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Peer Review Report on the Chromium Mode of Action Studies: 90-Day Rat Study

Expert Review Organized by Toxicology Excellence for Risk Assessment (TERA)
www.tera.org/peer

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Note

This report was compiled by scientists of Toxicology Excellence for Risk Assessment (TERA). The peer experts 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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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 rat 90-day study, which includes the toxicokinetic, biochemical and pathological analyses that have been completed on tissues from that study, has now been completed and is available for review. ToxStrategies has prepared a draft manuscript reporting the results of the rat 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 rat 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, TERA; Xianglin Shi, University of Kentucky; Dave Gaylor, Gaylor and Associates; and 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 July 12, 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. Appendix A contains Instructions to Reviewers and Appendix B is Reviewer 3's annotated draft.

Comments in Response to Charge Questions

Charge Question 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?***

Reviewer 1: 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. The use of GSH/GSSG ratio as an indicator for Cr(VI)-induced oxidative stress should be better justified.

Reviewer 2: 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 3: I do not see any issues with these items that would prevent submission of this paper for publication.

Reviewer 4: There should be determinations of Cr adducts to DNA (or other macromolecules). This is germane to the likely possibility that the MOA is Cr→ DNA Cr adducts→ mutation→ cancer. This possible MOA was discussed in the Chapel Hill meeting conducted by TERA. This may be data that is coming along with mutation data and cytogenetic assays. Based on the 2 Cr documents I have seen so far there has been zero attention paid to this Cr→ DNA Cr adducts→ mutation→ cancer hypothesis.

Some work should be done in tissues that are not tumor target tissues because otherwise you are left with associative type data saying oxidative stress is observed to occur and so does tumors so therefore one ASSUMES that oxidative stress caused the tumors. Oxidative stress may be happening in 18 out of 20 tissues and only one of these tissues is developing tumors. Then the association and the inference of causality is much weaker than if the oxidative stress is tumor tissue specific or somewhat so.

- ***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: 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 2: This is outside my area of expertise.

Reviewer 3: The endpoints monitored were very helpful. In the discussion section (or perhaps another paper), some text on the differences in the pH of the stomach in humans, mice and rats would be instructive. This is because I anticipate that reduction of chromium in the gut is more likely in humans because of a more acidic stomach.

Reviewer 4: See response to question above.

Charge Question 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?***

Reviewer: Yes.

Reviewer 2: Yes.

Reviewer 3: Based on the study descriptions, it appears that any one animal was used for multiple bioassays, which is fine, but please clarify this point in the methods section.

Reviewer 4: This reviewer did not respond to this question.

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

Reviewer: No.

Reviewer 2: No.

Reviewer 3: No.

Reviewer 4: This reviewer did not respond to this question.

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

Reviewer: Yes

Reviewer 2: Yes.

Reviewer 3: This is outside my area of expertise.

Reviewer 4: This reviewer did not respond to this question.

- **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: This reviewer did not provide an answer to this question

Reviewer 2: Adequate evidence was supplied.

Reviewer 3: The description and discussion of the various lesions are appropriate and support the conclusions wrought, with the exceptions noted in response to question 5 below.

Reviewer 4: There results look OK and the interpretations are reasonable.

Charge Question 3. Study Conclusions:

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

Reviewer 1: No

Reviewer 2: None were noted.

Reviewer 3: See response to question 5 below, but in general this report is a powerful statement on chromium effects in experimental animals and fills in an important gap in our understanding of its Mode of Action (MOA). I did not find any contradictory statements, nor observations. Critical issues associated with understanding chromium's MOA were addressed.

Reviewer 4: The three better possibilities for a Cr MOA are (a) cytotoxicity, (b) oxidative stress and (c) Cr DNA adducts→ mutation. It seems the Cr DNA adducts are being omitted from consideration and that this study may be biased because of favoritism for the oxidative stress hypothesis. DNA mutation is much more highly correlated with tumors than is oxidative stress. The Cr mutation MOA hypothesis needs to be eliminated or greatly diminished in likely hood before other possible MOAs (oxidative stress, cell proliferation) can be considered as likely MOA candidates.

Is there demonstrable oxidative stress in rat and mouse organs exposed to chromium that do not show tumor formation?

Why does not the oxidative stress in rat jejunum lead to tumors (Table 7)?

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

Reviewer 1: Although the studies performed is very important in understanding the MOA, based on the limited data obtained from the present study, it may be pre-mature to obtain the conclusion "The finding presented herein suggest that oxidative stress may play an important role in SDD-induced carcinogenesis (p. 16, second paragraph)".

Reviewer 2: Generally, yes. However, focusing only on statistically significant doses fails to indicate biologically significant doses, e.g., benchmark doses, that generally are lower.

Reviewer 3: Yes, with the enhancements shown in response to question 5 below.

Reviewer 4: If the study conductors do not know the results of the genetic orientated data yet, why are they publishing opinions about cell proliferation and oxidative stress as possible MOAs? Everything they are now saying can be rendered OBSOLETE as soon as positive genetic assay information arrives.

Charge 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***

Reviewer 1: 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 2: The data are amenable for benchmark dose analyses in order to calculate points of departure for setting non-cancer reference doses and for deriving a cancer slope factor.

Reviewer 3: I would prefer a chronic study for determination of a RfD/C, but this study might yield useful information for development of Chemical Specific Adjustment Factors (CSAFs). Results from this study give insights into chromium's MOA. I very much enjoyed reading it,

since what was brought into focus is the fact that an understanding of biology will yield more insightful dose response assessments. For chromium in particular, this will be important, since as a constituent of the environment it is found naturally in numerous places.

This study can be used to assist in the determination of a cancer slope factor for chromium's carcinogenicity. In brief, it supports a discontinuity in the dose response curve empirically evident for some tumors in the NTP bioassay and allows EPA and others to pursue a dose response assessment either by way of a dual mode of action (i.e., linear at low dose, oxidative stress at high dose), or with additional support, a tumor threshold. See Dourson et al. (2008) for an example of a tumor dose response assessment with a dual MOA. In this case, linearity in the dose response curve started at a lower point of departure, leading to a linear low dose risk that was lower than that starting the default point of departure of 10%.

Reviewer 4: The overall research is a good attempt to get a data base from which one can make inferences about MOA (and also non cancer reference dose) of Cr in 2 target organs. It is difficult to judge what an EPA regulatory office would do with the final results of this study.

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

Reviewer 1: 1) Tissue and serum iron levels. Better justification is needed for these measurements. The authors should make more effort to discuss how the results are related the MOA. 2) The authors performed various measurements of cytokine and chemokine. The results obtained are not well discussed. In the discussion section, these results are just briefly mentioned. The authors should provide a better rationale to carry these studies and how these results are utilized.

Reviewer 2: No additional comments.

Reviewer 3: The authors should consider the following points:

> Page 15, description of Figure 6.
>
> I did not see a statistically significant mark for the ileum at either 60 or 170 mg/kg-day in Figure 6, unlike what your text implies.
>
> Page 16. "Considering that hundreds of rats chronically exposed to very high Cr(VI) concentrations in drinking water (but lower than those herein) did not develop intestinal lesions or tumors in the NTP 2-year bioassay (NTP, 2008; Stout et al., 2009), it is conceivable that if lesions were present at earlier time points, they resolved before the study termination."
>
> I am not sure what is being added with this sentence. This paragraph reads fine without it. Please consider dropping it or placing it elsewhere.

Page 16. Please consider the following revision of text:

The overall findings presented herein and by Thompson et al. (2011) suggest that oxidative stress may play an important role in SDD-induced carcinogenesis. For example, **Table 7** provides a comparison of the target tissue effects in rats and mice at day 91 with the tumor outcomes reported in the NTP 2-year bioassay (NTP, 2008). For all tissues that developed tumors in the 2-year bioassay, oxidative stress was present at day 91, and in rats this stress is more severe at 91 days when compared with 8 days (compare Tables 5 and 6), a direction consistent with tumor formation at longer durations. Moreover, in two of three tissues without oxidative stress, tumors were also not present, despite the fact that, for example, dosimetry data in **Figure 7A** indicate that the tissue concentrations of chromium (Cr_t) in the rat oral mucosa with positive tumor results were similar (or lower) than the mouse oral mucosa with negative tumor results, yet oxidative stress was only present in the rat oral mucosa (**Figure 4A**). Furthermore, when the GSH/GSSG ratio is examined as a function of Cr_t levels in the oral mucosae (**Figure 8A**), it appears that rat oral mucosa may be more sensitive to SDD-induced redox changes than the mouse oral mucosa. Together, data in **Figures 4A, 7A** and **8A** suggest that the oral cavity tumors observed in rats is not simply a function of tissue dose, but may involve differential effects on redox status.

Page 17. Please consider the following revision of text:

In this regard, the incidences for atrophy and crypt hyperplasia at day 91 were lower than day 8 (**Table 3**, **Supplemental Figure S5**); this implies that if the animals had been allowed to survive and grow, the lesions might have resolved, which is consistent with the negative tumor findings at 2 years. As was observed in the oral mucosa, the differential effects of SDD on the rat and mouse duodenal GSH/GSSG ratio appears to entail more than tissue dosimetry (**Figure 8B**), and may involve species differences in duodenal responses to oxidative stress.

In the jejunum, oxidative stress was observed in both species (**Table 7**, **Figure 4C**). As was observed in the duodenum, the incidences for atrophy and crypt hyperplasia at day 91 were lower or even absent when compared with day 8 (**Table 3**, **Supplemental Figure S5**), and thus these tissues may have recovered if the study had continued for longer duration.

Page 19, last sentence: Whether (and how) this could manifest as oral tumors in rats is not known.

It seems that a brief literature survey on this topic would be beneficial, rather than just saying that this is not known. You give evidence that iron-deficient humans have more risk to oral cavity tumor in the following paragraph, but what about searching the International Toxicity Estimates for Risk (ITER) or NTP databases for oral tumors and then seeing if the experimental animals were iron deficient?

Page 20. "In humans, there is some evidence that metabolic conditions of iron deficiency or anemia are associated with changes in the oral epithelium and oral cavity cancers (Lucenteforte et al., 2009; Richie et al., 2008). For example, Richie et al. (2008) evaluated 65 hospitalized patients with oral cancer and 85 matched controls and found that mild iron deficiency and low GSH levels increased the risk of oral cavity cancer."

Very nice, now please describe what Lucenteforte et al found.

Page 20. "However, a recent occupational study reported that anemia was not associated with occupational exposure to Cr(VI) (Muller et al., 2011)."

Sorry, this latter sentence and the following ones are not helpful to the topic being discussed. You are discussing whether or not iron-deficient-induced-anemia is associated with oral cancers, not whether or not Cr is associated with oral cancers.

Page 25. Table 2.

The determined SDD doses cannot be more precise than the concentrations; please adjust them accordingly.

In general,

I also suggest a few minor editorial revisions as found in the attached strike and replace version of the text.

Reviewer 4: Pages 12 and 13, 15 there are two different fonts being used.

Table 1- the dose of 14 mg/L is not present in Table 1 and is included in other data Tables

Table 5 and 6 - I doubt that 4 significant digits can be justified in some of the GSH data in Table 5. For example are you sure there is not 0.01% water in the GSH standards that you weighed? That would be needed to justify a claim of four significant digits.

Similarly, I doubt that 5 significant digits can be justified in some of the delta E(mV) data in Table 5 and 6

Table 7 - Please use the whole word instead of abbreviations for OS, CP and T

Table 7 - is a good communicative concept summary.

Figure 2 - Is there no variation in 7 of the measurements that have lines and usually stars on them? Not detected? Not done? Reader does not know what you mean here. This looks mighty odd for quantitative data.

Figure 3 - Anything $P < 0.05$?

Figure 4 - Good figure and data

Figure 6 - should be given as a Table because 3 of the groups cannot be seen on the y-axis

Figure 7 - Rn = ___??? Mm = ___???? You need to say what Rn and Mm mean. The reader cannot figure this out.

Line numbers were not on my version of the paper so I could not use them in the review.

Appendix A: Instructions to Reviewers

Dear Reviewers,

Thank you again for your willingness to provide a peer review of the Rat 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 rat 90-day study. The final report of rat 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/Southern%2090%20day%20rat%20study%20report.pdf. Foll owing 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, Monday August 1, 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.

I will be out of town for the next week or so due to a family emergency. If you have immediate questions regarding this review, please contact Jacqueline Patterson (Patterson@tera.org; 513-542-7475, x29). 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 3's Annotated Draft

Comparison of the Effects of Hexavalent Chromium in the Alimentary Canal of F344 Rats and B6C3F1 Mice Following Exposure in Drinking Water: Implications for Carcinogenic Modes of Action

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Short title: Toxicity of Cr(VI) Following Exposures in Drinking Water

Abstract (250 of 250 max)
Chronic ingestion of high concentrations of hexavalent chromium (Cr(VI)), in the form of sodium dichromate dihydrate (SDD), in drinking water has been shown to induce oral mucosa tumors in rats and intestinal tumors in mice. To investigate the modes of action (MOAs) underlying these tumors, 90-day drinking water studies, with interim necropsy at day 8, were conducted in F344 rats and B6C3F1 mice with concentrations of 0.3-520 mg/L SDD. Blood and tissue samples were analyzed for chromium content, oxidative stress, iron levels, and gross and microscopic lesions. Results for the F344 rats are described herein and compared with results from B6C3F1 mice published previously. After 90 days of exposure, total chromium concentrations in the rat and mouse oral mucosae were comparable, yet significant dose-dependent decreases in the reduced-to-oxidized glutathione ratio (GSH/GSSG) were observed only in rats. In the duodenum, changes in GSH/GSSG were only observed in mice. Levels of 8-hydroxydeoxyguanosine and 8-isoprostane were not increased in the oral or duodenal mucosae of either species. Glutathione levels were increased in the duodenum but decreased in the jejunum of both species, indicating differential responses in the intestinal segments. Histiocytic infiltration was observed in the duodenum of both species, yet duodenal cytokines were repressed in mice, but increased in rats. Serum and bone marrow iron levels were more decreased in rats than mice. Collectively, the data herein suggest that Cr(VI)-induced carcinogenesis in the rodent alimentary canal involves oxidative stress; however, histopathological lesions, cytokines and iron status suggest contributions from species-specific differences as well.

Key Words: drinking water, oxidative stress, carcinogenesis, hexavalent Chromium, Cr(VI), mode of action (MOA)

Introduction (678)

Chronic ingestion of hexavalent chromium (Cr(VI)), in the form of sodium dichromate dihydrate ($Na_2Cr_2O_7$•$2H_2O$ or SDD), in drinking water has been found to induce oral mucosa tumors in rats at concentrations ≥ 172 mg/L, and intestinal tumors in mice at concentrations ≥ 57.3 mg/L (NTP, 2008; Stout *et al.*, 2009). The intestinal tumors observed in mice were preceded by cytotoxicity in the villous and hyperplasia of the crypt, whereas the oral tumors in rats were not preceded by obvious non-neoplastic lesions (NTP, 2008; Stout *et al.*, 2009). The differences in neoplastic and non-neoplastic effects suggest that the tumors in the two sites may have arisen through different carcinogenic MOAs. Although the NTP (2008) 2-year bioassay demonstrated that Cr(VI) was carcinogenic in rodents following chronic exposure to high concentrations in drinking water, the study did not provide adequate data for understanding how the tumors arose or why different tumors were observed in each species. Because mice and rats developed tumors in different tissues of the alimentary canal, comparisons of species-specific pathology and biochemistry in the target tissues (i.e. oral and intestinal mucosae) are expected to inform the MOAs for carcinogenicity in each tissue.

Several authors have proposed or discussed possible MOAs for alimentary cancers in rodents (McCarroll *et al.*, 2010; Stern, 2010; Thompson *et al.*, 2011). However, currently available data are insufficient to conclusively support any of the hypothesized MOAs. This is especially true for oral squamous cell carcinomas originating from the rat palate after 2 years of exposure to SDD, where no non-neoplastic lesions were reported to precede tumor formation (NTP, 2007; NTP, 2008; Stout *et al.*, 2009). The number of oral cavity tumors were significantly elevated over current and historical controls in the highest dose group (516 mg/L) in both males (6/49) and females (11/50), as well as historical controls in females in the 172 mg/L treatment group (2/50) (NTP, 2008; Stout *et al.*, 2009). Stout *et al.* (2009) noted that 21 chemicals have been shown to cause oral cavity tumors in rats, but no chemicals have been shown to cause oral cavity tumors in male mice, and only one caused oral cavity tumors in female mice. These findings may suggest an inherent susceptibility of rats to oral cancers.

In addition to oral tumors, Stout *et al.* (2009) reported that rats also exhibited a dose-dependent decrease in mean red blood cell (RBC) volume, mean cell hemoglobin (Hb) and hematocrit—indicating microcytic hypochromic anemia (NTP, 2008). Earlier studies reported similar anemic effects in rats exposed to Cr(VI), regardless of whether administered in feed or water (NTP, 1997; NTP, 1996; NTP, 2007). Although anemic effects were also observed in mice in the NTP (2008) study, the effects were milder. Interestingly, one study has shown that anemia can increase the risk of oral cancer in rats (Prime *et al.*, 1983). This suggests that the anemic effects in rats may play a role in the MOA of the oral tumors.

To better understand the MOAs for the alimentary cancers observed in the NTP 2-year bioassay (NTP, 2008), Thompson *et al.* (2011) conducted a comprehensive review of available data to develop a plausible MOA for the intestinal tumors in mice, identified key data gaps in that MOA, and designed a 90-day drinking water study to address those data gaps. Although a formal MOA analysis was not conducted for the oral cavity tumors observed in rats, data necessary to evaluate possible key events in the MOAs were collected from the target tissues of both species. These biochemical, toxicogenomic, and toxicokinetic data should inform the MOAs for Cr(VI) in the mouse intestine as well as in the rat oral mucosa. For the reasons described above, several endpoints related to tissue and serum iron levels were also collected.

The overall study design is depicted in **Table 1**, and is similar to that of the 90-day NTP study (NTP, 2007). Herein we report results from the 90-day rat study including descriptions of the histopathological findings, biochemical analyses related to oxidative status, and the chromium content in target tissues. Moreover, these results are compared with those previously reported for mice (Thompson *et al.*, in press) to provide information regarding key events in the MOA. Additionally, these data will be used to provide phenotypic anchoring for future toxicogenomic results, which will be published separately upon completion.

Materials and Methods

Test substance

SDD (CAS 7789-12-0) (99.95% pure) was obtained from Sigma-Aldrich, Inc. (Milwaukee, WI) and stored at room temperature and protected from light. The dose formulations were prepared at concentrations of 0.3, 4, 60, 170, and 520 mg/L SDD in tap water, which is equivalent to 0.1, 1.4, 4.9, 20.9, 59.3 and 181 mg/L Cr(VI)[1]. On the first, third, fifth, and seventh (final) batch preparations, samples of formulations for each dose group, including the control, were collected and shipped to Brooks Rand Laboratories (Seattle, WA) for analysis of Cr(VI) content. The first batch was also analyzed for total chromium. Samples were prepared and analyzed in accordance with EPA Method SW-7196A to confirm the Cr(VI) concentrations of the administered drinking water. In Method 7196A, Cr(VI) is complexed with diphenylcarbazide in an acidic solution and absorbance is measured at 540 nm. Batches found to differ from the target concentration by ±10% were not used. Prior to use, dose formulations of SDD were stored in sealed Nalgene carboys at room temperature protected from light, which has been shown to be stable for 42 days in dosed water formulations at a concentration of 41.8 mg/L when stored under these conditions (NTP, 2008).

Animals and husbandry

The in-life portion of the study was conducted at Southern Research Institute (Birmingham, AL), the same research facility that conducted both the NTP 13-week and 2-year bioassays (NTP, 2007; NTP, 2008). The water bottles, sipper tubes, cage size and type, number of animals per cage, bedding, environmental conditions, and food were the same as in the NTP studies. The same strain of female rats (Fischer 344/N) was used in the current study. However, the rats in the current study were obtained from Charles River Laboratories International, Inc. (Stone Ridge, NY), whereas the rats in the NTP studies were obtained from Taconic Farms (Germantown, NY). The rats were approximately 4 weeks of age when they arrived and were allowed to acclimate for approximately two weeks. At the start of the study, the rats weighed between 83.1 and 126.4 g. Irradiated NTP-2000 Wafers (Zeigler Bros.; Gardners, PA) were provided *ad libitum* during the pre-study and study periods. Water (dosed or control) was supplied in amber glass water bottles. Teflon®-lined lids with stainless steel, double-balled sipper tubes were used.

[1] The Cr(VI) concentration is equivalent to approximately 35% of the SDD concentration.

Water bottles were changed twice weekly, or as needed. Before the start of the study, samples of tap water from the animal facility were analyzed to determine if there were any contaminants present that could potentially interfere with or affect the outcome of the study. No such contaminants were detected.

Rats were group-housed (5/cage) in solid bottom, polycarbonate cages on a stainless steel rack in a room maintained at a temperature of 60.7–84.1 °F and relative humidity of 28.4%–100%. Excursions outside the desired temperature (69–75 °F) and humidity (35%–65%) ranges were brief in duration and did not adversely affect the health of the animals or outcome of the study. Fluorescent lighting provided illumination approximately 12 hours per day. Irradiated hardwood bedding chips (Sani Chips®; P.J. Murphy Forest Products, Corp.; Montville, NJ) were used as bedding material. No known contaminants were present in the bedding that would have been expected to interfere with or affect the outcome of the study. Cage size and animal care conformed with the *Guide for the Care and Use of Laboratory Animals,* the U.S. Department of Agriculture through the Animal Welfare Act (Public Law 99-198), and to the applicable Standard Operating Procedures (SOPs) of Southern Research Institute.

Study design

Given that male and female F344 rats responded similarly to Cr(VI) in the previous 2-year bioassay (NTP, 2008); only female rats were used in this present MOA study. Animals were assigned to their respective dose groups using a computerized randomization procedure designed to yield comparable group mean body weights. The body weights required for randomization were determined the week before the treatment began. After randomization, animals were assigned to treatment groups as indicated in **Table 1** and were provided with food and drinking water *ad libitum* until study termination at days 8 or 91 (**Table 1**). Water and food c***onsumption*** were measured weekly for each cage of animals throughout the study and values were reported as an average daily consumption (mL/animal or grams/animal, respectively). ***During treatment,*** animals were weighed on day 1, weekly thereafter, and prior to scheduled euthanasia. All animals were observed at least twice daily during the pre-study and study periods for signs of mortality and moribundity. Each animal was removed from its cage and examined for clinical signs of toxicity (e.g. alopecia, eye discharge, piloerection, hyperexcitability) on day 1 and weekly thereafter.

Pathology and histopathology

Pathology and histopathology was assessed in 5 animals from each treatment group on day 8 and 10 animals per treatment group on day 91 (Toxicology and Histopathology groups, **Table 1**). The animals were euthanized by CO_2 asphyxiation and subjected to a postmortem examination that included, but was not limited to, examination of the external surfaces of the body, all orifices of the body, and the cranial, thoracic, abdominal, and pelvic cavities and their contents. Based on findings of the NTP 2-year bioassay (2008), the oral cavity, duodenum, jejunum, and any gross lesions were collected from each rodent and saved in 10% neutral buffered formalin for histopathologic evaluation. Tissue sections (5 μm) were mounted on glass slides and stained with

hematoxylin and eosin (H&E) for microscopic examination. All slides were submitted to a veterinary pathologist for evaluation and diagnosis. Tissues were diagnosed and categorized using standardized nomenclature with lesions ranked for severity.

GSH and GSSG analyses

GSH and GSSG parameters were measured in 5 animals from each treatment group in the Biochemical Evaluations group in **Table 1**. GSH and GSSG were measured in plasma, as well as in oral mucosal tissue (from hard and soft palate) and duodenal and jejunal mucosae (scraped) on days 8 and 91. For the collection of blood samples, each animal was anesthetized with ketamine/xylazine (87 mg/kg ketamine; 13.4 mg/kg xylazine) injected intraperitoneally or with CO_2/O_2 by inhalation, and blood samples were collected from the retro-orbital sinus into tubes containing heparin as anticoagulant. Samples were gently mixed by inversion, placed on ice, then centrifuged at 4°C for 5 min. Plasma was collected and mixed in a 1:1 ratio with 2X Redox Quenching Buffer (RQB), to yield final concentrations of 20 mM HCl, 5 mM diethylenetriamine pentaacetic acid, and 1 mM 1,10-phenanthroline. The 2X RQB also contained 5% ultrapure grade trichloroacetic acid. Samples were snap frozen and stored at -80°C until analysis. Immediately following blood collection, each animal was euthanized using CO_2. Samples of oral, duodenal and jejunal epithelia were collected, immediately placed into tubes containing 0.5 mL 2X RQB on ice, and incubated for approximately 10-15 min to allow penetration of the buffer into the tissues. The samples were then snap frozen in liquid nitrogen and stored at -80°C until analysis. GSH and GSSG were determined fluorometrically using the *o*-phthalaldehyde procedure as previously described (Senft *et al.*, 2000). The calculation for redox potential (ΔE) is described in detail elsewhere (Dalton *et al.*, 2004), and is as follows: $\Delta E = -240mV - (61.5mV/2) \times \log([GSH]^2/[GSSG])$.

8-isoprostane analyses

Lipid oxidation was measured in 5 animals from each treatment group in the Biochemical Evaluations group in **Table 1**. One sample of oral cavity and one sample of duodenum from each rat were snap frozen and stored at -80°C until homogenization. Homogenization buffer solution was prepared as previously described (Thompson *et al.*, in press). Tissues were then homogenized in 0.3 mL using an Omni THQ homogenizer (Omni International, Kennesaw, GA) and a disposable hard tissue tip. After homogenization, samples were snap frozen and stored at -80°C until assayed. 8-isoprostane[2] was analyzed via OxiSelect 8-iso-Prostaglandin F2α ELISA Kit obtained from Cell Biolabs (San Diego, CA), and normalized to protein content via BCA assay (Pierce; Rockford, IL).

[2] 8-isoprostane is also known as 8-iso-Prostaglandin F2α (8-iso-$PGF_{2\alpha}$), 8-epi-$PGF_{2\alpha}$, 15-isoprostane F_{2t}.

Cytokine and chemokine analyses

Cytokines and chemokines were measured in 5 animals from each treatment group in the Biochemical Evaluations group in **Table 1**. One sample of oral cavity and one sample of duodenum were prepared as described above (for 8-isoprostane). For serum collection, each animal was anesthetized with CO_2/O_2, and blood samples were collected from the retro-orbital sinus into serum separator tubes containing no anticoagulant. The contents of the tubes were centrifuged to separate serum. The tissue and sera samples for rats were analyzed for 22 cytokines/chemokines via a Milliplex Rat Cytokine kit (Millipore, Billerica, MA) on a Luminex 200 (Austin, TX). The cytokines/chemokines included in this kit are shown in **Supplemental Table S1**. Cytokine levels were normalized to protein content.

8-hydroxydeoxyguanosine (8-OHdG) analyses

Oxidative DNA damage was measured in 5 animals from each treatment group in the Biochemical Evaluations group in **Table 1**. DNA was extracted from the oral cavity and duodenum tissue samples using Gentra PureGene Tissue Kits (Qiagen; Valencia, CA). Three hundred (300) µL of cell lysis buffer (included in the Qiagen kit) was added to each vial of tissue sample and the tissues were homogenized using an Omni THQ homogenizer and a disposable hard tissue tip. DNA was then extracted following the manufacturer's instructions. Genomic DNA samples were rehydrated with 50 µL of DNA hydration solution (included in the Qiagen kit), and concentrations were determined using the PicoGreen kit (Invitrogen; Carlsbad, CA) and a Bio-Rad Versafluor fluorimeter (Hercules, CA). 8-OHdG was measured via OxiSelect Oxidative DNA Damage ELISA Kit obtained from Cell Biolabs (San Diego, CA) per the manufacturer's instructions.

Iron status

Serum iron levels were measured in 10 animals from each treatment group (5 from the Toxicology and Histopathology group and 5 from the Mutation Analysis group; **Table 1**). Iron status was evaluated in half of the animals designated for macroscopic and microscopic pathological evaluation on day 91. Animals were anesthetized using CO_2/O_2, and blood samples (~0.4 mL) were collected from the retro-orbital plexus into tubes containing no anticoagulant. The contents of the tubes were centrifuged to separate serum. One aliquot of serum was used for measurement of serum iron, and another was snap frozen and stored at -80°C for potential future analysis. Serum iron was measured using the Cobas c501 Clinical Chemistry Analyzer (Version 04-02; Roche Diagnostics; Indianapolis, IN). Blood ferritin and transferrin were measured using commercial ELISA kits purchased from ALPCO (Salem, NH). The iron content in bone marrow smears was also assessed in the same 5 animals from the Mutation Analysis group (**Table 1**). In each animal, one bone marrow smear was prepared and stained with Prussian blue to assess the presence of iron in different treatment groups.

Measurement of total chromium and iron in tissues

Tissue levels of total chromium and iron were measured in 5 animals from the Toxicokinetic Analyses group in **Table 1**. Samples of the oral cavity, glandular stomach, duodenum, jejunum,

ileum, liver, plasma and RBCs were collected for evaluation of total chromium (Cr) and iron (Fe) content from animals exposed for 90 days. Animals were anesthetized using CO_2, and tissues were removed, flushed of contents, snap frozen, and stored at approximately -80°C. Samples were shipped frozen to Brooks Rand Laboratories where approximately 100 mg of tissue was digested in nitric acid in a controlled microwave digestion program. Samples were then brought to a final volume of 8 mL with deionized water. Analysis was performed using EPA Draft Method 1638 (modified) using inductively coupled plasma-mass spectrometry (ICP-MS) with Dynamic Reaction Cell (DRC™) technology. Digested samples were analyzed utilizing internal standardization with rhodium. This method incorporates ionization of the sample in an inductively-coupled RF plasma, with detection of the resulting ions by mass spectrometer on the basis of their mass-to-charge ratio. The limit of detection was 0.02 µg Cr/g tissue. Iron levels were simultaneously measured in these tissue samples, and the limit of detection was 0.2 µg Fe/g tissue.

Data management and statistical evaluation

During the in-life phase of the study, Provantis (Version 7; Instem Life Sciences Systems, Ltd.; Staffordshire, United Kingdom) was used for the direct on-line capture of most in-life and pathology data. In addition, Provantis interfaced with the Cobas c501 Clinical Chemistry Analyzer (Version 04-02; Roche Diagnostics; Indianapolis, IN) for capture of serum iron data. Environmental monitoring of animal rooms (i.e., temperature/humidity and light/dark cycles) was performed using the Edstrom Watchdog System (Version 5.13; Edstrom Industries, Inc.; Waterford, WI). The remainder of the data was collected manually.

For consistency with NTP practices, biochemical and clinical endpoints were first tested for dose-related trends using Jonckheere's test (Jonckheere, 1954). Biochemical datasets with a significant trend were then analyzed by Williams' (parametric) or Shirley's tests (non-parametric) (Shirley, 1977), whereas Dunnett's (parametric) or Dunn's (non-parametric) tests were run if there was not a monotone trend. Water consumption and bodyweight data were analyzed by one-way ANOVA followed by Dunnett's tests. Food intake was found to be non-normally distributed and was analyzed by Kruskall Wallis test, followed by Wilcoxon-Mann-Whitney test with Bonferroni adjustment. Statistical packages used included R (http://www.R-project.org), Prism 5 for Mac (GraphPad Software, San Diego California USA, www.graphpad.com), and Provantis. Microscopic lesion data were analyzed by Fisher's exact test (one-sided) using Prism 5 for Mac.

Results

Gross and histopathological findings

Administration of Cr(VI) as SDD in drinking water for 90 days had no effect on survival, nor were there any clinical signs of treatment-related toxicity. The bodyweights of rats in the 520 mg/L SDD treatment group were generally lower than those of control animals (**Figure 1A)**; however, the statistical significance was sporadic (weeks 5, 6, 12, and 13). Administration of SDD had no effect on food consumption in any treatment group. Water consumption was significantly lower than control animals every week at 170 and 520 mg/L SDD, and for most weeks at 60 mg/L (**Figure 1B**). The average daily doses of SDD to rats at day 8 and 91 of the study are shown in **Table 2**. The average daily doses of SDD in the companion 90-day mouse study are also provided for comparison (Thompson *et al.*, in press).

No treatment-related gross lesions were observed in the oral cavity or small intestine on day 8, nor were any microscopic lesions observed in the oral cavity. In the duodenum, villous atrophy, apoptosis, crypt cell hyperplasia, and histiocytic infiltration were observed at ≥ 170 mg/L SDD (**Table 3**, **Supplemental Figure S1**). Apoptosis and crypt cell hyperplasia were also observed at 60 mg/L, but only in 1 of 5 animals. No microscopic lesions were observed at ≤ 4 mg/L SDD. With the exception of histiocytic infiltration, similar lesions were observed in the jejunum in the 170 and 520 mg/L treatment groups. These results are summarized in **Table 3**.

Consistent with findings from a previous 13-week drinking water study conducted by the NTP (NTP, 2007), no treatment-related gross lesions were observed after 90 days of exposure to SDD. Also, there were no microscopic lesions observed in the rat oral cavity. In the duodenum, apoptosis was observed at ≥ 60 mg/L SDD, and crypt cell hyperplasia at ≥ 170 mg/L (**Table 3**). Histiocytic infiltration was present in almost all animals at ≥ 60 mg/L (**Supplemental Figure S2**). Apoptosis, crypt cell hyperplasia, and villous atrophy were observed in the jejunum at concentrations as low as 4 mg/L; however the incidences were not statistically different from control animals in any treatment group, and in many instances the lesions were not observed at higher concentrations (**Table 3).** In contrast, there were concentration-dependent increases in histiocytic infiltration beginning at 60 mg/L. Notably, histiocytic infiltration in the duodenum was the only histopathological lesion previously observed in the rat small intestine (NTP, 2007, 2008).

The histopathological findings in the rat small intestine were generally similar to those reported for mice in Thompson *et al.* (in press), with one notable exception. In mice, cytoplasmic vacuolization was observed at both day 8 and 91 starting at 170 and 60 mg/L SDD, respectively. At both time points, vacuolization occurred at lower SDD concentrations than villous atrophy and crypt cell hyperplasia. Whether the presence or absence of vacuolization is relevant to the sequalae of events leading to tumor formation in the mouse small intestine is presently unknown. Although there are many possible causes of vacuolization, it is generally regarded as an adaptive and reversible response (Henics and Wheatley, 1999).

Cytokine and chemokine analyses

Histiocytic infiltration was observed in the rat duodenum in the previous NTP studies (NTP, 2007, 2008) as well as this current study; therefore, several cytokines and chemokines (listed in **Supplemental Table S1**) were measured in the duodenum, oral mucosa, and plasma on day 91. In all tissues examined, the data were not normally distributed and exhibited inhomogeneous variance. None of the cytokines/chemokines exhibited significant concentration-dependent monotone trends in the oral mucosa. In the duodenum, only two of the examined proteins (IL-1α and IL-4) exhibited significant monotone trends. IL-1α was significantly elevated at ≥ 60 mg/L (**Figure 2A**). Interleukin-4 exhibited a significant monotone trend that was inversely proportional to dose (**Figure 2B**); however, no treatment groups were significantly different from control animals. Among the cytokines/chemokines that did not show a significant trend, only IL-6 had a treatment group that differed significantly from control, *viz.* the 60 mg/L SDD treatment group (**Figure 2C**). In serum, only three of the examined proteins exhibited significant monotone trends; none of the other cytokine/chemokines examined were significantly altered in any treatment group. As shown in **Figure 2D**, leptin appeared to be reduced by SDD exposure.

Both IL-12p70 and IP-10 also appeared to be reduced as a result of SDD exposure (**Figure 2E-F**).

Cytokine and chemokine levels in mice were assessed in Thompson *et al.* (in press). Similar to the data for rats, the data for mice were not normally distributed and exhibited inhomogeneous variance. **Supplementary Table S2** provides a summary of the effects of SDD on cytokines/chemokines in both species. Overall, there were few similarities in the effects on cytokines between the two species. In the oral cavity, several cytokines appeared to be elevated at the two highest treatment concentrations in mice, whereas there were no obvious effects in rats. In the duodenum, there were few changes in the rat, although there was an increase in the pro-inflammatory cytokine IL-1α. In contrast, all of the changes in the mouse duodenum were inversely proportion to SDD concentration, including the pro-inflammatory cytokines IL-1β and TNFα (Thompson *et al.*, in press). In serum, there were no clear effects of SDD on cytokines/chemokines in mice (**Supplementary Table S2**).

Tissue and Serum Iron Levels

Exposure to SDD was previously shown to induce mild microcytic hypochromic anemia in rats; therefore, iron status was assessed after 90 days of exposure to SDD. Bone marrow smears stained for iron content with Prussian blue indicated lower levels in rats exposed to 170 and 520 mg/L SDD (**Table 4**). Serum iron levels appeared to be increased relative to control at 0.3 mg/L SDD, but were decreased thereafter and significantly at ≥ 170 mg/L (**Figure 3**). Ferritin and transferrin levels exhibited no clear treatment-related effects (**Supplemental Table S3**).

Iron status in mice was described in Thompson *et al.* (in press). Although SDD was shown to induce mild microcytic hypochromic anemia in mice (NTP, 2008), there were no clearly discernable effects in iron stores in bone marrow or serum levels of iron, ferritin, or transferrin (Thompson *et al.*, in press). SDD exposure also reduced iron levels in the small intestine and liver of both species, although the effects in the intestine were generally more pronounced in mice (**Supplemental Figure S3**).

Effects of SDD on Oxidative Status

Low-molecular weight reducing agents such as GSH, cysteine, and ascorbate are thought to be important for reducing Cr(VI) to Cr(III) (Zhitkovich, 2005). Glutathione is estimated to be present in many cells at 1-10 mM (Schafer and Buettner, 2001), and is therefore likely to play a major role in Cr(VI) reduction. Because the ratio of GSH/GSSG is a key indicator of cellular redox status (Meister and Anderson, 1983; Moriarty-Craige and Jones, 2004; Schafer and Buettner, 2001), the GSH/GSSG ratio was examined in several portions of the alimentary canal to examine whether SDD exposure induced changes in redox status. **Table 5** presents the effects of SDD on the levels of GSH and GSSG in the rat oral, duodenal and jejunal epithelia, as well as in the plasma on day 8. Although statistically significant effects were observed for GSSG, GSH/GSSG ratio and ΔE in the rat oral mucosa at the 0.3 mg/L SDD, there were no effects at any other concentration, as such, this effect may not be treatment related. In both the duodenum and jejunum of the rat, there were significant increases in GSH at ≥ 170 mg/L and GSSG at ≥ 60

mg/L. However there were no changes in the GSH/GSSG ratio or ΔE in the duodenum in any treatment group. In contrast, the GSH/GSSG was significantly decreased at 520 mg/L in the jejunum. In the plasma, there were significant increases in GSH (≥60 mg/L), GSSG (≥170 mg/L) and redox potential (≥60 mg/L).

Changes in GSH, GSSG and redox potential in rats after 90 days of exposure to SDD in drinking water are shown in **Table 6**. In the oral mucosa, significant decreases in GSH and significant increases in GSSG were observed at ≥ 170 mg/L SDD. Significant decreases in the GSH/GSSG ratio and redox potential occurred at ≥ 60 mg/L. In the duodenum, statistically significant increases in GSH and GSSG occurred at ≥170 mg/L; however there was no apparent change in the GSH/GSSG ratio or redox potential. In the jejunum, a significant decrease in GSH, the GSH/GSSG ratio, and redox potential (i.e. increased ΔE) occurred at ≥60 mg/L SDD, while GSSG levels were significantly elevated at ≥170 mg/L. In the plasma, significant increases in GSH and GSSG were observed at ≥ 60 mg/L, and a significant decrease in the GSH/GSSG ratio started at 170 mg/L SDD (**Table 6**).

In addition to GSH and GSSG parameters, lipid and DNA oxidation were assessed in the rat duodenum and oral mucosa at day 91. Relative to controls, there were no statistically significant changes in 8-isoprostane, a measure of lipid oxidation, in the duodenum or oral mucosa at any exposure concentration (data not shown). Levels of 8-OHdG, a measure oxidative DNA damage, were not statistically altered in the duodenum or oral mucosa of rats at any SDD concentration (**Supplemental Figure S4**). Similar negative findings for 8-OHdG were reported in mice (Thompson *et al.*, in press).

Because tumors were observed in different portions of the alimentary canal of rodents following chronic exposure to SDD (NTP, 2008) and it had been hypothesized that oxidative stress may be an important key event in the MOA underlying SDD-induced carcinogenesis (Thompson et al, 2011), it is of interest to compare the effects of SDD on redox status in both species after 90 days of exposure to SDD. For comparison, the effects of SDD exposure on the GSH/GSSG ratio are plotted on a mg/kg bodyweight basis. In the oral mucosae, SDD exposure elicited significant decreases in the GSH/GSSG ratio in rats at concentrations ≥ 8 mg/kg, but had essentially no effect on the GSH/GSSG ratio in mice (**Figure 4A**). In the duodenum, SDD significantly reduced the GSH/GSSG ratio in mice at ≥ 3 mg/kg, but had no effect in rats (**Figure 4B**). In the jejunum, however, SDD reduced the GSH/GSSG ratio in both species (**Figure 4C**). Broadly, these data suggest that on a mg/kg bodyweight basis, the jejunum of both species respond similarly to SDD, whereas the mouse duodenum experiences more change in redox status than the rat duodenum. Interestingly, GSH levels were increased in the duodenal mucosae of both species at day 91, but were decreased in the jejunal mucosae (**Figure 5**), perhaps suggesting tissue-specific differences in response to SDD.

Tissue Concentrations of Total Chromium

To better understand chromium disposition in the rat alimentary canal, total tissue chromium (Cr_t) levels were measured in the oral cavity, stomach, duodenum, jejunum and ileum following 90 days of exposure to SDD in drinking water (**Figure 6**). With the exception of the glandular stomach, statistically significant increases occurred at ≥ 60 mg/L SDD. [Note to authors: I did

not see a statistically significant mark for the ileum at either 60 or 170 mg/kg-day.] Significant increases in the in the glandular stomach occurred at ≥ 170 mg/L. Within the intestinal segments, the Cr_t levels were higher in the more proximal portions (duodenum) than distal portions (ileum). Notably, Cr_t levels in the glandular stomach were never as high as the duodenum. Similarly, Cr_t levels in the oral cavity, the tissue that developed tumors in rats in the NTP 2-year bioassay (NTP, 2008), were much lower than in the duodenum.

Cr_t levels in mice are reported in Thompson *et al.* (in press). Comparison of the Cr_t levels in the oral mucosae of rats and mice indicate relatively similar tissues levels on a mg/kg basis of SDD dose (**Figure 7A**). It is also apparent, however, that Cr_t levels in the oral mucosae were higher in mice than rats in their respective 520 mg/L SDD treatment groups. Likewise, in the small intestine, Cr_t levels were lower in rats than mice within each intestinal segment (**Figure 7B**).

Discussion (1850)

This is the first report comparing the effects of repeated exposure to Cr(VI) in drinking water on histopathology and redox status in the oral and intestinal mucosae of rats and mice. The purpose of this drinking water study, and the companion one by Thompson *et al.* (2011), was to investigate the carcinogenic MOAs in the target tissue of both species by investigating possible key events that may precede tumor formation. Detailed results from the 90-day mouse study are described in Thompson *et al.* (in press). The current report focuses on findings in the 90-day rat study, and provides species comparisons of selected findings.

Consistent with the earlier NTP 90-day drinking water study (NTP, 2007), no obvious gross lesions were observed in any tissue. Likewise, there were no histopathological lesions in the oral mucosa of rats at day 8 or day 91. In contrast to previous studies, however, many of the intestinal lesions described in **Table 3** were not previously reported. One possible explanation for the differences in histopathology in the current and NTP studies is that the water intake for the rats in this current study, while consistent with published intake values (U.S.EPA, 1988), was higher than in the NTP studies (NTP, 2007, 2008). For example, the mean water consumption of rats in the 520 mg/L group at 13 weeks in this study was 15.5 g/day, whereas the values for female rats in the 500 mg/L group at 13 weeks in the NTP 90-day study was 9.1 g/day; this value was 8.4 g/day in the 516 mg/L group in the NTP 2-year bioassay (NTP 2007, 2008). The reason for the differences in water intake in these studies is not known; however, the differences suggest that at comparable mg/L SDD concentrations in the two 90-day studies, the mg/kg bodyweight doses in the current study were higher than in the NTP study. [Considering that hundreds of rats chronically exposed to very high Cr(VI) concentrations in drinking water (but lower than those herein) did not develop intestinal lesions or tumors in the NTP 2-year bioassay (NTP, 2008; Stout et al., 2009), it is conceivable that if lesions were present at earlier time points, they resolved before the study termination.] [Note to authors: I am not sure what is being added with this sentence. This paragraph reads fine without it. Please consider dropping it or placing it elsewhere.] Notably, this is the first study to examine the effects of SDD in the rat small intestine after only 7 days of exposure, and thus the findings herein cannot readily be compared with any previous study.

The overall findings presented herein and by Thompson *et al.* (2011) suggest that oxidative stress may play an important role in SDD-induced carcinogenesis. For example, **Table**

7 provides a comparison of the target tissue effects in rats and mice at day 91 with the tumor outcomes reported in the NTP 2-year bioassay (NTP, 2008). For all tissues that developed tumors in the 2-year bioassay, oxidative stress was present at day 91, and in rats this stress is more severe at 91 days when compared with 8 days (compare Tables 5 and 6), a direction consistent with tumor formation at longer durations. Moreover, in two of three tissues without oxidative stress, tumors were also not present, despite the fact that, for example, dosimetry data in **Figure 7A** indicate that the tissue concentrations of chromium (Cr_t) in the rat oral mucosa with positive tumor results were similar (or lower) than the mouse oral mucosa with negative tumor results, yet oxidative stress was only present in the rat oral mucosa (**Figure 4A**). Furthermore, when the GSH/GSSG ratio is examined as a function of Cr_t levels in the oral mucosae (**Figure 8A**), it appears that rat oral mucosa may be more sensitive to SDD-induced redox changes than the mouse oral mucosa. Together, data in **Figures 4A, 7A** and **8A** suggest that the oral cavity tumors observed in rats is not simply a function of tissue dose, but may involve differential effects on redox status.

In the duodenum, significant changes in redox status were observed in mice but not rats (**Table 7**, **Figure 4B**). Although histopathological lesions were observed in the rat duodenum at day 91 in the absence of oxidative stress, this observation might simply reflect that at the time of euthanasia, the cells had adapted to oxidative stress but that the tissue was still undergoing repair. In this regard, the incidences for atrophy and crypt hyperplasia at day 91 were lower than day 8 (**Table 3**, **Supplemental Figure S5**); this implies that if the animals had been allowed to survive and grow, the lesions might have resolved, which is consistent with the negative tumor findings at 2 years. As was observed in the oral mucosa, the differential effects of SDD on the rat and mouse duodenal GSH/GSSG ratio appears to entail more than tissue dosimetry (**Figure 8B**), and may involve species differences in duodenal responses to oxidative stress.

In the jejunum, oxidative stress was observed in both species (**Table 7**, **Figure 4C**). As was observed in the duodenum, the incidences for atrophy and crypt hyperplasia at day 91 were lower or even absent when compared with day 8 (**Table 3**, **Supplemental Figure S5**), and thus these tissues may have recovered if the study had continued for longer duration. However, given the higher doses in this study than in the NTP studies, it cannot be ruled out that the rats in this current study would have developed jejunal tumors if they continued to be exposed to high levels of SDD and oxidative stress. It is also apparent in **Table 7** that intestinal tumors may require both oxidative stress and cell proliferation. It is notable that in the rats in this study, the jejunum appeared to be more sensitive to SDD-induced redox changes than the duodenum. On day 8, there were significant increases in GSH and GSSG in both segments; however, the GSH/GSSG ratio was only decreased in the jejunum (significantly at 520 mg/L). At day 91, redox changes in the duodenum were minimal (not statistically significant), whereas changes in redox status were significant at ≥ 60 mg/L in the jejunum. Similar findings were also observed in mice (Thompson *et al.*, in press). At day 91, both the mouse duodenum and jejunum exhibited significant decreases in the GSH/GSSG ratio; however only the jejunum exhibited decreases in the GSSG/2GSH redox potential (i.e. increased ΔE).

One possible explanation for the apparent sensitivity of the rodent jejunum to oxidative stress may lie in the differential effects on GSH. In both rats and mice, GSH levels increased in the duodenum and decreased in the jejunum as a function of SDD concentration at day 91. Although Cr_t levels differ greatly between the duodenal and jejunal segments, data reported in Thompson *et al.* (in press) indicate that Cr_t levels are similar in the scraped epithelial cells from

the duodenum and proximal jejunum in mice. This suggests that the differences in GSH content are not related to dosimetry *per se*, but rather differences in redox chemistry (e.g. regulation or signaling). In this regard, previous studies using consecutive 10-cm sections of the rat small intestine indicated that duodenal segments could reduce oxidants infused into the lumen more efficiently than jejunal segments via release of the reductant cysteine (Cys) (Dahm and Jones, 2000). Other studies suggest that intestinal cells regulate the extracellular redox environment, and redox sensitive cell surface proteins can influence the intracellular gene expression (Go *et al.*, 2009; Mannery *et al.*, 2010; Moriarty-Craige and Jones, 2004). Thus, the differential effects of SDD on duodenal and jejunal GSH levels may reflect tissue-specific (i.e. segment-specific) differences in redox chemistry. Nevertheless, the dosimetry data in **Figures 6** and **7B** suggest that intestinal chromium levels decrease distally in the intestinal tract, and thus lower tissue chromium levels may offset the sensitivity of the jejunum as one moves distally from the duodenum toward the ileum.

Despite signs of oxidative stress in the rat oral mucosa and mouse duodenum, no increases in 8-isoprostane or 8-OHdG were observed in either species. A possible explanation for these negative findings is that repeated exposure to SDD resulted in the induction of genes involved in the repair of oxidative DNA damage. The toxicogenomic data from the animals in this study are expected to provide information to assess induction of DNA damage repair and will be evaluated as those data become available. Considering that oxidative stress and inflammation are interrelated (Kruidenier and Verspaget, 2002; Rahman and MacNee, 2000; Roberts *et al.*, 2009) and that histiocytic infiltration was observed in the small intestines of rats and mice (NTP, 2007; NTP, 2008; Stout *et al.*, 2009), we expected to see increased cytokine levels in mice following SDD exposure (Thompson *et al.*, 2011). However, contrary to expectations, duodenal levels of the pro-inflammatory cytokines IL-1β and TNFα were decreased in mice (Thompson *et al.*, in press). In the rat duodenum, SDD treatment increased the pro-inflammatory cytokine IL-1α, despite the apparent lack of oxidative stress. Interestingly, vacuolization can be a sign of proteasome dysfunction and proteasome function is required for pro-inflammatory signaling (Elliott *et al.*, 2003; Mimnaugh *et al.*, 2006; Neurath *et al.*, 1998; Reinstein, 2004). Whether the differences in cytoplasmic vacuolization in rats and mice partially explain the differences in duodenal cytokines/chemokines, or the differences in tumor outcome in the small intestine is currently unknown.

With regard to the different oral tumor outcomes in rats and mice, previous SDD drinking water studies have indicated anemic effects in both species that were more pronounced in rats (NTP, 2007; NTP, 2008; Stout *et al.*, 2009). Similar findings were observed in this current study and our companion study in mice (Thompson et al, in press); specifically, iron levels in serum and bone smears were lower in rats than mice. It has been previously reported that anemia can increase the risk of oral cancer in rats (Prime *et al.*, 1983). Chromium has been observed to compete with iron for Hb and transferrin binding (Ani and Moshtaghie, 1992; Gray and Sterling, 1950; Hopkins and Schwarz, 1964). The outcome of this competition could be a reduction in the rate of iron-consuming pathways, including hemoglobin synthesis, and the consequent appearance of anemia. Iron deficiency, which can produce free radicals and reactive oxygen species, has been linked to the etiology of oral cavity cancers (Lucenteforte *et al.*, 2009; Prime *et al.*, 1983; Richie *et al.*, 2008). In addition to oxidative stress, chromium may also alter the function of enzymes involved in chromosomal modeling. For example, Cr(VI) has been hypothesized to deplete ascorbate levels, thereby decreasing the ascorbate-dependent activity of

the histone demethylase JHDM2A (Sun *et al.*, 2009). Similarly, iron-dependent histone demethylases can be inhibited via competition with nickel ions (Chen *et al.*, 2010; Chen *et al.*, 2006). Thus, oxidative stress, iron depletion and/or competition, and depletion of reductants like GSH and ascorbate might alter critical enzymes involved in DNA structure and repair. Whether (and how) this could manifest as oral tumors in rats is not known. [Note to authors: It seems that a brief literature survey on this topic would be beneficial, rather than just saying that this is not known. You give evidence that iron-deficient humans have more risk to oral cavity tumor below, but what about searching the International Toxicity Estimates for Risk (*ITER*) or NTP databases for oral tumors and then seeing if the experimental animals were iron deficient?]

In humans, there is some evidence that metabolic conditions of iron deficiency or anemia are associated with changes in the oral epithelium and oral cavity cancers (Lucenteforte *et al.*, 2009; Richie *et al.*, 2008). For example, Richie *et al.* (2008) evaluated 65 hospitalized patients with oral cancer and 85 matched controls and found that mild iron deficiency and low GSH levels increased the risk of oral cavity cancer. [Note to authors: nice, now describe what Lucenteforte et al found.] However, a recent occupational study reported that anemia was not associated with occupational exposure to Cr(VI) (Muller *et al.*, 2011). [Note to authors: Sorry, this latter sentence and the following ones are not helpful. You are discussing whether or not iron-deficient-induced-anemia is associated with oral cancers, not whether or not Cr is associated with oral cancers.] Moreover, a systematic review of literature reporting on GI-tract cancers among workers with known occupational exposure to Cr(VI), and meta-analysis of 32 studies published since 1950, found no association between GI cancers and Cr(VI) exposure (Gatto *et al.*, 2010). Although the evidence for associations between iron deficiency and oral cancer is limited, and the evidence for associations between iron deficiency or oral cancer and Cr(VI) exposure are lacking in humans, it is conceivable that the Cr(VI)-induced iron deficiency and oral cancer in rats are mechanistically related.

In summary, the data herein together with that in Thompson *et al.* (in press), support that oxidative stress is a key event in the MOA for Cr(VI)-induced carcinogenesis. These studies also provide strong evidence for non-linearities in tissue chromium levels within the alimentary canal – with essentially no detectable increases at or below 4 mg/L SDD. Notably, evidence for carcinogenesis in the small intestine has only been shown at ≥ 60 mg/L SDD, and in the oral mucosa at ≥ 170 mg/L. Additional analyses currently underway with tissues collected from the rat and mouse 90-day studies (**Table 1**) should provide additional data for informing key events in the MOAs. Moreover, the pharmacokinetic data collected in these studies will improve our understanding of the species difference in Cr(VI) disposition, and will be used to develop physiologically based pharmacokinetic models for extrapolation between species and from the very high concentrations of Cr(VI) that induced tumor in rodents to environmentally relevant levels.

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