TERA

Peer Review Report on the Chromium Mode of Action Studies: Mutation Studies

Expert Review Organized by Toxicology Excellence for Risk Assessment (TERA)
http://www.tera.org/Peer/index.html

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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 (Cr(VI)) 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 the release of the peer report, ToxStrategies incorporated the panel's recommendations and engaged several research laboratories to conduct the proposed studies.

Currently, the mouse and rat 90-day studies have been completed, including the toxicokinetic, biochemical, pathological, toxicogenomics and cytogenetic analyses that have been conducted using tissues from the 90-day studies. In addition, the mutation analyses on mouse and rat tissues have been completed and the manuscript reporting these studies is the subject of this review.

Toxicology Excellence for Risk Assessment (TERA) arranged for a written peer review of ToxStrategies' draft manuscript on the mutation analysis (*Evaluation of cytotoxicity, genotoxicity and mutagenicity of hexavalent chromium in the mouse intestinal epithelium following 90-day drinking water exposures*. Travis J. O'Brien, Hao Ding, Mina Suh, Chad M. Thompson, Mark A. Harris, Laurie C. Haws, William A. Winkelman, Jeffrey C. Wolf, J. Gregory Hixon, Arnold M. Schwartz, Barbara L. Parson, Meagan B. Myers, Deborah M. Proctor· October 2012). This review was conducted by four risk assessment experts with expertise in mode of action analysis, genotoxicity/mutagenicity, and biostatistics**:** Michael Dourson, TERA; Toby Rossman, New York University of Medicine School of Medicine; Dave Gaylor, Consultant; Bhaskar Gollapudi, Consultant . 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 TERA. This work was done under contract to ToxStrategies and was sponsored by the American Chemistry Council.

The experts received the draft manuscript and charge questions on October 16, 2012. 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 reviewers' written comments, organized by charge question. Appendix A contains the Instructions to Reviewers.

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

Reviewer 1:
The statistical analyses were appropriate and conducted correctly. The other issues are outside of my area of expertise.

Reviewer 2:
Since a major question that was being addressed by this study was "Is there an early increase in intestinal mutagenesis during treatment of mice with chromate" (which might indicate a mutagenic mode of action for intestinal tumors), it was disappointing that a suitable genetic marker was not chosen. In order to detect increased mutagenesis, one should use a marker that allows detection of many different types of mutation. Instead, these authors chose an extremely narrow end point, i.e., a single mutation in K-ras. They attempted to justify this on the grounds that K-ras is mutated in many tumors (although not necessarily in mouse intestine tumors). While K-ras might be a "target", looking for a specific mutation that may or may not be induced does not make a good marker. While a positive result might be informative of a mutagenic MOA, a negative result is not sufficient evidence of a non-mutagenic MOA.. Also, one cannot conclude from this study that K-ras mutation is not an early event. Other mutations in K-ras could have occurred.

In addition, the extremely high rate of spontaneous mutagenesis at this position of K-ras does not bode well for observation of increased mutagenesis. Something appears to be amiss here.

Let me state that I do not consider chromate [via Cr(III)] to be significantly mutagenic in animal cells at non-toxic concentrations, but this study does not test this sufficiently in animals.

There is no need for the genetic marker to be related to the oncogenesis when testing the hypothesis in question. The most important feature should be a low background and a good possibility to detect a wide variety of mutations. The HPRT gene would be more suitable, or any other gene that would be neutral with respect to survival and recovery of mutants. Methodology similar to that used to study K-ras could have been developed for another gene.

For more information about oncogenes that might be important to the tumorigenesis, immunohistochemical staining for gene product (protein) could have been used to study the APC/beta catenin pathway, which does play a role in mouse intestinal tumors (see papers on min mouse). Loss of APC and/or upregulation of beta catenin could be studied *in situ*, and if positive results were seen, followed by analysis of mutations or epimutations. Similar studies could have been done with mismatch repair genes, which also play a role (and which might have been epigenetically downregulated under conditions of toxic exposure to chromate). This would allow one to distinguish between two different MOAs: mutagenesis vs epimutagenetic effects.

I did not see enough on the criteria for apoptosis. Could a picture be included? Other papers use a different stain (methylene blue). There was no biochemical confirmation of apoptosis (or necrosis).

Figure 4 is superfluous, and thrown in at the Discussion. Proliferation was already established. Immunohistochemical staining for Ki-67 could have been a better confirmation of where the proliferation took place.

I have no problem with the statistical analysis.

Reviewer 3:
On the whole, the study was thoughtfully planned and carefully executed. The report is very well written with informative tables and figures. The appendices are very helpful to contextualize the data presented in the main report. There are a few significant issues as summarized below:

1. Page 7: There were no statements in the Methods section on whether the studies were conducted under Good Laboratory Practice Standards to include analytical characterization of the test material, verification of the stability and concentration of the test substance in the drinking water, and quality assurance audits of the in-life procedures as well as the final data.
2. Page 8, line 147: The methodology employed for the identification of micronuclei is neither widely accepted nor internationally validated. The single reference from 1983 (Goldberg et al.) will not be adequate to demonstrate whether the laboratory performing this analysis has validated the end point using a list of positive and negative control chemicals. This is even more important given that a concurrent positive control was not employed in the present study to demonstrate the laboratory proficiency. In the absence of concurrent positive control data, the authors should document their laboratory's experience with chemicals known to induce micronuclei in duodenum.

3. The absence of increased mutant frequency at a single base pair of a single codon of the K-*Ras* gene was used as a suggestive evidence for a MoA that does not involve mutation as an early key event. The selection of this target as the reporter gene for mutagenicity can be questioned on the basis of 1) high mutant frequency in control animals (two to three orders of magnitude higher than the generally accepted background mutant frequency for endogenous genes), 2) the use of a single sampling time for assessing mutagenicity in a target gene that is not neutral when mutated which might influence the recovery of any increased MF at longer intervals such as the one use in the study, and 3) lack of information showing that K-*Ras* was mutated at codon 12 or any other codon in duodenal tumors induced by chromium (VI).

4. Page 11, line 211-212: The methods used to statistically compare the K-*Ras* mutant frequencies were not presented. Simply mentioning that that Excel and Prism 5 were used is not informative. In addition, the statistical power of the study design to detect a specified increase should be mentioned not only for K-ras, but also for the MN induction. I am not sure that the mentioning of the sensitivity of ACB-PCR to detect 3 mutant alleles in 300,000 wild-type alleles (page 14, line 279) satisfies this query.

5. For assessing genotoxicity in the tumor target tissue, the Comet assay would have been a good choice for acceptance of the data in a regulatory context since this assay has undergone extensive international validation and is currently under review for an OECD guideline. This end point can also help in the identification of genotoxicity, if any, at non-cytotoxic dose levels.

6. For mutagenicity evaluation, and given the large body of literature suggesting mutagenicity of chromium (VI) both in vitro and in vivo, I am surprised that the authors did not use transgenic B6C3F1 Big Blue mice for the study so that they could have evaluated mutations in a neutral transgene (cII or *lacI*) in the tumor target tissue by employing an internationally accepted and OECD guideline-compliant protocol/methodology; this is in addition to the other endpoints that were evaluated in the study. The advantage of selecting a neutral gene is that the mutation does not impart either selective advantage or disadvantage to the cell.

Reviewer 4:

Study design: Overall, there are no apparent problems with the test article, system, statistical analysis, etc. although I am not familiar with the R software package cited by the authors for statistical analysis of the data.

The authors used allele-specific competitive blocker-PCR (ACB-PCR) to identify mutations of the *Kras* gene in mice. This is an unconventional PCR technique developed and published by McKinzie and Parsons (2002 and 2005) to identify rare mutations or alleles in the presence of a much more common genotype. The ACB-PCR approach uses a mutant-specific PCR primer with more 3′-terminal mismatches to the wild-type sequence than to the mutant sequence. Because background can be a problem, ACB-PCR reactions also include a nonextendable blocker primer to reduce the amount of background signal generated from the abundant wild-type template. Together the mutant specific-primer with wild-type mismatches and blocker primer discriminately amplify the rare allele in the presence of the wild-type.

One concern with the unique PCR approach used in this manuscript is that, unlike the chemicals tested in the papers by McKinzie and Parsons (2002, 2005 and 2011), SDD (Cr(VI)) did not appear to causes dose-dependent *Kras* mutations. Rather, , *Kras* mutations were present in all samples, including untreated controls. Although the lack of a dose response is supportive of the authors' contention that Cr (VI) is not mutagenic, it is not possible to conclude whether the PCR signal indicates a high-level of *Kras* mutation in mouse duodenum generally, whether signal nonspecific background due to nonspecific amplificationwild-type template in the PCR reaction, or if dose-dependent mutations are actually present but masked by high background signal. Inclusion of 3 controls would resolve these questions: a negative control from genomic DNA isolated from tissue/cells that do not possess the *Kras* mutation, a positive control from tissue/cells possessing the *Kras* mutation, and, ideally (but not critical), a control for PCR and gel loading.

The standards used with the PCR assay clearly demonstrate the sensitivity and specificity of the PCR technique, and provide a positive control for the amplification portion of the experiment - that is, we know the primers amplify their intended targets. However, the standards appear to be synthesized DNA sequences corresponding to the mutant and wild-type *Kras* exon targets recognized by the PCR primers; they do not contain genomic DNA and are not processed, purified or isolated in the same manner as the genomic DNA being analyzed. Reassuringly, the standards are a 1:1 mixture of wild-type *Kras* DNA and different concentrations of mutant *Kras* DNA.

Because no mutation was observed using ACB-PCR, a positive control for mutant *Kras* in purified genomic DNA is important for this assay and the manuscript in general. In the McKinzie and Parsons studies, there were samples tested that were clearly positive for *Kras* mutation and these contrasted with samples that were negative. These data, when considered in the context of the chemical treatments or tumors types, negated the need for positive controls. However, in this manuscript there is no treatment-related effect on *Kras*. Rather, compared to the standards all samples are apparently positive. The authors do include B[a]P data in figure 2D, but this data is from another lab (Meng et al. 2010) and a different tissue (lung); this is not the same as having a positive control for this experiment but demonstrates the authors' awareness that a clearly positive signal would be helpful for data interpretation. Without a positive control, the possibility exists that although PCR technique works with the pure synthesized DNA templates (standards), the overall approach, which is complicated by the presence of genomic DNA and sample processing, might not have worked. I believe that if the authors could provide DNA isolated using the same protocol from cell lines/mouse lines that have the mutated *Kras* gene (or which has been transfected with a plasmid expressing *Kras* mutation) this would suffice. Conversely, the same would apply to a negative control from animals or cells with the wild-type gene. Inclusion of a figure containing a positive control and the negative control PCRed using exactly the same conditions and run on a PAGE gel alongside a few representative samples from Fig 2 would satisfactorily clinch the argument.

To establish there is no variability in the amount of DNA included in the PCR reaction mixture or loaded on the gel, the mutant gene data should be normalized to another gene, or perhaps the wild-type Kras gene. Given the MF variability observed among samples (Fig 2C and D) even within the same treatment groups, this control would substantiate that equal amounts of DNA were amplified and run on the gel for each sample. Another "housekeeping" gene could be easily selected from the genomic DNA samples or the authors can justify why is it is not necessary.

Question: Is there any chance that the method of scraping the epithelium off the underlying layers could result in a loss of cells at the base of the crypts and decrease the power of the approach to detect mutation?

Other Endpoints - There are other targets and approaches that might complement the ACB-PCR approach used in this manuscript. APC mutations are common in colon cancer and might also be a consequence of Cr(VI) exposure (mutation associated with intestinal tumors is outside my area of expertise). An advantage of assessing additional mutations common to intestinal cancers or Cr-related cancers is that the conclusion about the mutagenic effects of Cr(VI) would be more robust.

There is a commercially available TaqMan system designed to detect *Kras* mutations, and other mutations as well. It is technically similar to the method used here with the advantage that it is quantitative, since it is based on real-time PCR.
http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/PCR/real-time-pcr/real-time-pcr-assays/taqman-mutation-detection-assays/somatic-mutation-real-time-pcr.html?s_kwcid=TC|17846|kras%20mutation%20detection||S|b|14982233450

With regards to the histopathology, the authors present the hypothesis that cytotoxicity in the villus causes a dose-dependent proliferative response in the crypts. This hypothesis is supported by an accumulation of enterocytes in the crypts; however the mitotic index, which does not change with dose, does not coincide with this conclusion. Other markers, particularly immunohistochemistry cell proliferation markers, could be useful to support the conclusion that proliferation is increased in crypt enterocytes. BrDU incorporation might also have been useful prior to sacrificing the animals and probably should be considered for any future studies.

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

- ***Were the data correctly summarized?***
- ***Are there nomenclature issues that need clarification?***
- ***Was adequate statistical information provided for quantitative dose-response analyses?***
- ***For each finding presented in the study, please comment on the strength of the evidence supporting the authors' conclusions that the finding is, or is not, treatment-related.***

Reviewer 1:

The data were adequately summarized in order to conduct statistical analyses. There were no nomenclature issues. The authors provided adequate statistical information in order to conduct quantitative dose-response analyses and the statistical analyses conducted support the authors' conclusions regarding whether or not a finding is treatment related.

Reviewer 2:

The data seemed to be correctly summarized, but for reasons stated above, I do not consider the mutagenesis results to be relevant. The studies on crypt structural changes seemed reasonable, but that is outside my area of expertise. I was puzzled as to why apoptosis was measured in crypts and not in villi.

Nomenclature issues: I usually hesitate to use the term "genotoxicity" when I really mean DNA damage-inducing. If that's not what is meant, use a different descriptive term.

Dose-response relationships were reasonable for the cytotoxicity. Statistics seemed OK, but that's not my field of expertise.

Data other than mutagenesis are more believable, but somewhat incomplete (e.g. lack of validation of apoptosis, lack of distinction between MN with and without centromeres). However, I do believe they got it right about the cytotoxicity localized to the villi and not the crypts. I see no reason to doubt that these effects were treatment-related.

Reviewer 3:

- Were the data correctly summarized?

Ans: Yes.

- Are there nomenclature issues that need clarification?

Ans: At several places in the report (e.g., page 5, line 71), the wording "heritable mutations" was used. Isn't mutation by definition a heritable change in DNA? I suggest that the authors chose some other verbiage to communicate their point.

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

Ans: Yes.

- For each finding presented in the study, please comment on the strength of the evidence supporting the authors' conclusions that the finding is, or is not, treatment-related.

Ans:

1. Micronuclei: Page 6, line 94-95: If enterocytes in villi do not proliferate, how can we expect to see the induction of micronuclei as observed in Table 2? Cell division is obligatory for the formation of micronuclei. Alternatively, these micronuclei might have arisen in mitotically active crypts and the cells with MN might have migrated upwards into the villi. It is noted that the authors have speculated in the Discussion that these MN might have been induced in the transition cells in the villi - an equally plausible hypothesis assuming that such cells do undergo cell division. It does not, however, preclude the possibility of MN induction occurring in the crypts as well.
2. Micronuclei, Table I: Given the low background incidence of MN, the number of cells enumerated in the crypts (approx. 2000-3000/group) is not considered to be adequate to conclude that there was no influence of treatment on MN formation in this compartment. With such a low incidence, a minimum of 4000 cells/animal (20,000/group) seems to be a desirable number to draw statistically meaningful conclusions. The recording of zero MN counts for all treatments in Table 1 is an indication that the sample size used was not adequate as the background MN frequency is seldom zero.
3. Micronuclei, Page 13, line 250-251: The fact that MN occurs only at cytotoxic doses should not be viewed as exceptional, but rather an anticipated association since chromosomal breakage/spindle malfunction responsible for MN formation also lead to cytotoxicity. In fact, in the regulatory guidelines for the conduct of a micronucleus assay, the top dose/concentration is selected to induce significant level of toxicity, for example a 50% inhibition in cell proliferation.
4. Chromium binding to DNA, Pages 15-16: Given the justified statement "…. *the Cr-DNA binding data were deemed to be uninformative with regard to the carcinogenic MOA*", it is not reasonable to make the following two speculations "….. *any in vivo Cr-DNA binding probably occurred in the villi and might have contributed to cytotoxicity, MN formation, and karyorrhexis which occurred in the villi. As noted earlier, nuclear damage was not observed in the crypts at any dose suggesting that Cr(VI) did not penetrate the crypt compartment at levels that would induce toxicity*". The authors should have attempted other

means of assessing intracellular concentrations of chromium in order to draw any firm conclusions on the availability of chromium to different compartments.

5. Chromium binding to DNA, Appendix B, Supplementary Figure 1: If chromium were only available to the cells in the villi (which slough off thereby precluding bioaccumulation) and not to the crypt compartment as speculated in the Discussion, what is the explanation for the 10X higher levels of the putative Cr-DNA binding observed at Day 91 when compared to Day 8?
6. Page 16, line 331: In my opinion, the following statement "*The higher than expected K-Ras codon 12 GAT MFs observed in the intestinal mucosa, at all doses including controls, suggests long-term Cr(VI) exposure-induced effects may facilitate tunorigenesis through accumulation of K-Ras mutations*" is only valid if the particular K-*Ras* mutation was demonstrated in the tumors induced by chromium.

Reviewer 4:

- Were the data correctly summarized?

In my opinion the data was correctly summarized with the exception that I think the controls are inadequate for the ACB-PCR data to be properly interpreted.

- Are there nomenclature issues that need clarification?

The actual gene symbol for K-*Ras,* following standard nomenclature rules, is *KRAS* in humans and *Kras* in mice.

- Was adequate statistical information provided for quantitative dose-response analyses?
- It appears that the statistical information provided was adequate in the methods. It is not clear if there are no error bars in the Ki-67 figure or if variability is so low the error bars are not distinguishable. Please add error bars or please indicated in the figure legend that error bars are present.

- For each finding presented in the study, please comment on the strength of the evidence supporting the authors' conclusions that the finding is, or is not, treatment-related.

The major finding in this study was that 0.3-520 mg/L Cr(VI) in drinking water for 90 days did not cause *Kras* mutation or genotoxicity in mice. Two lines of evidence support this conclusion: ACB-PCR, which is discussed above; and histopathology. The conclusions that Cr(VI) does not increase cell proliferation or cause genetic damage in the crypts seems well supported by the histopathology evidence presented. The conclusion that cytotoxicity damages the villus is also well supported by evidence including disruption of cellular arrangement, desquamation, nuclear atypia and villus blunting, as well as increased karyorrhectic nuclei and micronuclei. The evidence presented in Fig. 2 D & E strongly support a treatment related effect on villus blunting and crypt widening. Although toxicologic responses of the intestinal epithelium are outside my area of expertise, I suspect that villus shortening and crypt widening is a generalized response to toxicological insults across an array of agents so I wonder how much these changes actually say about compensatory enterocyte proliferation.

The authors suggest that Cr(VI) causes a dose-dependent proliferative response in the duodenal crypts. This is evidenced by an increase in crypt area and increased number of crypt enterocytes (Figure 2). The rationale is that damaged villi increased cell turnover that trigger compensatory cell proliferation of crypt enterocytes. Ki-67 is one gene, the expression of which is indicative of proliferation. Ki-67 expression in figure 4 (data obtained by an earlier study) is elevated at 91 days for the 2 highest dose groups, but is below control levels for all dose groups <170 mg/L at 91 days, suggesting that if a proliferative response exists, it only occurs at the highest exposure levels, and even then is more predominant early in treatment. Together these data provide evidence that there is increased proliferation, which might be a cytotoxic compensatory response, however direct evidence of increased mitosis in the crypts is not presented.

Charge Question 3. Study Conclusions

- ***Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made?***
- ***Do the data and analyses presented support the author's conclusions? Could the data and analyses reasonably support alternate conclusions? If so, describe here.***

Reviewer 1: This question is outside of my area of expertise.

Reviewer 2:

I already discussed why I consider the mutagenesis experiments to be lacking. Thus, any statements that conclude that mutagenesis was not increased early in treatment should be disregarded.

I found most of page 15 unintelligible or wrong. Line 290: "These data (referring to previous sentence) were interpreted as chemically-induced amplification of spontaneous mutagenesis".. On what basis? Was a mutational spectrum of spontaneous mutation done? The sentence on line 292 starting "Therefore, if….." makes no sense. I assume that the authors are not referring to gene amplification here, but do they mean what they call "amplification" of spontaneous mutagenesis? This is confusing language. Spontaneous mutagenesis can be INCREASED. The same confusing language appears on page 19, line 388 ("amplified with mutational loading"?)

I also fail to see how any of this relates to lack of "early tumors", nor do I know what "early tumors" means. Define!

At the bottom of page 15 going into page 16, a statement is made that the Cr-DNA binding (which was a very crude assay indeed) is "not representative of pre-mutagenic DNA damage". That might in fact be true, but one cannot base this on lack of mutagenicity at a single site in one gene.

Reviewer 3:

1. Page 15, line 291: Isn't chemically-induced amplification of a spontaneous mutant a hallmark of promotion rather than an indication of a mutagenic MoA?
2. With prior knowledge that cells in villi a) do not proliferate, and b) are not important to tumorigenic process as they slough off into the lumen, why did the authors even spend resources trying to quantify chromium(VI)-induced micronuclei in that compartment?
3. The conclusions are stated as follows, "*Taken together, the multifaceted data in this study support that Cr(VI)-induced intestinal carcinogenicity is unlikely to involve early mutations or cytogenetic damage to crypt enterocytes, but instead these data support a MOA based on chronic wounding (i.e., cytotoxicity) of the intestinal villi, thereby placing persistent proliferative pressure on intestinal crypt cells*". I am not convinced that the evidence presented in the study has adequate weight to rule out the contribution of mutagenicity or cytogenetic damage as an early key event. As indicated earlier, the use of a neutral reporter gene in a transgenic model combined with the analysis of K-*Ras* mutations at more than one sampling time (e.g., 28 days and 90 days) would have gone a long way in ruling in or ruling out a mutagenic MoA.

Reviewer 4:

It is not clear to me if proliferation increases in the crypts or not. Table 1 shows there is no change in the mitotic index of crypt cells across the different treatment doses; direct evidence that there is no dose-related change in crypt-cell division. However, Figures 2 and 4 provide indirect evidence of increased crypt cell proliferation. This point is made several times in the paper, for example:

> *"transcripts support that Cr(VI) increased cell proliferation as early as day 8 of exposure and that Ki-67 was induced throughout the 13 week exposure period. Consistent with the findings presented in this paper"*
>
> *"Under the proliferative conditions induced by 170 and 520 mg/L SDD…"*
>
> *"…cytoplasmic vacuolization in villi at lower concentrations than crypt hyperplasia suggests that toxicity to the villi cells trigger compensatory cell proliferation of crypt enterocytes."*

"The transcripts support that Cr(VI) increased cell proliferation as early as day 8 of exposure and that Ki-67 was induced throughout the 13 week exposure period"

Although the mitotic index clearly indicates that Cr(VI) did not cause "cytostasis" or "cellular arrest in the crypt compartment", it is also clear that there is no direct evidence that cells are responding to "persistent proliferative pressure on intestinal crypt cells". Could the authors more clearly resolve the apparent inconsistency between evidence supporting proliferation (Fig. 2D & F, Fig 4) and evidence supporting no change in proliferation (Table 1).

Although there are inherent weaknesses, I believe the evidence presented in this manuscript is important for understanding the MOA of Cr(VI) and, taken as a whole, generally supports the authors' conclusions that Cr(VI) is cytotoxic and does not cause cell cycle arrest within the proliferative compartment. The conclusion that Cr(VI) does not induce genotoxicity within the proliferative compartment of the duodenum is a higher hurdle, since it must be proven that the event does not occur. I believe the data on this are equivocal given the background and variability in the ACB-PCR data already discussed. It is also notable that there is a significant increase in micronuclei observed in the villi that might reflect earlier damage occurring during cell replication, which according to the authors occurs only in the crypts. Given the shortcomings of the manuscript already discussed, more finessed interpretations regarding the role of mutation and proliferative pressure in the mode of action of Cr(VI) do not appear to be well substantiated.

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

Reviewer 1:

The results described in this paper should be considered in the derivation of EPA IRIS quantitative health benchmarks and the qualitative characterization of cancer risk. No major uncertainties are apparent that would preclude the study from being used to determine the weight-of-evidence for the potential cancer risk of chromium VI.

Reviewer 2:

The mutagenesis experiments are not informative, and thus, this study fails to support a non-mutagenic MOA completely. On the other hand, the dosimetry for cytotoxic effects does indicate that a MOA dependent on cytotoxicity and subsequent cell proliferation is reasonable.

Reviewer 3:
The major strength of the study is its robust dose-response data on a number of endpoints which are important to tumorigenic process. The application of a non-standard methodology to measure cytogenetic damage, *albeit* in the tumor target tissue, may limit its utility when presented with an overwhelming evidence on the mutagenicity of chromium (VI) both *in vitro* and *in vivo* as reviewed in McCarroll et al. (2010).

Reviewer 4:
This manuscript supports the argument that Cr(VI) is not mutagenic over the wide range of doses tested relevant to the point of entry (intestines), but a strong conclusion cannot be drawn. I believe this study's reliability and strength would be increased dramatically with the addition of PCR controls for the detection of *Kras* mutations. The study does not directly link Cr(VI) to increased crypt cell proliferation via mitosis or other markers of cell proliferation, so a compensatory increase in cell proliferation due to Cr(VI) is not well supported. Genotoxicity at some point in the life-cycle of duodenal enterocytes is supported by micronuclei in the villi but the relationship between MN and the cytotoxic effects of Cr(VI) is not obvious. Due to uncertainties associated with this study, I am not convinced it is a reliable study for derivation of EPA IRIS quantitative health benchmarks

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

Reviewer 1:
Table 1. Define the ± values. Presumably, they are standard deviations.

Table 3. Define SEM (standard error of the mean).

Reviewer 2:
I was disappointed that results were not confirmed by immunohistological techniques. For example, apoptosis (or its lack) could be confirmed by immunohistochemical staining with an antibody against active caspase 3 (there are other choices as well), and proliferation could have been confirmed with BrdU labeling and anti-BrdU antibody. This would have demonstrated unequivocally whether these events took place in the villi or crypts.

Reviewer 3:

I would strongly recommend a 28-day drinking water study in transgenic Big Blue B6C3F1 mice according to OECD Guideline 488 to further examine whether mutagenicity is or is not an early key event in the carcinogenicity of chromium VI.

Reviewer 4:
I would suggest moving figures 2A – 2C to supplementary data since there figures are not discussed in the text and are presented only to support and document the analysis in panels D – F. In the Supp. Data section the figures could be used to enhance the methodological discussion.

Appendix A

Instructions to Reviewers

Dear Reviewers,

Thank you again for your willingness to provide a peer review of a genotoxicity/mutagenicity analysis for hexavalent chromium following drinking water exposure. This paper has been developed based on data generated in a 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 genotoxicity and mutagenicity studies that were conducted. Following your peer review, ToxStrategies will be submitting the draft manuscript for publication.

For this peer review, the reviewers are asked to carefully review the manuscript and provide written responses to the charge questions. Please address each charge question (as appropriate given your expertise). Also, please comment on any aspect of the study that is not specifically addressed by the charge. For all comments, 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).

If possible, please send your written review by email to me by Friday, November 2, 2012. After the reviews are submitted, we may schedule a follow-up with ToxStrategies to resolve any issues or to answer any clarifying questions that ToxStrategies may have for the reviewers. The need for follow up will be determined upon receipt of the reviews. A draft compiled report with the other experts' comments on this study will be forwarded to you and you will be provided the opportunity (albeit brief) to revise your comments if you feel that is needed. At the completion of this review, please destroy any copies of the review materials, as they are draft and are not for distribution outside of the review panel.

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

Joan Strawson
Review Coordinator
910-528-9768