuring the difference between total chromium and Cr(III). By using gavage dosing, the study is looking at reduction *rate*, rather than tissue concentration. A panel member asked why the authors are imaging the stomach in the drinking water study. Dr. Clewell replied that it was a tissue that was measured in the NTP study. By waiting several hours to empty chromium from lumen, they will measure just stomach tissue Cr(III).

One panel member noted that MRI has limited resolution such that it will not be possible to measure tissue levels of chromium; the tissues are small and the method needs lots of pixels to identify the tissue of small intestine or stomach wall. This reviewer suggested that the authors will have a better chance of seeing signal change by measuring chromium in lumen of stomach or small intestine. Dr Clewell replied that with the multiple dose drinking water study, they are trying to build up the concentration of chromium in tissues. By waiting several hours to empty chromium from lumen, they will measure just tissues. In the gavage study, they *are* looking at lumen and at the *rate* of reduction of Cr(VI) to Cr(III). One reviewer suggested using a mini-pump and evaluating chromium at steady state. An observer asked if the imaging study would be conducted in male or female mice. Dr. Kaimal indicated that they would use female B6C3F1 mice.

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5. Panel Discussion

Overall, the panel appreciated the cutting edge nature of the research strategy as a basis to integrate information from both evolving and traditional toxicity testing for both the chromium assessment and risk assessment in general. The panel considered that the author/research team is highly qualified and expressed appreciation for the quality of their work on both the written material and presentations. The panel noted that the presentations by the research team were important for helping the panel understand purpose of research and recommended that additional information be included in the proposal so that it is self-explanatory.

In evaluating the research program, the panel considered the series of charge questions that are presented in Appendix B, page B-4. After discussing all of the questions, the panel noted some technical concerns with the research and made recommendations on how the proposal could be improved. Therefore, the report is organized according to the major areas of discussion and recommendation, rather by charge question. These areas include MOA evaluation, the 90-day study, microarray analysis, HCA analysis, the PBPK modeling, and the MRI imaging.

5.1 Mode of Action

The panel opened the discussion by clarifying the definition of a key event. The panel noted that key events are quantifiable endpoints that are necessary in a hypothesized MOA, but are not necessarily sufficient by themselves. One panel member cautioned that it is important for the research strategy to provide support for any hypothesized MOA; therefore this reviewer recommended that the research define the key events as those that are *measured*. Several panel members pointed out that not every step in the progression toward cancer is a key event; for example detoxification is not a key event. Therefore, the panel suggested that the research strategy should focus on all of the quantifiable key events, and not just those that may cause nonlinearities. The research strategy needs to describe which events are key and why, and then explain why each event is being measured. The ultimate goal is to determine those key events that are the rate-limiting early events driving the response. One panel member also suggested that the research strategy should analyze the MOA following guidance provided by ILSI, IPCS, and EPA. This reviewer noted that using tables to organize the data would help to clarify the key events. Specifically, the panel is seeking the concordance tables as outlined in the ILSI/IPCS framework for evaluating human relevance. In these tables, the hypothesized key events are delineated, along with the supporting data from the experimental animal studies regarding the dose-related and time-related progression of the hypothesized key events, as well as information on the supporting data and biological plausibility of the key events in humans. (If it would be helpful to the research team, panel members offered to provide the template). Dr. Andersen noted that he does not expect to see qualitative differences between humans and rodents, but

there may be important quantitative differences; evaluating such potential differences is one of the purposes of the HCA.

The panel also discussed the issue of multiple MOAs. Dr. Clewell pointed out that the concept of a MOA as a series of cause and effect steps is not necessarily always true. He noted that several chemicals cause chronic oxidative stress or DNA adducts without causing tumors. Rather, tumor development is a multi-factorial process requiring a combination of events. Therefore, the research process cannot focus on testing just one hypothesis; it needs to consider major possibilities in parallel.

A panel member noted that mutation is necessary for tumor formation regardless of the MOA, and that genotoxicity is usually a step in a proposed MOA. Mutations could arise from either chromium-induced proliferation or from chromium-DNA-reactivity. The question is whether genotoxicity is a rate-limiting early key event. That is why it is critical for the authors to determine what is limiting and driving the response. Other panel members suggested that inflammation, cytotoxicity, and direct DNA reactivity are likely key events in the mode of induction of the observed tumors. The panel agreed that cellular uptake of chromium (of unknown oxidation state), oxidative stress, DNA adducts, DNA inter- and intrastrand crosslinks, mutation, cytotoxicity, and cell proliferation are all important key events and should be examined simultaneously to understand dose-response and temporal relationships. Oxidative stress could lead to any of those. Another panel member agreed, noting that oxidative stress is the most important key event in Cr(VI) carcinogenicity. This panel member stated that essentially any effect of Cr(VI) can be inhibited by antioxidants, so all subsequent effects are related to oxidative stress. However, the research proposal does not focus on oxidative stress. A different reviewer noted that reduction of Cr(VI) to Cr(III) may result in either oxidative stress or in ternary adducts; however the research proposal does not include experiments to evaluate those endpoints.

One panel member described his understanding of the early events important to the chromium MOA. This reviewer noted that Cr(VI) in a cell without reducing agents does not bind to DNA; thus, Cr(VI) itself is not a carcinogen. Cr(III) does not enter the cell well, while Cr(VI) does enter the cell, so a key event is the reduction of Cr(VI) to Cr(III), resulting in reactive oxygen species and Cr(V). Cr(V) is unstable and can bind to DNA. Overall, this process supports the oxidative stress response as a key event. Therefore, this panel member suggested that the two important processes in chromium carcinogenicity are oxidative stress and formation of Cr(III), which can react with DNA. The reviewer stated that available data on chromium show that if free radical generation is inhibited, 80-90% of DNA damage is also inhibited. However, other panel members suggested that the tumor response of chromium may be equally related to the presence of Cr(III) from Cr(VI), since Cr(III) can bind to guanine alone, and even more strongly to adjacent guanines, as well as binding to DNA in ternary complexes with other ligands. The

first reviewer noted that there are two potential ways for chromium to cause DNA damage, and suggested that they can be distinguished experimentally. The reduction of Cr(VI) to Cr(III) results in reactive oxygen species and oxidative stress; antioxidants and cellular reducing agents would protect from DNA damage resulting from this oxidative stress. The second approach if for Cr(III) to interact directly with DNA; reducing agents would not provide protection from that interaction.

One reviewer indicated that the information provided from these studies will not be enough to determine the appropriate approach for low-dose extrapolation. The panel member noted that the research team is hoping to generate data that will support a non-linear low-dose extrapolation. This panel member noted that cells have a large capacity for reducing agents and enzymes, and cells will continue to produce reductants such as glutathione (GSH) while Cr(VI) is being reduced. Therefore, it will be unlikely for Cr(VI) to fully saturate or overpower a cell's reducing capacity. The concentration of Cr(VI) would have to exceed >10 mM in cell before it would saturate its reducing capacity; however this concentration would result in cytotoxicity. Even at the high doses tested in the NTP study, this reviewer would not expect Cr(VI) to overwhelm the reductive capabilities of cells. This concept is supported by the available data, which, according to this reviewer, do not demonstrate clear nonlinearities for DNA damage, apoptosis, or any other effects; this reviewer stated that there is no evidence of a threshold for Cr(VI)-induced cell injury for any type of injury.

The panel members agreed that the research proposal does not adequately address the role of oxidative stress, and that measures of oxidative stress are critical to understanding the key events for Cr(VI) carcinogenicity. One reviewer also indicated that the research proposal is deficient in quantifiable measures of mutation and genotoxicity – particularly Cr(0)-DNA adducts. The panel agreed that the proposal was weak on evaluating genotoxicity and oxidative stress. Specifically, the panel recommended that the research program include the following analyses:

- one or more of the following measures of oxidative stress:
    - 8-OHdG
    - Measures of lipid oxidation such as malondialdehyde (measured by hydrazine derivitization), isoprostanes, or 4 hydroxy-nonenol
    - protein oxidation
- a measure of anti-oxidant status in animal such as glutathione peroxidase or superoxide dismutase or catalase, to see if these enzymes are becoming saturated.
- a measure of key oxidant-response proteins such as AP1, p53, or N-kappaB.
- a measure of DNA adducts using techniques such as inductively coupled mass spectroscopy
- a measure of DNA inter or intra-strand crosslinks, which can be done with existing technology in mouth or small intestine.

One panel member asked about whether the research plan will measure cell proliferation rate, as a potential key event. Dr. Thomas replied that there is a very high background of cell turnover in buccal cells and in the small intestine, and so it would be very hard to measure any increase in cell proliferation.
The panel also discussed whether it would be useful to evaluate inflammation as a potential key event. For example, cytokines (e.g., IL6) could be measured as an inflammatory marker. Dr. Thomas noted that there is a difference between the significance of circulating cytokine levels and levels of tissue cytokines. The microarray study would identify inflammatory changes in the target tissue if those changes were regulated transcriptionally. He stated that changes in circulating cytokines may not be identified in the microarray study. One panel member thought it would be useful to measure circulating cytokines in blood, since that measure reflects changes in multiple tissues.

The panel noted that EPA's cancer guidelines promote the concept that a chemical's MOA must be considered before a dose-response approach is selected; the guidelines emphasize the use of data over default decisions. One reviewer stressed that the guidelines ask for an understanding of MOA for risk assessment purposes, not a complete understanding of the mechanism of action. Therefore, it is important to remember that it is not necessary to have all of the mechanistic details in order to evaluate MOA. The questions are whether there is enough information to define key events in a hypothesized MOA, for which the experimental protocol will provide data relevant to consideration of the weight of evidence based on the modified Hill criteria.

Overall, the panel agreed that the research strategy is consistent with spirit of the guidelines; however, as discussed earlier, there are some areas of the program that should be expanded including evaluation of speciation, oxidative stress, and mutation. The panel also stressed that the point of conducting research such as this is to measure changes that are related to cancer, not just to understand what is happening at the mechanistic level. It is important to not assume that the research can lead to an understanding of the underlying mechanism while at the same time addressing risk assessment questions. Therefore, the panel stressed again the importance of defining the key events as those that are directly related to the endpoint of concern, that is, tumor formation, laying out all of the possible MOAs, and then prioritizing the research approach based on the available evidence.

An observer, Dr. Richard Sedman of California EPA, noted that a key question of the research relates to the issue of dose response and whether there is a threshold. He observed that in the NTP study, tumors were not observed at low doses, but that doesn't mean that there is a threshold, since the study may not have been sensitive enough to detect a small increase. He also asked whether the dose-response information being collected for the key events will be sufficient to evaluate whether there are thresholds as opposed to just not being able to detect the effect.

For example, if genotoxicity is not detected at a certain dose, is that informative regarding threshold for more than tumors? In response, one panel member noted that risk assessors need to distinguish among background, adaptive, and adverse effects, so that regulators can protect the population. The goal is to describe what is going on at lower doses; both "linear" and "non-linear" low-dose extrapolation are artificial approaches, not fully describing reality. Another reviewer agreed, noting that it will be difficult, if not impossible to identify a threshold dose. The best that risk assessment can do is to statistically define the confidence limits on response. Key events can be measured, but not translated directly to tumor incidence. However, if confidence limits describing a precursor are available, then one can be more precise about how the precursor response changes with dose and can determine if risk is "low enough" to be "acceptable/ negligible." Dr. Sedman noted that, ultimately, if a chemical's MOA is not relevant to humans, there is no risk. So, regulators need to develop dose response data at doses to which the public is exposed, and need to relate the dose-response for the endpoints being evaluated to tumor response.

In contrast, one panel member noted that, when considering the issue of MOA and precursor events occurring at lower doses, the objective is to define when the observed changes are not adverse and to be confident that dose-response-severity relationships have been adequately characterized. Then, the risk assessor will have confidence that if the concentration remains below a certain level or severity of response, subsequent key events will not occur and the population will be protected from cancer.

The panel briefly discussed the need to prioritize endpoints and assays being conducted. Dr. Andersen noted that the research team needs to evaluate which studies provide the biggest scientific payoff, both based on the number of animals used, and recognizing the limitations of the number of assays that can be done with small amounts of tissue. Panel members agreed in principle, recognizing the expertise of the research team in this area, but also noting the need for evaluation of certain additional endpoints to evaluate critical hypothesized key events.

In summary, the panel directed the research team to clearly define the hypothesized key events in the MOA within the context of risk assessment. The research program must then generate data on those key events. Identifying the rate limiting early key event will be critical for determining the overall MOA. The panel agreed that cellular uptake of chromium (of unknown oxidation state), oxidative stress, DNA adducts, DNA inter- and intrastrand crosslinks, mutation, cytotoxicity, and cell proliferation are all important key events and should be examined simultaneously to understand dose-response and temporal relationships. Oxidative stress could lead to any of those, and evaluating oxidative stress markers is a key element that needs to be enhanced in the research framework.

The panel discussed the risk assessment process for carcinogenicity and how risk assessors might incorporate the MOA data into a regulatory effort. Currently, the methods available to

regulators focus on counting tumors, so the research proposal needs to also include information that demonstrates how to use the MOA data directly in risk assessment, by focusing on the low dose region. The issue for the research team will be anchoring data on key events and the hypothesized MOA to traditional risk assessment approaches for carcinogens. One panel member explained that since the research team is proposing evolving technology, it needs to provide additional effort on the front end to explain how that technology can be used.

5.2 90-day Study and Microarray Analysis

Because the study designs of the 90-day study and microarray analysis are so closely related, this report will present the discussion of both studies together. Key issues under discussion were the dose spacing, the number of animals, the appropriate exposure durations, inclusion of non-target tissues, and use of only female animals.

5.2.1. Dose spacing

The panel started this discussion by considering the appropriateness of the doses and dose spacing for the proposed studies. The research team scientists explained that their intent is not to match all doses used in the NTP studies. Rather, they had considered using the 516, 172, and 14 mg/L dose groups plus two lower doses, an environmentally relevant dose and a control. The panel agreed that 6 doses plus controls should be adequate to evaluate the dose response; however, one reviewer noted that it is crucial for the proposed doses to be in the range where tumors are starting to develop and doses below that. Panel members were not sure if the environmentally relevant dose would inform the shape of the dose-response curve because it would be too low to have detectable effects. One panel member suggested using the doses 516, 172, 57/60, 14 mg/L and then adding 2 lower doses and a control. Other panel members agreed.

The panel then discussed the advantages of using higher doses than used in NTP bioassay. Dr. Andersen noted that higher doses were limited by the poor palatability of drinking water containing such high Cr concentrations, resulting in decreased drinking water ingestion and decreased body weight gain. In addition, the highest dose in the NTP 90-d study led to erosion of the stomach, so a somewhat lower dose was used as the high dose in the 2-year study. One panel member suggested that doses for the in vivo mutation analysis should be high enough to ensure a positive result, noting that if mutations are not observed at the doses tested, there will be questions about what would happen at 2 years. This would raise the question of whether mutations would be increased if the high dose in the 90-day study were extended to two years, or whether the mutations would only be increased at higher doses. Another panel member agreed that testing a higher dose may be worthwhile. If it fails, at least it has been tried; if it succeeds, then more information is available.

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5.2.2. Number of animals

The panel then discussed recommendations regarding the number of animals proposed for the research program. One reviewer noted that in order to evaluate the shape of the dose-response curve statistically, the studies need to have an adequate number of animals; 10 animals/dose group is probably adequate for continuous data, but is marginal for statistical testing of quantal data. Therefore, this panel member expressed concerned that the research only proposed 5 animals/dose group for the histopathology analysis. The proposal indicates that histopathology will be evaluated only in the high dose and controls and that histopathologic examination will continue on lower dose groups until a NOAEL is clearly recognized (page 9, line 10 of proposal). However, the proposal does not specify how a NOAEL will be identified with only 5 animals. This reviewer indicated that one cannot identify a NOAEL with only 5 animals, since an effect in 3 out of 5 animals (60%) would not be statistically significant. This high incidence certainly cannot be considered a no effect level even though it does not achieve statistical significance. Dr. Thomas indicated that the primary use of the histopathology in this study is to phenotypically anchor the genomics data. The reviewer indicated that this type of categorical data is better than yes/no data, but that 5 animals per group is still not enough; 10 animals per group would give better statistical results if resources are available. This reviewer also recommended against the approach of conducting histopathology on the control and high dose, and evaluating lower doses until a NOAEL is identified. Instead, the reviewer recommended conducting histopathology on all doses and controls, except perhaps not at the two lowest doses. Another panel member suggested that a power analysis should be performed in order to provide justification for the number of animals and for the choice of using female animals. Overall, the panel recommended that the number of animals used for the histopathology analysis in the 90-day study should be increased to 10/group in order to improve the statistical evaluation of the shape of the dose-response curve.

Regarding the histopathology, Dr. Haws asked if there is a utility in doing pathology analysis of tissues from the lower dose groups if no histopathology was observed in the high-dose tissues. One panel member agreed that this would not be useful; however, he also explained that this does not define a no effect level.

The panel also discussed the number of animals proposed for the mutation analysis. One reviewer suggested that, based on the proposed study design, 5 animals/group would be adequate, but that there would be more confidence in the response if the sample size were increased to 10 animals/group rather than 5. Dr. Thomas explained that the research proposal calls for exposing 10 animals, but they will be performing the microarray analysis on approximately 5 animals per treatment group. However, they will run microarray analysis on all

10 control animals to better define the control for statistical comparisons. Other panel members agreed that this was sufficient.

5.2.3. Appropriate exposure durations

The panel then discussed the issue of exposure duration and the proposed time points for the studies. In order to understand the time course effects, the panel asked when tumors first occurred in the two-year study. Dr. Proctor indicated that the first adenoma in male mice occurred at day 451; carcinomas in males were only found at termination, day 729. The first adenoma in female mouse small intestine was at day 693; the first carcinoma was observed at day 625.

One panel member suggested that the rationale for using a 90-day exposure needs to be articulated in the proposal, since it could be that a 3-week, 1-month, or 6-month exposure could be more relevant to the endpoints being examined. Another panel member agreed, noting that De Flora et al. (2008) did not observe cross-links at 9 months. Dr. Haws replied that the 90-day study is the only study available to anchor genomics to the preneoplastic lesions. The research team is concerned that if they only have shorter exposures, integration of results from the 90 day study with the existing data would be more difficult. The panel agreed that this is a good rationale for doing the 90-day study, but the rationale should be articulated in the research plan.

One reviewer questioned whether a 90-day exposure was long enough to detect mutation endpoints in the microarray analysis. Another reviewer replied that the length of exposure needed to see an effect varies depending on the endpoint being monitored; however, generally, a 90-day exposure should be sufficient to assess mutations.

One panel member asked why the authors were considering a 1-day exposure. Dr. Andersen replied that the broader literature on compounds that affect the stress compounds demonstrate significant effects at 1 day that resolve by 5 days. For example, hypochlorous acid shows up-regulation of stress-response pathways; the 1-d response reflects what is happening in naïve animal, so it is important to evaluate 1-day and 7-day exposures separately. One panel member agreed with the rationale for the 1-day exposure but suggested that the 7-day exposure might be too soon because the responses may still be leveling out a bit at 7 days. Dr. Andersen replied that, based on their experience from formaldehyde, a clear adaptive response occurs at 5 days but beyond that there is not the same response. Therefore, 5 days seems to reflect some alteration in tissues; based on formaldehyde data, they believe that 21 days would be too long, but they are not sure.

Dr. Haws asked if the research design should include an evaluation of recovery/reversibility. One reviewer suggested that the research team consider the risk assessment criteria for Weight of Evidence and MOA in determining this issue. There may be more important questions, but it would be of interest if a recovery period prevented a key event. This reviewer suggested that risk assessors are more comfortable with recovery data for histopathology when compared with genomics and suggested that the research team consider adding a recovery period after the 90-day exposure and hypothesizing whether the effects are expected to be reversible with discontinuous exposure. Another reviewer indicated that there would not be any value in doing a recovery period for the genomics studies because we already know there is a high capacity for DNA repair.

5.2.4 Inclusion of non-target tissues

One reviewer also suggested including non-target tissues where tumors did not develop because it is difficult to evaluate *in vivo* mutation parameters in the target tissues. A different panelist asked whether using a different species as control would overcome these difficulties, for example, using mice as the non-target control for the rat study. The first reviewer thought that this approach would work, but could increase cost. This person suggested using liver as a non-target tissue, and also using lung, since that is a target for the inhalation route. Examining lung as the negative tissue may provide more information than examining the oral cavity in mice or small intestine in rats. Other panel members agreed, noting that primary human exposures are drinking water and occupational exposure, which clears to GI tract.

5.2.5. Use of only female animals

The panel then discussed the issue of gender and whether conducting studies in only females will provide sufficient information about MOA. One panel member noted because there is typically so little difference between males and females in tumor development, the fact that the females were more sensitive in the NTP assay means that it makes sense to focus on females. However, another reviewer stated that if the studies showed dramatic differences in the precursor lesions between males and females, this would be useful to know. Therefore, this reviewer thinks it would be useful to also generate data in males. Ms. Proctor noted that a New Jersey risk assessment for chromium evaluated the dose response in both sexes separately and combined. The cancer slope factor was based on the male mouse data based on fit of the BMD modeling. While the panel generally agreed that there is no reason to expect different MOAs in males compared to females, one panel member suggested that the proposal provide additional information on the rationale for using only females. Other panel members suggested contacting New Jersey to find out why they used data from males. One reviewer noted that the NJ report

shows that the dose response curve fit the male mouse data a little better than the female data; however, no values for assessing curve fit were provided.

During the course of the meeting, *TERA* contacted NJ to ask why they based risk values on male mouse data. Dr. Alan Stern of the NJ Department of Environmental Protection gave the following response:

> As stated in our risk assessment document, the male mice were used in preference to the female mice because the female mouse data could not be well fit to any of the standard dose-response models. Although some of the models gave higher potencies for the female mice than for the male mice, those models all gave weak fits to the data. This made the potency estimates derived from those models suspect. Among the models that fit the female mouse data better, the potency was comparable to that found with the male mice. In contrast, nearly all of the available dose-response models gave strong fits and gave essentially the same potency when applied to the male mice. Furthermore, the data suggest that at the highest dose, the female mice exceeded the MTD.

Based in part on the NJ response, the panel concluded that using only female mice was reasonable.

5.2.6. Role and Interpretation of Microarray Data

In response to a panelist question, Dr. Thomas stated that the analysis of the microarray data will group genes based on function (e.g., apoptosis, DNA repair, cell proliferation, oxidative stress, etc.), and evaluate how the effect levels for changes in gene expression of the different functional categories changes with dose and time. They will then compare those effect levels with those for the tumor response, to see if the data are consistent or inconsistent with hypothesized MOAs. For example, if genes related to a specified functional category (key event) change at a higher dose than the tumor response, then this observation is inconsistent with the hypothesis that the functional category plays an early causal role in tumor formation. A panel member noted that it is important to anchor such observations to other measures of key events in order to increase the level of comfort of risk assessors with genomics data. Another panel member noted that genomics data provide only correlative information, not cause and effect. Dr. Thomas agreed that mechanistic studies are needed to evaluate cause and effect. In conducting such mechanistic studies, panel members agreed with the research team that it makes sense to conduct pilot studies evaluating certain key endpoints (e.g., GSH) before extending to other endpoints.

5.2.7. Summary

The panel recommended adding 5 animals/dose (i.e., n=10, at least for the histopathology study). The panel noted that the proposed number of animals was probably adequate, from a statistical perspective, for defining the shape of the dose-response curve for continuous data, but it is marginal for statistical analysis of quantal data. The panel noted that the purpose of doing histopathology is to identify phenotypically anchor the genomics data. However, with only 5 animals per dose, there were concerns both about the study sensitivity to identify an effect, and the approach of starting at the high dose and conducting histopathology evaluation until a NOAEL is identified. Therefore, the panel recommended an increased number of animals to improve the phenotypic anchoring. Several panel members also recommended that the histopathology approach be modified to evaluate the controls and dose groups (except perhaps not the two low-dose groups), unless no histopathology at all is seen at the high dose. The increased number of experimental animals may also allow some of the suggested testing for oxidative stress endpoints, but the panel recognizes that some of these requested tests may lead to technical challenges.

The panel agreed with the authors to run the 90-day study using 6 dose groups and a control. Instead of using three doses lower than the lowest dose in the NTP bioassay, however, the panel recommended two unspecified low doses plus the top three doses in the NTP bioassay. Therefore, the dosing regimen should have the following approximate mg/L doses: 520, 170, 60, 14, two unspecified lower doses and a control.

Panel members concurred with the authors' suggestion to add a 1-day interim sacrifice in addition to the 7 and 90-day sacrifices for the genomics analyses.

The panel recommended the consideration of recovery time points for some key events, if experimental findings suggest that such analyses will provide data that are critical to risk assessment.

The panel agreed with the authors in that it did not believe that the MOA would be different between males and females or that there was any statistically significant difference between males and females in the dose-response. Therefore, using only females in the research is sufficient.

No specific panel recommendations were made for the microarray analysis, separate from what was recommended for the 90-day study.

5.3 HCA Analysis

One panel member noted that the HCA studies appear to be designed to look at proteins related to cell cycle control, and asked how these relate to MOA hypotheses that have been proposed. Dr. Thomas explained that they are related to DNA damage response/repair, not cell cycle per se. Changes in H2AX foci has been correlated with double strand DNA breaks; changes in nuclear P53 binding protein correlate with increased single strand breaks, DNA damage by alkylating agents, and, potentially, oxidative stress. The proposal suggests conducting these studies in human and rat cell lines related to target tissues because the methods have not been developed to conduct the studies in the target tissues themselves. PBPK modeling will inform dose selection based on target tissue dosimetry and the studies will assess quantitative difference between species. Dr. Thomas stated that an advantage of HCA is that it is possible to evaluate more dose levels and replicates than would be possible *in vivo,* so they can better define the shape of the dose-response curve. One reviewer suggested that the proposal should differentiate between agents that look at single strand vs. double strand breaks. This reviewer also stated that the selection of endpoints will not allow for evaluating accumulating mutations, an important distinction, because if repair is 100% successful, there will be no tumor formation. Dr. Thomas agreed, noting that the endpoints selected relate to DNA repair processes and not the tumor response. The HCA studies are meant to complement *in vivo* genomic and mutation studies, not stand alone.

Another panel member asked for more information about the cell lines to be used in these studies. Dr. Thomas explained that they had chosen cell lines in order to match the affected cell type for the intestinal tumors seen in mice. To his knowledge, there is no rat buccal cell immortalized cell line; they would have included one if it were available. The Caco2 cell line has been widely used in the pharmaceutical industry, but the proposed mouse epithial cell line is not widely used and would need to be characterized prior to use for the HCA studies. The panel agreed that using the proposed cell lines would give reasonable information, given the limitations of conducting the studies in the primary target tissues.

The panel concluded that the choice of doses and dose spacing, exposure duration, and examination time points were all appropriate. One panel member noted that these studies could easily be modified to include more endpoints relevant to evaluating MOA. For example, all of the endpoints related to oxidative stress that were mentioned for the *in vivo* study and an antioxidant block could also be evaluated in the HCA studies. In addition, HCA can be used to evaluate the implications of inhibiting specific pathways. However, another reviewer noted that assessing oxidative stress and inflammation endpoints is more important in the *in vivo* studies than the HCA studies. The panel recognized these differences, but suggested that these additional endpoints also be evaluated in the *in vitro* systems because it would be easy to do so.

Another reviewer suggested that the *in vitro* cell cultures could also provide information about various valence states of chromium. Dr. Haws asked if reducing agents could be used to understand the valence state issues. Another reviewer thought that this approach would not generate any additional information, but since it was so easy and cheap, it could not hurt to try it. Generally, application of antioxidant will be more informative because antioxidants can block pathways and selectively block the formation of Cr(V) and Cr(III). In addition, this reviewer suggested that the research team conduct a literature search for HCA studies using reducing agents such as GSH or ascorbate, and HCA studies on Cr(VI) and Cr(V).

In a related issue, the panel considered whether the research should include *in vitro* genomics using the same cell lines as for the HCA studies. However, the panel agreed that such *in vitro* studies would not be useful for a risk assessment and that the *in vivo* data would be preferred.

5.4 PBPK Model and MRI Analysis

Because development of the PBPK model and the outcome of the MRI studies are so closely related, the report discusses them together.

Overall, the panel concluded that the existing PBPK model is well worked out in rats and that key issue for model development is differentiating Cr(VI) and Cr(III). The panel agreed that the approaches proposed by the research team are appropriate, and the main question is whether the research team can measure Cr(III) with current technology. One panel member suggested that it could be valuable to get direct experimental measures in the mouse. Dr. Clewell responded that they currently have limited mouse data and that additional kinetic data in the mouse would be good. In addition, they do not have data on clearance, just on accumulation. Therefore, an oral gavage, single-day study could collect clearance data. In addition, NTP has data on total Cr in urine and feces in male rats and female mice from the 2-year study, which will be published soon. A reviewer suggested that NTP be contacted regarding the availability of the data.

One panel member gave a short presentation regarding approaches for measuring different chromium species/valence states. The presentation explained that Cr(VI) not paramagnetic, so it cannot be measured using MRI. Electronic spin resonance (ESR) measures free radicals. Cr(V) has one free electron, so it can be measured with ESR; however, Cr(IV) cannot be measured with ESR. Cr(III) can be measured with ESR but gives very broad lines so it is difficult to evaluate. This reviewer has collaborated on an approach to measure Cr(V) *in vivo* with ESR. In this project, the animals were anesthetized, then injected with 10 uL of 100 mM Cr(VI). By measuring every 30 seconds, it is possible to follow Cr(V) formation in real time in the whole animal. This method will resolve Cr(V)-GSH vs. Cr(V)-NADPH and can follow changes with

time. Using this approach, they have determined that there is a sharp increase in Cr(V) followed by a rapid decrease, with most Cr(V) gone by 2-3 hours. This is an effective approach to evaluate the effects of antioxidants on chromium levels and valence state.

The discussion then focused on the proposed imaging studies. One panel member stated that with MRI, measuring relaxation times in different tissues will result in a variability of ~10%. Therefore, this reviewer expects that the effects of Cr(III) on tissue relaxation time will be small. Because other naturally-occurring elements in body (Fe, Mn) have a larger effect on relaxation time, these other elements may swamp out effects of the chromium. This reviewer's impression of the proposal was that the research would be looking at small changes using instrumentation that is not designed to look at small changes, especially *in vivo*. This reviewer suggested that it would be useful to conduct another pilot, using Cr(VI) administered by gavage, rather than the current drinking water study, and measuring the signal from Cr(III). Use of the more controlled exposure scenario would allow the researchers to define reduction of Cr(VI) to Cr(III) more precisely. This methodology could also include different dose levels and exposure durations (e.g., compare reduction rate at 7 day vs. 90 day). This reviewer recommended starting with the more basic studies and pilot studies that are more likely to succeed, and allowing the pilot studies to suggest potential further studies.

One panel member noted that animals are currently being dosed for the pilot study and discussed approaches for determining the best route forward with those animals. This reviewer suggested that looking at accumulation of Cr(III) in intestine is not feasible. In addition, this reviewer suggested that the authors also conduct relaxation time mapping to obtain quantitative data that would be directly related to tissue Cr(III) concentrations. However, this reviewer suggested that a more realistic approach would be to continue earlier pilot studies with oral gavage to develop data on the rate of reduction of Cr(VI); these data can be used to refine the PBPK model and to determine what additional information is needed to further improve the model in the future. Dr. Haws proposed several options for continuing with the pilot study, and asked the panel which of these options were appropriate: 1. Continue to expose animals via drinking water for longer time, such as 180 days, to increase the likelihood for appreciable accumulation. 2. Stop the drinking water study and begin dosing animals via gavage. 3. Terminate the study, collect the tissues, and measure total chromium. The team could also explore conducting the imaging at earlier times, after the dark period, to increase the signal.

One panel member responded that, based on Dr. Clewell's presentation, chromium levels in intestine should have reached steady state, so one would not expect that a longer dosing period would lead to increased levels in intestine. This reviewer also disagreed with the proposal to sacrifice the animals now, because it is possible to generate additional useful data from them. Therefore, this reviewer suggested that the authors try the oral gavage exposure with the animals in the Charles River pilot that had drinking water exposure, to look at the rate of reduction. However, the authors should not use the control animals for the gavage experiment. Rather, the

reviewer suggested using the controls for an *ex vivo* imaging study, in which the small intestine is removed for imaging, in order to compare with animals exposed by drinking water. In the feasibility stage, it is not critical if chromium from tissues affects the results from the gavage study. After preliminary results are available, the study would need to be repeated with appropriate controls for quantitative use. In addition, this reviewer suggested that the authors also conduct relaxation time mapping to determine the rate of formation of Cr(III).

The panel also discussed use of ESR technology to accomplish some of the research goals. Panel members acknowledged that this technology could help the research team gather the necessary data, but that it may be difficult to find someone capable and willing to do the study because ESR machines are not as available as MRI machines and special expertise is needed. Therefore, the panel was hesitant to suggest pursuing this, given the unknowns. In addition, the panel voiced the opinion that the MRI experiments seemed to be the most risky in the proposal and the research teams should evaluate the time and cost of solving these issues relative to other more easily accomplished aspects of the proposal.

Panel members with expertise in MRI offered to assist the research team in study design issues off-line after the meeting, as needed.

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6. References

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