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

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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Contents

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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. The mutation analyses on mouse and rat tissues have been completed, but the manuscripts for these studies are still in development. Now that the majority of the analyses are complete in both species, ToxStrategies has evaluated the overall study data collectively using a weight-of-the-evidence-based approach to determine if the data are sufficient to draw conclusions about the MOA(s) underlying the toxic/carcinogenic responses.

Toxicology Excellence for Risk Assessment (TERA) arranged for a written peer review of ToxStrategies' draft manuscript on the mode of action analysis (*Assessment of the Mode of Action Underlying Development of Rodent Small Intestinal Tumors Following Oral Exposure to Hexavalent Chromium and Relevance to Humans*; Chad M. Thompson, Deborah M. Proctor, Mina Suh, Laurie C. Haws, and Mark A. Harris. August 2012). This review was conducted by seven risk assessment experts with expertise in mode of action analysis**:** Michael Dourson, TERA; Elaina Kenyon, U.S. EPA; Bette Meek, University of Ottawa; Xianglin Shi, University of Kentucky; Toby Rossman, New York University of Medicine School of Medicine; Kirk Kitchin, U.S. EPA; Lucy Anderson, National Cancer Institutes, Emeritus. 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 September 7, 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.

This report includes the following appendices:

Appendix A - Instructions to Reviewers

Appendix B – References cited by Reviewers

Appendix C – Reviewer 4 Comments in Manuscript

Appendix D – Reviewer 5 Comments in Manuscript

Reviewers' Comments

General Comments

Reviewer 1:

None submitted.

Reviewer 2:

None submitted.

Reviewer 3:

This manuscript presents a detailed argument for a proposed mode of action (MOA) for the carcinogenicity of hexavalent chromium in murine small intestine. This research team has worked with a high degree of focused productivity. The narrative is carefully and clearly laid out, starting with the main step-by-step arguments, followed by sections of Additional Supporting Information. The latter are often repetitive and redundant and could be substantially shortened. The authors could consider incorporating supporting information into the step-by-step sections. There are a few minor corrections to the text needed, noted below.

easier for me first to comment on it step by step. I will then extract relevant parts of this commentary as answers to the specific questions on the list.

Section-by-section comments:

Key Event 1: Saturation of Reduction and Absorption from the Lumen. The data and the argument here are convincing: there is more Cr(VI) in the intestines of mice at carcinogenic doses, compared with rats, and the biological effects of the metal occur only when detectable amounts reach the target tissue. These results have been published in peer-reviewed journals (1-3). It should be made clear in the legend to Fig. 3 that the intestines were flushed before analysis.

Key Event 2: Villous Cytotoxicity. The argument made is that the Cr(VI) is toxic to the villi and not to the crypts, and that crypt hyperplasia occurs in response to villous damage. It is clear and reasonable that the villous cells are experiencing toxicity. What is not proven is that there is no toxicity to the cells of the crypt; in fact the data suggest toxicity to the proliferating cells of the crypt, as follows.

There are four parts to this argument for lack of toxicity to the crypt cells.

a. *Absence of an increase in mitotic and apoptotic indices (Table 3).* Table 3 is not completely clear. The variations in the indices are very large (S.D. or S.E.?). The exposure time is not given, but is presumably 90 days. Hyperplasia without an increase in mitosis seems a contradiction. The explanation given is that stem cells may have increased, based on a report by Snow and Altmann (4). However the latter publication tells a different story. It was based on a study of carcinogenesis in rat duodenal epithelium by dimethylhydrazine. Both crypt size and mitoses/crypt increased with dimethylhydrazine, but mitotic index did not. Therefore total number of cycling cells increased, but Snow and Altmann believed that small intestine has no reserved stem cells, according to an earlier publication (5). They concluded that the hyperplasia resulted from an increase in the number of cell cycles in the proliferative progenitor cells before terminal differentiation.

However, in the modern era there is new evidence that there may indeed be two stem cell types in small intestine, one involved in routine homeostatic regeneration, and a second population that is normally quiescent but responds to radiation injury with vigorous proliferation (6). Radiation, like Cr(VI), causes generation of free radicals. If the Cr(VI)-exposed small intestine experiences hyperplasia without an increase in the mitotic index, as claimed from Table 3, this may indicate activation of the reserve stem cells and provide proof that the Cr(VI) does in fact reach and damage stem cells. Intestinal stem cell biology is currently an active area of research, with molecular markers and special models available (7). These methodologies could be used advantageously, to investigate the details of interaction of Cr(VI) with the stem cells.

b. *Presence of micronuclei (MN) and karyorrhectic nuclei in the villi but not the crypts*. These data in Tables 3 and 4 are convincing. However there is a problem with the interpretation. MN occur during cell division; abnormal mitotic segregation of damaged chromosomal material is the basis of the test (8). The proliferating cells in the small intestinal mucosa are in the bottom two-thirds of the crypt. MN may require a lag time for expression, and may persist in end-stage cells (9, 10). Therefore the MN in the villi almost certainly arose during the proliferative phase in the crypt and indicate genotoxic damage there by Cr(VI).

c. *Oxidative stress*. There is sufficient evidence from previous publications for oxidative stress in the duodenum as a result of the higher doses of Cr(VI), as indicated by GSH/GSSG ratio in particular, and also gene expression changes. It is reasonable that the observed cytological changes in the villous cells were due at least in part to oxidative stress. But these likely facts do not inform as to what might be happening in the stem cells of the crypt. As noted above, injury to both normal and reserve stem cells in the crypt may be likely.

d. *Inflammation*. Published data are reviewed, showing differences in mice vs. rats with regard to cytokine/chemokine responses. It is not clear how these observations are meant to relate to this Key Event.

Key Event 3: Crypt Cell Proliferation. Several points are made in this section. These are paraphrased in italics, followed by my comments.

a. On day 8 of exposure, villous cytoplasmic vacuolization was seen in 3/5 mice at the 170 mg/l dose, but crypt hyperplasia in 0/5 mice. This difference did not pertain at the higher dose or at the later time point. The values, 3/5 vs 0/5, are not statistically different (P=0.17, Fisher Exact Test). Even if the difference is real, it does not prove lack of toxicity in the crypt.

b. *No focal hyperplasia was noted.* Focal hyperplasia, while a useful indicator in tissues such as liver, kidney, and lung, is not a usual endpoint in carcinogenesis studies of the gut. No example of increased focal hyperplasia in the small intestine during carcinogenesis is found in the published literature (PubMed search). Possibly the rapid turnover of the intestinal epithelium limits stability of such foci. Also, since focal hyperplasia may be regarded as a stage in pre-neoplastic progression, it might not be expected as early as day 91.

c. If mutation were involved, this would be driving the hyperplasia. Since no increase in K-ras mutation was found (see below), the hyperplasia was not due to mutation but was in response to villous damage. There are several problems here. Hyperplasia does not require mutations; it is a normal process. The fact that mutant K-ras targeted to intestine in transgenic mice resulted in increased cell numbers (11) is beside the point. Secondly, there may be problems with the choice of K-ras mutation for analysis (see below).

Key Event 4: Expansion of Spontaneous Mutations. The rationale here is that, if early mutation is part of the MOA as proposed by the EPA, then an increase in K-*ras* mutations should be found. K-*ras* codon 12 GGT to GAT mutations were assessed by the ACB-PCR technique for tissues taken on day 90. Details have not been published yet. This mutation was found in very high frequency, up to 1/10, with no differences among doses (Fig. 6).

There are numerous possible problems here.

a. K-*ras* was evidently chosen because mutations in this gene occur in human small intestinal tumors. The obvious question is: are there K-*ras* mutations in mouse small intestinal tumors caused by Cr(VI)? If so, then this argument could have merit. If not, then this effort is fruitless. It is stated on p. 37 that the NTP has not made the tumor

samples available. The NTP has the capability to assay K-*ras* mutations and hopefully is in the process of doing so.

b. The GGT to GAT mutation was chosen because this change was found in rat colon tissue after exposure to azoxymethane, a colon carcinogen. Azoxymethane is an alkylating agent, which typically causes G to A transition mutations. However, reactive oxygen species, which are putative players in Cr(VI) genotoxicity, cause G to T transversions, so perhaps GGT to GTT mutations in K-*ras*. G to T transversions occurred in the lungs of *LacI* transgenic mice treated intratracheally with Cr(VI) (12). GTT mutations in codon 12 of K-*ras* should also be assessed in the small intestines.

c. The extremely high apparent spontaneous mutation rate is concerning. Though such high rates have been reported before (13), this ACB-PCR assay for K-*ras* mutations has been used by only one laboratory. Confidence in it will be increased when it is in more general use. No experimental details are given, and there are no positive controls shown (materials with K-*ras* mutations proven by other methods), nor any negative controls (water blanks carried through all steps).

d. No K-*ras* mutations were found in human lung cancers associated with Cr(VI) exposure (14). In view of extensive knowledge about mutagenic interactions of Cr(VI), chromosomal changes, genetic instability, epimutation, etc. may be more likely markers of Cr(VI) mutational effects in small intestine (15, 16).

e. When mutant K-*ras* was targeted to intestine in transgenic mice, it caused hyperplasia, but not expansion of stem cells or neoplasia (11). In human colon cancers K-*ras* mutation is thought to contribute more to tumor progression than to initiation (17).

This section of the manuscript also calls into question the binding of Cr(VI) to DNA of the intestine. Evidently their unpublished results suggest that this is artifactual and occurs during collection of the intestinal tissues. Since no details are given, the strength of this argument cannot be evaluated. There can be little doubt about the fact the Cr(VI) binds to and alters the DNA of living cells (18). Most work has been done in cell culture. The troublesome issue of secondary artifactual interactions during extraction might be circumvented by tissue imaging to show Cr in the cells and their nuclei (19, 20).

Also mentioned in this section is lack of early tumors, metastases, and mortality in the 2-year study. Evidently the idea is that early mutations would have led to these findings. Numerous carcinogenesis models could be cited, wherein mutations were established early, but not expressed in tumors until much later, and in tumors that rarely progressed or spread.

After the presentation of the main supporting evidence for Key Events 1-4 (pages 9-21), the manuscript contains sections of Additional Supporting Information. The first section deals with toxicogenomic responses as discovered by microarray analysis. Conclusions related to Key Event 1, tissue dosimetry, are reasonable. With regard to Key Event 2, cytotoxicity, various expression changes are described related to redox effects, DNA damage, and inflammation. As these results were obtained with extracts of entire small intestinal lining, they are not useful for the attempts in this paper to distinguish between villus and crypt cytotoxicity. However they do show increased expression of enzymes involved in repair of oxidative DNA damage. Lack of apparent increase in 8-OHdG, reported previously (1), may have reflected inadequacy of the method used. This question could be re-visited, with their impressive histochemical high content analysis technique (21).

Increased expression of genes related to proliferation (Key Event 3) was noted, and expected. Finally, in the context of Key Event 4, cluster analysis of 116 genes grouped the Cr(VI) results with those from four non-mutagenic carcinogens, distinct from those from four mutagenic carcinogens (22). The outcome (Fig. 7) is impressive, though limited to day 8 and the highest Cr(VI) dose. It should be stated that the cluster analysis shown in Fig. 7B is for this time and dose, and that the comparison is with rat liver. The heat map in Fig. 7A shows that entirely different results were obtained at day 90, at least for these 15 genes. Expression patterns at day 90 would seem to be more relevant to an MOA based on chronic hyperplasia. Also, of the 15 DNA repair enzymes shown in the heat map, six of them are clearly and markedly upregulated at the two highest doses on day 8. Clearly a lot of DNA damage is taking place at this point. *In situ* hybridization studies could be undertaken to establish which cells in the small intestine are up-regulating these genes: crypt or villus?

These microarray results must be interpreted with additional cautions. The four genotoxic carcinogens are all organic compounds, and all form DNA base adducts and cause mutation via mispairing. They are thus distinct from Cr(VI), the genotoxicity of which is ascribed to oxidative DNA damage and/or non-specific DNA adducts and binary/tertiary cross-linking, strand breaks, error-prone double-strand break repair, etc. One would expect the gene expression profiles to be different even if they were due mainly to DNA damage effects. It would be of interest to repeat the exercise with a known inorganic carcinogen such as cisplatin, with DNA interactions more similar to those of Cr(VI). Also, the non-genotoxic carcinogens all cause ROS to some degree, to which their effects are often imputed. Stimulation of ROS-responsive genes by Cr(VI), early and at low doses, was established. Commonality in gene expression profiles is thus not surprising. It would be useful to have more detailed and specific information about which genes, in the set of 116, most clearly distinguish the two categories of carcinogens.

The next section of Additional Supporting Information presents *in vitro* micronucleus formation. These data mainly show that MN formation dose-response parallels cytotoxicity. Since in the small intestine the MN, though seen in the villi, must have been initiated in the proliferating cells of the crypt, the results with the CHO-K1 and A549 cells (page 25) confirm likelihood of cytotoxicity of Cr(VI) in the crypt.

An *in vitro* intestinal Caco-2 cell model is described (pages 26-27), wherein effects are studied of Cr(VI) on proliferating vs confluent more differentiated cells. These results were published recently (21) and show a commendable, useful new approach: immunohistochemical quantitative imaging in single cells. The focus is on γ-H2AX and 8-OHdG staining. (Please add here a note on the significance of γ-H2AX, that it is a highly sensitive marker for double-strand DNA damage.) In the publication, the staining for 8-OHdG appears to be more cytoplasmic than nuclear, suggesting damage to the nucleotide pool. This is interesting, since 8-OHdG in the nucleotide pool has been suggested to contribute to carcinogenesis (23).

The dose-response for the nuclear staining, which includes hydrogen peroxide and rotenone as positive controls, is convincing. The proliferating cells were much more sensitive than the differentiated cells to the oxidative and DNA damages by all agents. As the current manuscript states: "These *in vitro* results predict that Cr(VI) exposure *in vivo* might induce more adverse effects in crypt enterocytes than villous enterocytes." This correct conclusion is then countered by the repeated invocation of the mitosis, apoptosis and MN results, an argument of questionable strength (see above).

This exciting high content imaging method has a lot of advantages relative to extract-and-quantify approaches, especially for 8-OHdG, where artifacts are a serious problem. It could be usefully applied to tissues after *in vivo* exposure, to discover exactly which cell populations are experiencing increases in 8-OHdG and in double-strand DNA breaks.

On the bottom of page 26 it is suggested that "crypt enterocytes were minimally (or not at all) exposed to Cr(VI) *in vivo*. In fact, the intestinal mucosa is covered by a protective mucus layer that serves as a barrier…". This seems quite unlikely, that no Cr(VI) would find its way into the crypt during chronic exposure to high doses. The imaging techniques available for looking at Cr species in tissues (19, 20) could help resolve this question.

Sections on Concordance of Dose-Response, Temporal Association and Plausibility (pages 27-30) do not present any new data or arguments.

Results for Cr(VI) are compared with those for captan on pages 30-31. Captan had effects in rodent small intestine similar to those for Cr(VI), and was re-classified by EPA as a "not likely" human carcinogen, based on a non-mutagenic MOA that

"required prolonged irritation of the duodenal villi as the initial key event". Captan has not been studied as extensively as Cr(VI). The conclusion of lack of *in vivo* genotoxicity in small intestine appears to be based on a single study of nuclear aberrations. Captan is genotoxic in many *in vitro* systems and in mouse bone marrow *in vivo*. It binds thiols and might be expected to cause increases in reactive oxygen species, yet no studies of oxidative stress or DNA damage by captan have been reported. The classification of captan may need to be re-visited yet again.

With regard to Alternative MOAs, pages 31-34, the key question is whether mutation in the target tissue is an early event. The arguments presented against an early mutation as part of the MOA, listed in summary in the first paragraph on page 32, are absence of cytogenetic damage in the crypts, of K-*ras* mutations and of pre-neoplastic lesions; late tumor onset; and lack of increased mortality. Issues with these arguments are elaborated above, and are, in summary: likelihood of cytogenetic damage in the proliferating cells of the crypt, resulting in MN in the villi; incomplete assessment of K-*ras* mutations and questions about the suitability of this endpoint for establishing whether any mutations have occurred; rarity of pre-neoplastic lesions in this tissue in general; and many examples of long latency of animal tumors with mutations. Tumor-related mortality is a separate issue and relates more to the tumor type than to mutations or latency. For example, lung tumors in mice are rarely fatal, even though they are caused by mutations and have relatively short latency.

Properties of Mutagenicity as related to Cr(VI) are summarized in Table 7 (page 60). The conclusion of lack of mutations is based on one target nucleoside base, in one gene, with results yet unpublished. Direct DNA reactivity of Cr(VI) is listed as "equivocal". Certainly DNA reactivity of Cr(VI) is well known. While some of the lesions caused by Cr(VI) in DNA are not likely to be mutagenic (bottom of page 33), some of them clearly are mutagenic. DNA reactivity in the cells of the small intestine may be equivocal but imaging could resolve this. The topics of direct exposure in the crypts, cytogenetic damage, preneoplastic lesions, and latency/lethality are discussed above.

The section on Potentially Susceptible Subpopulations (pages 34-36) is well done.

Data Gaps (pages 36-37) are all sensible. Additional gaps have been noted along the way in this commentary. Human Relevance (page 38-39) will in fact be determined in part by pharmacokinetics, as suggested. Detailed molecular comparisons of human and Cr(VI)-induced animal tumors could also be very revealing. Much could be learned by additional tumor-endpoint studies in mice, with samples taken at frequent intervals until tumor appearance for assessment of histological, cytological, and molecular changes. Initiation/promotion protocols with the *min*/+ mouse could reveal whether Cr(VI) initiates small intestinal tumors by genotoxicity, promotes initiated cells through ROS production, stimulation of proliferation, etc., or has both actions.

The brief Discussion (pages 39-40) does not introduce anything new.

A few minor corrections

-p 6, line 14: **comprised**, not compromised

-p 35, line 9, and p 36, line 7, **bowel**, not bowl

-p 35, lines 10, 11: **that** are sufficient to cause… **in** villi

- p 56, Table 3. Title: there are data in the table in addition to total number of aberrant nuclei. More details are needed for this experiment. Make it clear that the indices were obtained by counting total nuclei and mitotic and apoptotic cells and aberrant nuclei in 10 crypts from each of 5 animals. The exposure time should be given, and it should be stated how many of the 5 crypt samples at each dose were classified as hyperplastic. In the legend, MI = total mitotic, not apoptotic. State whether S.D. or S.E. are shown.

There are a lot of typos in the References.

Reviewer 4:

None submitted.

Reviewer 5:

None submitted.

Reviewer 6:

None submitted.

Reviewer 7:

Two time points are not a lot to go on. More time points would be useful. There are enough exposure concentrations included in the experimental design.

In general the authors tend to focus on some of their data and advocate for a 100% cytotoxic MOA. They downplay or ignore significant other evidence both in their studies and in the published Cr literature that lead to the conclusion of genotoxicity/mutation as a Cr MOA. It may be that both mutational events and cytotoxicity are mutual drivers of carcinogenesis in mouse small intestines, but the authors are not suggesting mutation as a possible MOA. The authors are putting forward an absolutistic extreme position on MOA, a position of 100% cytotoxicity. I do not agree with their MOA views.

Even when this experimental work is eventually published, I do not think it will be considered that highly by regulatory agencies. I expect they will give a lot of weight to the positive data on what is already known about the DNA-Cr interactions.

Chromium's well known genotoxicity and clastogenicity suggest and argue that mutation has a role in chromium carcinogenesis. For some reason, the authors do not favor that hypothesis. Although the 5 parameters cited do not suggest mutation as a major component of chromium carcinogenesis, many other experimental results do suggest a role for mutation/genotoxicity. Putting forth a MOA which is mixed (both nongenotoxic and genotoxic key events) is a better scientific choice than trying to advance a 100% nongenotoxic MOA.

Responses to Charge Questions

Charge Question 1: Does the MOA analysis consider all relevant data appropriately? If key data were not considered in the analysis, please discuss them here.

Reviewer 1:

The MOA is presented in Fig. 2. Oxidative stress is considered to be very important in the overall mechanism of Cr(VI) carcinogenesis. This manuscript has discussed its role. The importance of oxidative stress is not reflected in MOA in Fig. 2.
The discussion in the text and MOA in Fig. 2 provide an indication that reduction of Cr(VI) to Cr(III) is a "good" process because Cr(III) is unable to enter the cell. This is correct but two more issues has to be considered: (i) If Cr(III) is produced inside the cell, it will also generate hydroxyl radical via reaction with hydrogen peroxide (Shi, X., Dalal, N.S., and Kasprzak, K.S. Generation of free radical from hydrogen peroxide and lipid hydroperoxide in the presence of Cr(III). *Archives of Biochemistry and Biophysics* 302, 294-299, 1993); and (ii). During the Cr(VI) reduction process, enzymatic Cr(VI) reduction in particular), molecular oxygen is consumed and reactive oxygen species (superoxide radical, hydrogen peroxide, and hydroxyl radical) are generated.
In the text, it is stated that the most studied biological reducing agents for converting Cr(VI) to Cr(III) are GSH, ascorbate, and cysteine, and that enzymatic and non-enzymatic reduction by proteins also likely plays a role in Cr(VI) reduction. Using *in vivo* living animal ESR, it has been shown that reduction of Cr(VI) in living animal generated Cr(V)-NAD(P)H complex and NAD(P)H is the major cellular Cr(VI) reducing agent (Liu, K.J., Shi, X., Jiang, J.J., Goda, F., Dalal, N.S., and Swartz, H.M. Chromate-induced chromium(V) formation in live mice and its control by cellular antioxidants: an L-band EPR study. *Archives of Biochemistry and Biophysics* 323, 33-

39, 1995; Liu, K., Jiang, J., Swartz, H.M., and Shi, X. Low frequency ESR detection of chromium(V) formation by one-electron reduction of chromium(VI) in whole live mice. *Archives of Biochemistry and Biophysics* 313, 248-252, 1994; and see Dalal NS's publications for cellular studies).

Reviewer 2:

a. I have little quarrel with the majority of the contents of this document, and I compliment the authors for their thorough (for the most part) study and analysis. I agree that a good case has been made for the proposed MOA. However, there are 2 areas that I have problems with: 1) *in vivo* mutagenicity studies; 2) the distinction between focal and diffuse hyperplasia.

b. The original meaning of genotoxicity was "reaction with DNA by a chemical or its metabolites", but this meaning has since been stretched to include any positive result in any test now considered a "genotoxic assay". Some of these do not measure heritable events, but rather measure evidence of DNA damage or its consequences including chromosome aberrations, micronuclei, comet assays, DNA lesion measurements, and DNA repair assays.

c. In contrast, a mutagen is an agent that can cause heritable changes in DNA sequences (order or amount). While most mutagens (or their metabolites) do this by causing damage to DNA that is converted to mutation via error-prone polymerases that bypass unrepaired DNA damage, mutagens can also act in other ways. These include base analogs (by mis-incorporation followed by mispairing), or various types of indirect mutagenic events that frequently are consequences of reactions with proteins rather than DNA. Another mutagenic event, gene amplification (increased copy number) also may result from protein modifications such as altered p53 activity. Other indirect modes of mutagenesis include oxidative damage/glutathione depletion and interference with DNA replication. Aneuploidy (which alters gene copy number) can be caused by protein damage or interference with proper function of critical proteins or subcellular structures (e.g., spindles).

d. Genotoxicity data <u>alone</u> cannot support or refute a mutagenic MOA. That is why mutagenicity data is so crucial. While I agree that chromate is a very weak mutagen *in vitro* (and is unlikely to act by a mutagenic MOA), unfortunately this study has chosen too narrow a test of its *in vivo* mutagenicity to target tissue.

Activation of proto-oncogenes and inactivation of tumor suppressor genes are considered to be critical molecular events in chemical carcinogenesis. Mutations in the Ras family of proto-oncogenes (comprising H-Ras, N-Ras and K-Ras) are commonly found in human and rodent cancers. Ras is a small GTPase that hydrolyses GTP into GDP and phosphate. Ras is activated by growth factor signaling (e.g. EGF, TGFβ) and acts like a switch (on/off) in growth signaling pathways, most of which in turn regulate

genes that mediate cell proliferation. Activating mutations of K-ras occurring early in colorectal tumorigenesis are thought to abolish GTPase activity, leading to increased and unregulated cellular proliferation and malignant transformation. These mutations occur almost exclusively at codons 12, 13, and 61 in both animal and human tumors resulting in any 1 of 19 possible point mutations in a given tumor. *Thus, looking at only one specific mutation in one gene misses the boat.* In addition, the choice of GGT to GAT mutations was not well justified. (Wicki et al. is an abstract). Polycyclic aromatic hydrocarbons cause mutations at G because they bind there. It is not a valid justification for choosing this mutation in this case.

Mutations at multiple sites in more than one gene should have been studied. Since p53 mutations are seen so frequently in many tumor types, and because a wide variety of mutation sites have been mapped, it would have been a better choice. Furthermore, in humans, p53 and K-ras mutations rarely occur in the same colorectal tumor (Conlin et al., *Gut* 2005 54: 1283-1286). It is possible that this is the case for intestinal tumors as well.

Another choice might be the adenomatous polyposis coli (APC) gene. In mice, inactivation of the adenomatous polyposis coli (APC) gene is observed at early stages of intestinal tumor formation (Smits et al., Gastroenterology 2000, 119(4):1045-53). Many mutations have been mapped here also.

In addition, it would also be informative to look for mutations in a control (non-oncogene) marker, such a *hprt*. Choose one or a few exons where mutations have already been mapped. If a late increase (after proliferation has increased) in mutant frequency occurs, it would support the MOA.

2) On pages 17 and 29, focal hyperplasia is described as “preneoplastic” whereas diffuse hyperplasia is not. Frankly, I do not find this distinction meaningful. If the focal hyperplasia is not an adenoma, then it is just hyperplasia and not a pre-neoplastic lesion, whether the NTP considers it so or not. I also showed figures of the slides from the NTP study to a Pathologist friend (without disclosing the source), and this person could make no distinction between them other than the amount of hyperplasia.

Reviewer 3:

a. Appropriate consideration of relevant data.

i. Saturation of reduction, and absorption from the lumen

ii. Dose-dependency of hyperplasia similar to that for tumorigenesis

iii. Oxidative stress as a component of the MOA

iv. Toxicogenomics, basic analysis showing changes in expression of genes related to oxidative stress and proliferation

b. Inappropriate consideration of relevant data.

i. Absence of micronuclei in the crypts, but dose-dependent occurrence of these in the villi, are presented as evidence that toxicity to the villus cells is primary, and hyperplasia in the crypt is secondary. This is not an appropriate interpretation. The micronucleus test is based on effects in proliferating cells. Therefore the nuclear damage, though visualized in the villi, actually occurred in the crypts.

ii. Lack of GGT to GAT mutations in codon 12 of K-*ras* in the small intestines after 90 days of treatment is interpreted to mean that tumor-generating mutations did not occur early in the process. This is a weak point.

- K-*ras* mutations have not been demonstrated in the murine small intestinal tumors.

- Mutant K-*ras* expressed in intestines of transgenic mice caused hyperplasia, but not stem cell expansion or adenomas.

- Mutant K-*ras* is considered to be a collaborative, not an initiating, mutation in human colon cancers.

- Cr(VI)-associated human lung cancers do not display mutant K-*ras.*

- Many studies of the mutagenicity of Cr(VI) suggest that chromosome rearrangement, genetic instability, and epimutation may be more likely genotoxic events than DNA point mutations.

- GGT to GTT mutations should also be assayed, since these could be caused by ROS from Cr(VI).

iii. Cluster analysis of the microarray data suggested grouping Cr(VI) with four nongenotoxic carcinogens, rather than with four genotoxic ones. This is interesting, but was limited to only the highest dose and the early time point (8 days). Changes at the later time would be more relevant to an MOA based on chronic proliferation. The mutagenic actions of Cr(VI) are based on different DNA interactions, compared with the four organic carcinogens that form simple DNA base adducts; this was not discussed.

c. Key data not considered in the analysis.

- Nature of the dose-response curve is a key aspect of MOA analysis, with a linear relation suggesting mutation and lack of a threshold, and with a sublinear more consistent with a chronic-proliferation mechanism. Dose-response for the Cr(VI)-caused small intestinal tumors (from the NTP report) is linear for both females ($r = 0.91$, $P = 0.03$) and males ($r = 0.99$, $P= 0.0005$), and for both genders ($r = 0.88$, $P = 0.0008$). These data should be considered.

Reviewer 4:

The potential for a mutagenic mode of action needs to be more fulsomely considered in the context of the available data on the genotoxicity of Cr VI. Development of benchmark doses for concomitant dose-response/temporal concordance analysis and additional consideration of specificity/consistency in documenting weight of evidence would strengthen the case considerably as would tabular concordance analysis for human relevance.

Reviewer 5:

The authors do a good job at stepping through the various areas of data related to key events within a well-constructed key event framework. Although I have several suggested revisions of the text to enhance clarity and strengthen arguments on the attached text, I am quite taken by the overall impact of the data. One area for the authors to perhaps expand their already good text is in the link between EPA (2005) guidelines and the biology of evoked tumors, and the types and timing of the tumors found with chromium. From a biologist's view, a mutagenic carcinogen would have a very different pattern of tumor development than what is exhibited by chromium.

Reviewer 6:

No. What is missing here, and was not really covered in sufficient detail in earlier pharmacokinetic analyses, is the possibility of alternative model forms and structures that might have explained some of the pharmacokinetic data. Specifically, it would be desirable to provide some evidence that a second order pharmacokinetic process was the optimal choice among possible forms. In looking back at some earlier papers, I realize that some of the data were on a log scale which makes it hard to evaluate the fit which appeared to be not as good at the higher end of the exposure levels based on Figure 2 (see figure 1 and 2, Proctor et al., Chemosphere 2012). No discussion of species differences in regards to pharmacokinetic and pharmacodynamic differences renders the analysis incomplete.

Reviewer 7:

The authors state; “Thus, the hypothesis that mutation is an early initiating key event in the inconsistent with the absence of cytogenetic damage, *Kras* mutations, or preneoplastic lesions (NTP, 2007; NTP, 2008a; Thompson *et al.*, 2011b), as well as the late tumor onset and lack of increased mortality (NTP, 2008a).“ intestinal carcinogenesis is

However, Chromium’s well known genotoxicity and clastogenicity suggest and argue that mutation has a role in chromium carcinogenesis. For some reason, the authors do not favor that hypothesis. Although the 5 parameters cited do not suggest mutation as a major component of chromium carcinogenesis, many other experimental results do suggest a role for mutation/genotoxicity. Putting forth a MOA which is mixed (both nongenotoxic and genotoxic key events) is a better scientific choice than trying to advance a 100% nongenotoxic MOA.

The authors state that: “Taken together, the *in vitro* and *in vivo* findings suggest that crypt enterocytes were minimally (or not at all) exposed to Cr(VI) in vivo.”

However, I agree in the two year bioassay the animals are quite likely to have been oxidatively stressed for all the duration of the experiment. Then some Cr- exposed animals developed tumors in some sites by the end of the two year exposure. This is a good argument for a genotoxic MOA of Cr carcinogenesis.

The authors indicate that the findings in the crypt enterocytes, as well as high Cr-DNA binding levels in the non-target tissue of the mouse liver following drinking water exposure to SDD (O’Brien *et al.*, in prep), calls into question the reliability of Cr-DNA binding data, and furthermore, the data do not support the role of Cr-DNA adducts as pre-mutagenic lesions relevant in the MOA for small intestinal tumors. However, tt is common to observe carcinogen-DNA binding in tissues that do not develop cancer (e. g. benzo(a)pyrene in liver). Cr-DNA effects may play a role in carcinogenesis in the mouse small intestine. There are many organs with high mitosis rates that do not develop tumors in all individuals including the GIT and bone marrow in many people and rat skin for example.

The authors concluded: “In summary, the DNA reactivity of chromium is well established (Chiu *et al.*, 2010; Nickens *et al.*, 2010; O'Brien *et al.*, 2003; Zhitkovich, 2011; Zhitkovich, 2005). Known and/or suspected forms of DNA damage associated with Cr(VI) exposure include DNA adducts, single and double strand DNA breaks, inter- and intra-strand crosslinks, oxidative DNA damage, and replication blockage. However, as discussed in the Eastern Research Group peer-review panel comments to the Draft EPA Toxicological Review for Hexavalent Chromium (ERG, 2011), most of these many genotoxic lesions are not likely to be mutagenic, and the WOE supports that Cr(VI) is, in fact, a very weak mutagen. Further, it is important to note that much of this

evidence comes from *in vitro* systems, often comprising acellular *in vitro* reactions and possibly *ex vivo* Cr-DNA adduct formation." However, the authors cite 7 types of DNA effects caused by chromium and then claim that these are not mutagenic or weakly mutagenic. The evidence is opposite the view of the authors; there is plenty of evidence that some of these lesions particularly double strand breaks are mutagenic and clastogenic. Genotoxicity is all you need to get tumors in mammals. Cr is known to produce cancer in at least three species – man, rat and mouse and in at least three tissues lungs, oral cavity and GIT. This is consistent with a genotoxic mode of action or at minimum a MOA that includes genotoxic components and it not 100% driven by prior cytotoxicity as the author claim.

Charge Question 2: Have the hypothesized and alternative MOAs been adequately described, with the key events identified? Is there anything missing for these MOAs? Are there other MOAs or pathways that should be considered?

Reviewer 1:

In addition to Cr(VI)-induced inflammation, Cr(VI)-induced cell transformation and tumorigenesis should be discussed. A recent study has shown that Cr(VI)-induced ROS generation plays a key role in Cr(VI)-induced cell transformation and tumorigenesis (Wang, X., Son, Y.O., Chang, Q., Sun, L., Hitron, J.A., Budhraja, A., Zhang, Z., Ke, Z., Chen, F., Luo, J., and Shi, X. NADPH oxidase activation is required in reactive oxygen species generation and cell transformation induced by hexavalent chromium. *Toxicological Sciences* 123, 399-410, 2011). Cr(VI)-induced angiogenesis should also be mentioned.
The alternative MOAs have been adequate described. There is nothing major missing.

Reviewer 2:

The additional mutations that presumably arise as a result of damage-induced proliferation might also be a result of selection for chromate-resistance in the surviving cells. *In vitro*, cells grown in the presence of chromate show selection for mismatch repair-deficient cells that are chromate-resistant (Salnikow and Zhitkovich, Chem. Rev. Toxicol. 21:28-44, 2008). These cells are mutators (having a high spontaneous mutation rate) and show microsatellite instability, as do chromium-induced lung cancer cells (Takahashi et al., Mol. Carcinogenesis 281:150-158, 2005).

Another factor might be epigenetic alterations. Klein et al. (Environ. Health Perspect. 110, Suppl. 5:739-743) showed that treatment of cells *in vitro* with 40 µM chromate

(resulting in about 40% survival) induced apparent mutations at 3-times background levels. Further analysis showed that 53% of these were deletions and the most of the rest were epimutations due to DNA methylation. Thus, the actual increase in mutant fraction reached only about 1.5-fold over background. The ability of Cr(VI) to cause changes in DNA methylation can also be related to the selection of mismatch repair-deficient cells, since turning off mismatch repair genes by methylation is known to be an important mechanism in carcinogenesis. This is a testable hypothesis *in vivo*. A discussion of Cr(VI)-induced epigenetic alterations can also be found in Nickens et al. (Chemico-Biological Interactions 188: 276–288, 2010).

The manuscript is also confused about the meaning of "epigenetic". On pg. 11, line 14, there is a statement "…. cytotoxicity can result from epigenetic and genotoxic effects…" I thought what the authors meant to say is "cytotoxicity can result from DNA damage or damage to other parts of the cell" , which is true. However, a few lines down, there is mention of oxidative stress and inflammation as "epigenetic mechanisms". They are not, although since inflammation is associated with oxidant stress, it is true to say that such stress can lead to epigenetic effects (it can also lead to mutagenesis). For the record, epigenetic effects refer to changes in the methylation of DNA (at CpG sites) or to changes in histone modifications (e.g. methylation, acetylation) that change gene expression. These changes have been shown to be important in carcinogenesis.

Reviewer 3:

The hypothesized and the main alternative MOA are clearly described and their key events noted. More emphasis could be placed on the fact that the alternative MOA, i.e., early mutation encouraged to tumor development by chronic hyperplasia, is a tumor initiation-promotion scenario. Tumor promotion has been demonstrated in the large bowel.

Reviewer 4:

It is suggested that a rationale be included at the outset indicating basis for the focus on a cytotoxic mode of action, in light of available data on other possibilities. A template for early consideration of potential modes of action is provided in one of the references included in the detailed comments (Meek & Klaunig, 2010).

Hypothesized key events are those for which there is experimental measurement to investigate weight of evidence through application of the Bradford Hill considerations. Absorption is rarely considered a key event and there is need for reference to ***sustained*** proliferative response (in the presence of continuing exposure) in the favored hypothesized mode of action.

Reviewer 5:

Before reading this paper, I suspected that these intestinal tumors might be evoked by several modes of action, including mutagenesis. However, the totality of the data clearly does not show this. The overall impact of the data is to completely support the oxidative stress Mode of Action (MOA) for causing tumors. I do not see any other MOA that even comes close to Occam's Razor.

Reviewer 6:

See comments above in regards to pharmacokinetics. In regards to Figure 3, the authors present adequate information for Cr(VI) in mouse vs. rat, but it would be more compelling if comparisons were provided for the mouse with Cr(III) vs. Cr(VI) and if this were discussed in the text of the document.

Collins et al. (2010) have made the statement in their abstract in regards to mice that "resulting in intestine epithelial neoplasms in mice at a dose equivalent to or within an order of magnitude of human doses that could result from consumption of chromium-contaminated drinking water, assuming that dose scales by body weight$^{3/4}$". While a PBPK model is the preferred approach for species extrapolation, this statement and the assumptions that underlie it need to be addressed explicitly since this is an alternative way of evaluating the data that has been published in a well-respected peer-reviewed journal.

Reviewer 7:

The authors state "Furthermore, Cr(VI) reduction can result in the production of Cr(V) and Cr(IV) reactive intermediate species and the generation of reactive oxygen species (ROS) (Liu and Shi, 2001; Nickens *et al.*, 2010)." However, these data actually form one basis of a genotoxic MOA – oxidative attack on DNA. There are many known Cr-DNA interactions that can contribute to a mutagenic MOA, not just oxidative attack. Oxidative redox status changes precede tumors so oxidative stress may be the major cause either via DNA or via other pathways.

The authors state that "These data indicate tissue oxidative stress that could not be mitigated even after relatively long durations of exposure (7-90 days), implying that the mice exposed to Cr(VI) in the 2-year NTP bioassay (NTP, 2008a) experienced increased intestinal oxidative stress nearly their entire lifespan. " I agree in the two year bioassay the animals are quite likely to have been oxidatively stressed for the duration of the experiment. Then some Cr-exposed animals developed tumors in some sites by the end of the two year exposure.

The authors indicate "*Kras* was selected because it is often mutated early in human intestinal tumors (Wicki *et al.*, 2010), and codon 12 GGT to GAT mutation is one of the most commonly reported mutations in human duodenal tumors (Nishiyama *et al.*, 2002; Sutter *et al.*, 1996; Wicki *et al.*, 2010; Younes *et al.*, 1997), and accounts for 12.6% of colon tumors in the COSMIC database (Forbes *et al.*, 2010)." However, 100% minus 12.6% leaves 87.4% colon tumors coming from other genetic/mutational causes. That is the remaining work to be done (by ToxStrategies or someone else) before other possible genetic/mutational causes of mouse GIT tumors can be strongly argued against. At present the authors have only a weak argument against mutation and genotoxicity for Cr induced tumors.

Charge Question 3: Are there any significant questions or inconsistencies related to the proposed MOA?

Reviewer 1:

In the proposed MOA, there is no molecular mechanism indicated. It is unclear from 1 to two, what is the molecular event involved; from 2 to three, what is the key event involved to move from villous cytotoxicity to crypt hyperplasia; and from 3 to 4, how crypt hyperplasia leads to spontaneous mutation. The MOA is too general and not very much useful information is provided.

Reviewer 2:

No inconsistencies. Questions already addressed.

Reviewer 3:

a. The interpretation that toxicity does not occur in the crypt cells, based on the appearance of micronuclei in the villus cells, is not consistent with the fact that micronuclei form during cell division, which occurs in the crypt, not the villus (as noted above).

b. Current literature indicates the presence of reserve stem cells in the crypt, which are activated by injury to proliferate. Activities of these cells during Cr(IV) treatment could be followed histochemically by use of specific markers.

c. Information is needed on occurrence of K-*ras* mutations in the Cr(VI)-caused small intestinal tumors (as noted above).

d. Data published by this research group (21) indicate the high likelihood of 8-OHdG and γ-H2AX in the nuclei of proliferating intestinal cells (e.g. crypt cells) exposed to Cr(VI). This same technique could be applied to intestinal epithelium, and, in combination with markers for the several classes of intestinal stem cells, used to obtain conclusive evidence as to whether there is early DNA damage in the stem cells or their proliferating immediate descendants.

e. It is suggested that Cr(VI) may not reach the crypt cells because of protective mucus. This question could be answered definitively by use of *in vivo* imaging techniques.

f. The expressions of the multiple DNA repair enzymes, which were upregulated early in the small intestinal epithelium by the highest Cr(VI) doses, could be localized as to cell type by use of *in situ* hybridization.

Reviewer 4:

There are some critical uncertainties in a weight of evidence context that have not been articulated.

This includes the lack of counterfactual evidence related to prevention of tumors, in the absence of a key event. It also includes lack of data on early response to contribute to combined analyses of dose-response and temporal aspects. Inclusion of benchmark dose analysis would strengthen the dose-response/temporal concordance analysis for key events and critical supporting data in the paper by O'Brien et al. (apparently in preparation) need to be published prior to release of the MOA analysis.

Reviewer 5:

I have a few questions that the authors need to address. These are found on:

Page 13 "Given that crypt stem cells are the most likely clonal sources of tumor formation (Barker et al., 2009; Potten and Loeffler, 1990), increases in the numbers of crypt stem cells may increase the chance of spontaneous tumor formation (Feng et al., 2011)." [Note to authors: ok, but if the MI is not increasing in crypt cells, this is evidence against the regenerative hyperplasia MOA, correct?]

Page 14 “This pattern of toxicity, specifically limited to the villi, supports that toxicity to the villi caused the regenerative response in the crypt in the absence of any direct genotoxicity or cytotoxicity in the crypt. [Note to authors: ok, but the MI does not increase in the crypt (Table 3)]

Page 18, top paragraph. This paragraph is very confusing. I agree with what appears to be this paragraph’s intent, but please rewrite it for clarity. I have made some suggested changes in the attached document, but may not have hit the mark.

Page 19 “Thus, Kras codon 12 is a likely candidate oncogene for early mutations in intestinal carcinogenesis following exposure to mutagenic carcinogens.” [Note to authors: any data in mice for Kras mutations?]

Page 33 “The evidence herein supports involvement of both cytotoxicity and oxidative stress as key events in the MOA.” [Note to authors: I believe that this argument might be enhanced, if the gene array data do not suggest DNA replication due to regenerative hyperplasia rather than DNA repair. Do they?]

Page 39, top paragraph. This paragraph needs to be revised. I suggest “The Cr(VI) concentrations that induced tumors in mice (≥ 20 mg/l Cr(VI)) are orders of magnitude higher than typical drinking water exposures, which average 0.001 to 0.005 mg Cr(VI)/l. Given the lack of epidemiological evidence for increase in the risk of intestinal cancers in humans at these environmental levels (Gatto et al., 2010; Fryzek et al., 2001; Gatto et al., 2010; Morgan, 2011), it would be necessary for human pharmacokinetics (or dynamics) to differ substantially from mice in order for typical exposures to pose a cancer risk. In this regard, the physiologically based pharmacokinetic models… [Note to authors: SHOW WHAT?]

Reviewer 6:

There were none that I identified per se, other than lack of information concerning the rat.

Reviewer 7:

No response provided.

Charge Question 4: Are there any counterfactual data that should have been considered but were not included?

Reviewer 1:

No.

Reviewer 2:

I don't think so.

Reviewer 3:

Dose-response curves for the small intestinal tumors (as noted above).

Reviewer 4:

Counterfactual evidence (i.e., experimental support for the necessity of a key event) relates to "specificity" in application of the Bradford Hill considerations. This includes, for example, experimental evidence in sufficiently exposed null animal models that prevention of a key event precludes development of the end (adverse) effect. Similarly, if following cessation of repeated exposure for extended periods, effects are reversible (i.e., late key events or the end (adverse) effect is prevented), this constitutes relatively strong evidence that key events are causal. Unfortunately, it appears that studies relevant to development of counterfactual evidence were not conducted; this detracts somewhat from the weight of evidence for the case.

Reviewer 5:

The authors did a good job of tying together their hypothesized MOA for chromium tumorigenesis to the captan story. Counterfactual evidence would mean reviewing the NTP database for strong mutagens and comparing the types of tumors evoked by these strong mutagenic carcinogens with the types of tumors evoked by chromium. I know that the difference would be dramatic, but the authors may wish to document this.

Reviewer 6:

No not exactly, but the evaluation would be more complete if some information were included that explained the interspecies differences, in at least a summary form. In a

true MOA analysis, one does not just consider information from a single species in isolation from findings in other species.

Reviewer 7:

No response provided.

Charge Question 5: Is the approach used consistent with current guidance documents (e.g., EPA 2005; WHO 2007) for evaluating MOA and human relevance?

Reviewer 1:

There is no major concern.

Reviewer 2:

Yes

Reviewer 3:

The approach is consistent with both guidance documents, and systematic.

Reviewer 4:

While consistent with current guidance documents, the case doesn't reflect evolution of frameworks to more meaningfully assimilate the data in a weight of evidence context. This includes comparative consideration of potential modes of action early in the analysis, concomitant consideration of dose-response and temporal concordance, explicit consideration of the Bradford Hill considerations of consistency and specificity including counter-factual evidence in contributing to overall weight of evidence for the preferred hypothesized MOA and tabular concordance analysis for human relevance.

Reviewer 5:

Yes. The authors have used these guidelines as the guideline authors intended, by incorporating more biological data into the carcinogenicity assessment.

Reviewer 6:

Yes the approach is definitely consistent. In terms of the toxicodynamic aspects and taking the analysis at face value since I am not cancer biologist, the analysis does what appears to be an outstanding job of presenting alternative explanations and explaining why they are not supported by the data.

Reviewer 7:

No response provided.

Charge Question 6: Are the conclusions reached consistent with current guidance (e.g., EPA 2005; WHO 2007) documents for evaluating MOA and human relevance?

Reviewer 1:

There is no major concern.

Reviewer 2:

Yes

Reviewer 3:

a. EPA 2005. Mode of Action – General Considerations and Framework for Analysis

Under General Considerations, EPA lists 13 "important factors to review" (2-37 to 2-38 in the EPA document).

Some of these have been directly addressed in the current document. More attention could be given to

i. "effect of dose and time on the progression of lesions from preneoplastic to benign tumors, then to malignant"; and "time of appearance of tumors after commencing exposure". A longer time series, including several points between 90 days and 2 years, would be required to obtain this information.

ii. "tumors at organ sites with high or low background historical incidence in laboratory animals". Small intestine has a low background incidence of tumors. The significance of this was not discussed.

iii. "biomarkers in tumor cells". The need for assessment of K-*ras* mutations in the tumors was noted. Various additional molecular studies could be listed.

iv. "shape of the dose-response curve". This was not discussed.

Also, there are listed five ways by "which information from chronic animal studies influences mode of action judgments". Several of these may need attention.

i. "late onset of tumors" was invoked, but 90 days is too short a time. Very few experimental animal tumors present within that time frame.

ii. "more than one mode of action in a single tissue" . Both mutation and hyperplasia could be relevant to the MOA, as separate mechanisms in different tumors, or as part of the same MOA in a tumor initiation-promotion scenario.

The EPA's Framework for Analysis has been followed, including definition of key events, and discussion of dose-response concordance, temporal relationships, biological plausibility, and human relevance. Consideration of issues associated with each of these Framework steps are given above.

b. WHO 2007

The components of the framework in the WHO document are basically similar to those in the EPA directive. A similar schematic for the human relevance framework is utilized (Fig. 10). The key component is the first: Is the weight of evidence sufficient to establish a mode of action (MOA) in animals? The current document concludes "that the MOA in mice likely involves the key events described in Fig. 2". I cannot concur, based on issues with the data for the Key Events and their interpretation (see above). However, there are some new experiments that could be done, that could clarify the situation and have been noted above. Also, both EPA and WHO emphasize desirability of experiments interfering with Key Events, an approach that has not yet been tried with this model.

Reviewer 4:

There are a number of weaknesses in the hypothesis and consideration of weight of evidence of (the) proposed mode(s) of action. These relate to lack of consideration of full datasets for weight of evidence for potential alternative modes of action (e.g., a mutagenic MOA) and more fulsome consideration of the data relevant to application of the Bradford Hill considerations, including concomitant analyses for dose-response and temporal concordance and consistency and specificity (including counterfactual evidence and whether or not the incidence of the toxic effect is consistent with (i.e., less than) that for the key events). Also, some critical data gaps have not been noted.

Reviewer 5:

Yes. EPA's guidelines sets the direction that the authors followed in their chromium research plan, and the WHO guidelines gave a specific framework that the authors have used their analysis of their data. The overall result is a tour de force, and one for which risk scientists should strive with other carcinogenicity risk assessments.

Reviewer 6:

In terms of the application of the MOA framework as I understand it, the answer would be yes with the caveats mentioned in regards to pharmacokinetics and discussion of interspecies differences in pharmacokinetics and pharmacodynamics.

Reviewer 7:

No response provided.

Charge Question 7: 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:

The data and analyses presented support the author's conclusions.

Reviewer 2:

I have already discussed this with regard to the *in vivo* mutagenicity data and the problem with focal vs. diffuse hyperplasia. However, I do not think either problem leads to an alternate conclusion.

In the discussion of "aberrant nuclei" (page 13), aside from having problems with the use of the term "genotoxic" instead of DNA-damaging, I also do not think that the aberrant nuclei described (karyorrhectic nuclei and micronuclei) reflect only DNA damage. This is acknowledged in the case of MN. But apoptosis, no matter how it is caused, should give rise to "abnormal" nuclei. Also probably necrosis. By what mechanisms are the cells killed?

Reviewer 3:

The data and analyses support the conclusions regarding Key Event 1, Saturation of Reduction and Absorption from the Lumen. They do not support Key Event 2, Villous Cytoxicity; Key Event 3, Crypt Cell Proliferation as secondary to villous cytotoxicity; or Key Event 4, Expansion of Spontaneous Mutations. Detailed concerns about these conclusions are given above.

One alternate conclusion that seems rather likely is that there was toxicity, including genotoxicity, to the crypt cells, and that this may have contributed to the observed hyperplasia. Beyond this, one cannot conclude whether, where, or when mutations occurred, and the extent to which these resulted in tumors. Given the genotoxicity of Cr(VI) in the crypt cells, the known mutagenicity of Cr(VI) in cultured cells and *in vivo*, and the linear tumor dose-response curve, an MOA with early involvement of mutation seems at least as likely as the MOA proposed in this document. There are many additional experiments that could be done to clarify this issue, as described above.

Reviewer 4:

Weaknesses in the current analysis would need to be addressed, prior to meaningfully responding to this question. This includes expansion of consideration of a potential mutagenic mode of action and additional analyses to support the weight of evidence determination for the preferred, hypothesized mode of action and potential human relevance and additional consideration of critical data gaps. The lack of counterfactual evidence to support specificity in application of the Bradford Hill considerations for the preferred hypothesized mode of action is an important weakness.

Reviewer 5:

I was not convinced at the outset of the author's research program that the appropriate MOA was related to oxidative stress. Rather, I hypothesized that several MOAs were likely occurring, including mutagenicity. However, the authors' comprehensive, multi-year project is developing data that unequivocally support the oxidative stress MOA. Although the authors could probably do a few more studies to further solidify these findings, for example additional mutation targets in intestinal cells, I find myself abandoning my hypothesis and defending their theory.

Reviewer 6:

For the most part yes, other than the need for some detail in regards to the pharmacokinetic analyses and data presented as detailed above. Overall, the discussion of folpet/captan is not strongly compelling because they are not structurally related to Cr(VI) and data that would lend stronger support are missing, i.e. no comparable GSH depletion data for Cr. It needs to be more clearly acknowledged that there are superficial similarities that while intriguing, have limited mechanistic information available.

Reviewer 7:

The authors indicate that "These findings are particularly interesting when contrasted with Cr-DNA binding data from genomic DNA isolated from scraped duodenal epithelia. Although increases in Cr-DNA binding were observed with increased SDD dose, there was no correlation with *Kras* mutation frequency (O'Brien *et al.*, in prep). Moreover, we determined that the Cr-DNA isolation technique, while similar to that used in other published studies (Zhitkovich, 2005), was likely confounded by *ex vivo* chromium binding. Specifically, the addition of chromium chloride to scraped intestinal tissue from untreated animals prior to DNA isolation resulted in Cr-DNA levels that were comparable to those in treated animals—indicating that chromium present in (or on) scraped duodenal cells from treated animals was likely binding to genomic DNA *ex vivo* during the DNA isolation assay (O'Brien *et al.*, in prep)." I agree with the authors that chromium is strongly genotoxic, clastogenic, and mutagenic. Administered Cr chloride to a DNA isolation procedure from untreated animals is capable of giving positive Cr-DNA binding because it so strongly interacts with DNA. The combination of high enough water solubility, sulfate mimic, and DNA reactivity suggest that a genotoxic/mutagenic MOA should be considered and included as part of the MOA and not excluded.

The authors should consider performing either an alkaline elution assay or a comet assay for genotoxicity, or both. With an alkaline elution assay the water soluble Cr will be continuously washed away from the DNA by the moving liquid phase maybe giving you a cleaner preparation than the Cr-DNA DNA isolation adduct procedure that you are using. With the comet assay, there might be fewer ex vivo artifacts than are present in the current studies. Including some controls of added Cr chloride to untreated tissue will demonstrate how much ex vivo effects are possible with these two alternative DNA damage techniques.

The authors indicate that "Alternative MOAs include mitogenic and mutagenic MOAs. With regard to the former, there is no evidence to suggest that Cr(VI) stimulates crypt hyperplasia in the absence of cytotoxicity, as hyperplasia occurs subsequent to villous cytotoxicity in both dose and time. With regard to the latter, prior to the conduct of the studies described herein, some investigators hypothesized that the intestinal tumors observed in mice arose by a mutagenic MOA—based primarily on data from non-target tissues and *in vitro* systems (McCarroll *et al.*, 2010)." While the authors offer evidence for a nongenotoxic cytotoxic MOA, there is substantial evidence from other systems that suggest a genotoxic/mutagenic MOA are part or most of the overall MOA for oral chromium. I

think the scientific reception and impact of this type of paper, as it is currently written, is going to be limited.

Charge Question 8: Please comment on other issues that have not been addressed by this charge.

Reviewer 1:

From literature and studies described in this manuscript, it appears that Cr(VI)-induced oxidative stress seems to play a key role in Cr(VI)-induced carcinogenesis in general and rodent small intestinal tumors in particular. Future studies should use the "whole-life" chronic Cr(VI) exposure model to investigate Cr(VI)-induced formation of tumors, including the rodent small intestinal tumors and the protection of antioxidant supplement. Breeder animals of males and females will be treated with Cr(VI) with or without antioxidant supplement before breeding. The pregnant females will be treated during the entire pregnancy, and the dams will be treated while lactating and offspring will be treated for up to 2 years of age. Tumors will be assessed in the offspring. This "whole-life" chronic arsenic exposure model was recently developed by the laboratory of Dr. Michael P. Waalkes at NIEHS (Tokar, E.J., Diwan, B.C., Ward, J.M., Delker, J.W., and Waalkes, M.P. (2011) Carcinogenic effects of ''whole-life'' exposure to inorganic arsenic in CD1 mice. Toxicol. Sci. 119, 73-83). This group investigated arsenic rather than Cr(VI) and no prevention study has been carried out using this model. Accumulating data suggest that early-life events can be critical in causing disease later in life. It is likely that in certain population, humans would have full life exposure. Chronic exposure of animals to Cr(VI) during their ''whole life'' is very relevant to that to be expected in exposure of humans to Cr(VI).

Reviewer 2:

A problem with this manuscript is that it often did not clearly differentiate between mutagenicity and genotoxicity. Because individual scientists have varying, and sometimes conflicting, definitions of genotoxicity, the term should be replaced by exactly what is meant (e.g. DNA damage). This problem appears mainly in the Abstract and on page 13.

Reviewer 3:

No other comments.

Reviewer 4:

The manuscript represents consideration of an impressive array of experimental work conducted to address mode of action of CrVI including characterization of effects at several levels of biological organization; consideration of mutation in the target tissue is particularly helpful. Lack of counterfactual evidence for support of specificity in application of the Bradford Hill considerations is an identified weakness, as is the lack of information on very early incidence of key events following exposure (e.g., after one day).

I was also very pleased to see an attempt to assimilate the data early in the manuscript in an MOA context, thereby emphasizing patterns at different levels of biological organization with effective use of tables and figures, in this context. However, this has led to a number of experimental details important to interpretation being missing, particularly those related to dose-response for various strains. Potentially, this information could be added to the tables, so as not to increase the complexity of the text.

I've tried to flag in the background sections where data assimilation in a slightly different context would contribute much more convincingly to consideration of weight of evidence for hypothesized modes of action. This would include more fulsome and separate consideration of hypothesized modes of action at the outset and concomitant consideration of dose-response and temporal aspects for the most promising MOA. The latter would be best presented in revised (combined) Sections 3.3 and 3.4 In addition, considerations relevant to specificity and consistency of the supporting data for the preferred hypothesized MOA need to be added.

The abstract does not represent well the content of the paper and should be expanded to emphasize those observations which contribute to weight of evidence for the hypothesized mode of action based on the Bradford Hill considerations, outcome of the human relevance (concordance) analysis and implications for dose-response analysis.

Reviewer 5:

I have additional thoughts that the authors might consider. These are found on:

Page 20 "This observation, as well as high Cr-DNA binding levels in the non-target tissue of the mouse liver following drinking water exposure to SDD (O'Brien et al., in

prep), calls into question the reliability of Cr-DNA binding data, and furthermore, the data do not support the role of Cr-DNA adducts as pre-mutagenic lesions relevant in the MOA for small intestinal tumors." [Note to authors: ok, so now you explain why the chromium binding is important. I believe this is better handled as a footnote. The important thing to state is that mutations are not preceding hyperplasia.]

Page 59, Table 6. Please consider adding the bolded text below to the description of "plausibility":

Early mutation in highly proliferative intestinal mucosa is not consistent with absence of cytogenetic damage, absence of Kras mutations, absence of preneoplastic lesions (e.g. focal hyperplasia) (NTP, 2007, 2008a; Thompson et al., 2011b), late tumor onset (NTP, 2008a), **lack of tumors in non-portal of entry tissues**, and lack of effect on survival (NTP, 2008a).

Reviewer 6:

One major problem I see is that the presentation of the MOA analysis for mice, completely separate from that of rats tells a story that is sufficiently incomplete that it leaves too much unsaid to really form an analysis with which I am completely comfortable. Consider adding some details in regards to the rat at least as an appendix or in a figure with reference to forthcoming rat analysis. This is a serious deficiency.

What follows are some comments of an editorial nature that impact the readability or accessibility of analysis as currently presented:

Introduction, page 5, first full paragraph, 4th sentence beginning with "In contrast to mice… - the latter part of this beginning with, "suggesting that the intestinal tumors…" sentence is a non-sequitor based on the first, i.e. it is unclear how tumors not being observed in rats suggests a cytotoxic MOA. It is the 3rd sentence that suggests the conclusion articulated in the latter part of the 4th sentence of this paragraph.

Section 3.1.2.3, p. 14, first paragraph – Change the word "predicts" in the 3rd sentence to "suggests the possibility that"

Section 3.8, p. 37 – it is sufficient to simply say that no analysis of these tissues is currently available.

Section 4, last paragraph, last sentence, top of p. 39. This is an interesting point, but not particularly compelling as evidence unless dose-response analyses were

done. If this is the case, then discuss in more detail. The question will always remain as to whether the studies are sufficiently sensitive (or put another way, have the statistical power) to detect elevated risks of intestinal cancer that might still be of public health concern.

For Figure 4, it needs to be clarified whether or not the comparisons are day 8 vs. day 91 or day 8 and day 91 vs. their comparable controls.

Reviewer 7:

The authors indicate that "Although significant increases in 8-OHdG were not evident in duodenal tissues from mice exposed to Cr(VI) for 90 days (Thompson *et al.*, 2011b), it is conceivable that changes in genes related to redox signaling and DNA repair prevented 8-OHdG formation/detection." 8-OHdG is just one of many clastogenic/mutational events. Even if there is not a demonstrated increase in this parameter it does not mean that the genotoxic (in part or in whole) MOA theory is not true. It is difficult if not impossible to prove something is not genotoxic. 8-OHdG is technically hard to measure as dG → 8-OHdG while the samples are stored, DNA is isolated and even during the measurement of 8-OHdG. The problem of adventitious oxidation of dG is well known. True Cr effects might be lost in the amount of additional 8-OHdG formed subsequent to sampling.

The authors identified the following key events:
Key Event 1 - saturation of reduction and the absorption of Cr(VI) from the intestinal lumen;
Key Event 2 - villous cytotoxicity due, in part, to oxidative stress;
Key Event 3 - compensatory crypt hyperplasia to repair/replace the damaged intestinal mucosa; and
Key Event 4 - expansion of spontaneous mutations in the crypt cells as a consequence of the constant proliferative pressure, ultimately leading to tumorigenesis.

For Key event 1, it seems the author's data better argues for saturation of absorption and not of saturation of the reduction of Cr6 --> Cr3. Thus they might want to remove the word reduction from their key event 1. Where is the evidence of saturation of reduction? Rather, the capacity of the Cr uptake system into GIT cells appears to be saturated. For Key event 4, the authors need to provide evidence that the 7 types of DNA-Cr interaction that they cite do not contribute to mutation and that 100% of the mutation is "spontaneous" and not due to the Cr exposure.

Appendix A

Instructions to Reviewers

Dear Reviewers,

Thank you again for your willingness to provide a peer review of a mode of action analysis for hexavalent chromium following drinking water exposure. This MOA analysis has been developed 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 MOA analysis. 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 MOA analysis 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, October 5, 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

Appendix B

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Reviewer 3

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Appendix C

Reviewer 4 Comments in Manuscript

Assessment of the Mode of Action Underlying Development of Rodent Small Intestinal Tumors Following Oral Exposure to Hexavalent Chromium and Relevance to Humans

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Short title: MOA for Cr(VI) in Mouse Small Intestines

Abstract (248 of 250)

Although chemicals may act by multiple modes of action (MOAs) See comment in discussion. Avoid use of this term.
at different tumor sites and at different doses, knowledge of the MOA that is specific to the tumor-response used for risk assessment is critical for low-dose extrapolation. In fact, MOA doesn't inform us about low dose extrapolation. Rather, it informs us about key events, which, if prevented, will ensure prevention of the adverse effect For a mutagenic MOA, a chemical or its metabolites interacts directly with DNA to induce heritable mutations, whereas nonmutagenic carcinogens display a wider range of mechanisms that may involve genotoxicity and/or nongenotoxicity (e.g. cytotoxicity- or hormonally-induced cell proliferation) that result in tumorigenesis after prolonged exposure. This sentence not very clear. The important point is that for mutagenic MOAs, mutation is an important early key event; for non-mutagenic MOAs for carcinogens, it is secondary to some other (generally rate limiting) key eventChronic exposure to high concentrations of hexavalent chromium (Cr(VI)) in drinking water was shown to cause intestinal adenomas and carcinomas in mice, but not rats, and the potential MOA underlying these tumors has been the subject of recent research. Although Cr(VI) is known to be genotoxic, subchronic studies indicate that Cr(VI) causes blunting of intestinal villi and crypt hyperplasia in mice as early as 8 days of exposure—suggesting that the tumors may have arisen from chronic mucosal injury. To better understand the MOA of Cr(VI) in the intestine, a 90-day drinking water study was conducted to collect histological, biochemical, toxicogenomic, and pharmacokinetic data in target tissues. Using MOA analyses and human relevance frameworks There is one framework which continues to evolve proposed by national and international regulatory agencies, the weight-of-evidence supports a nonmutagenic cytotoxic MOA with the following key events: a) saturation of luminal reductive capacity and elevated intestinal absorption, b) toxicity to villi, c) sustained crypt hyperplasia See comments in text on proposed key events
and d) clonal expansion of spontaneous mutations within the crypt stem cells, resulting in late onset tumorigenesis. Based on? The critical aspects of this analysis should be included in the abstract including Bradford Hill considerations for WOE & human relevance concordance analysis

Key Words: Risk Assessment, Cancer, hexavalent chromium, Cr(VI), Mode of Action

Table of Contents

1. INTRODUCTION (742 of 750)

Chromium is a naturally occurring element that primarily exists in two oxidation states: hexavalent chromium (Cr(VI)) and trivalent chromium (Cr(III)). Cr(III) is a putative micronutrient that may have a role in insulin sensitivity (Anderson, 2000; Di Bona *et al.*, 2011). Oral toxicity studies indicate that Cr(III) has only limited acute and chronic toxicity and is not carcinogenic (IARC, 1990; NTP, 2008b; Stout *et al.*, 2009b). In contrast, occupational exposure to high concentrations of Cr(VI) has ~~been shown to~~ caused lung cancer in workers in certain industries (IARC, 1990), and lung tumors in rodents when administered by inhalation or intratracheal instillation (Glaser *et al.*, 1986; Steinhoff *et al.*, 1986). Differences in toxicity between Cr(III) and Cr(VI) are primarily due to the lower cell permeability of Cr(III). However, Cr(VI) is reduced to Cr(III) in many bodily fluids thereby mitigating Reducing? the effects of Cr(VI) exposures, especially via ingestion due to acidic reducing conditions of the stomach (De Flora *et al.*, 1987; De Flora *et al.*, 1997; Febel *et al.*, 2001). Recognizing Based on recognition of? differences in kinetics, biochemistry, and toxicology of Cr(III) and Cr(VI), health risk assessment of chromium compounds has historically been both route- and valence-specific (U.S. EPA, 1998).

In 2008, the National Toxicology Program (NTP) reported findings from 2-year cancer bioassays for both Cr(III) and Cr(VI) in drinking water (NTP, 2008b; NTP, 2008a). Consistent with the low bioavailability, ~~NTP found that~~ for Cr(III) (administered as chromium picolinate), there was ~~demonstrated~~ "no evidence of carcinogenicity in mice or female rats", and only "equivocal evidence for preputial gland adenomas in male rats" (NTP, 2008b; Stout *et al.*, 2009b). By contrast, Cr(VI) (administered as sodium dichromate dihydrate; SDD) produced a dose-related increase in tumors in the small intestine of mice and oral mucosa of rats (NTP, 2008a; Stout *et al.*, 2009a). It has been questioned ~~A significant question arising from this bioassay is~~ whether the high administered Cr(VI) concentrations ~~employed~~ exceeded the reductive capacity of the mouse stomach and intestinal lumen in this bioassay (Collins *et al.*, 2010; De Flora *et al.*, 2008; Proctor *et al.*, 2011; Stern, 2010) supported by ~~. R~~recently published studies on species-specific elements of Cr(VI) toxicokinetics~~suggest that the carcinogenic concentrations in the 2-year bioassay indeed exceeded reductive capacity~~ (Kirman *et al.*, in press; Proctor *et al.*, 2012).

Hexavalent chromium can induce DNA adducts, DNA damage, mutation, and transformation (Chiu *et al.*, 2010; Holmes *et al.*, 2008; Nickens *et al.*, 2010; Zhitkovich, 2011). It has therefore been argued that the tumors observed following chronic oral exposure to Cr(VI) arose by a mutagenic mode of action (MOA) involving DNA mutation as an early ~~initiating~~ Mutation is never the initiating event key event in the carcinogenic process (McCarroll *et al.*, 2010; U.S. EPA, 2010; Zhitkovich, 2011). However, the NTP study authors described the non-neoplastic lesions in the mouse intestine (*viz.* diffuse hyperplasia) as secondary to previous epithelial injury (NTP, 2008a). In contrast to mice, diffuse hyperplasia and tumors were not observed in rats (NTP, 2008a), suggesting that the intestinal tumors in mice may have occurred via a cytotoxic MOA Explain? Where cytotoxicity is an important early key event?. Until recently, there was insufficient information to determine the MOA underlying the development of intestinal tumors in mice with any degree of certainty because data describing the dose-response and sequence of potential key events in the target tissue were not available~~did not exist~~.

To investigate the MOA underlying the intestinal tumors in mice, an ~~MOA~~ analysis was conducted by applying the MOA framework outlined in the U.S. EPA *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005) to: a) hypothesize a plausible MOA for the mouse intestinal tumors; b) identify critical data gaps; and c) design and conduct a 90-day drinking water study to in? acquire critical data to fill these gaps (Thompson *et al.*, 2011a). In this study, ~~The study design~~, described in detail in Thompson *et al.* (2011b and 2012c), groups of species/strain? ~~employed~~ were exposed to the same drinking water concentrations as in ~~from~~ the NTP 2-year bioassay (NTP, 2008a) as well as two lower drinking water concentrations; one of which was 100 µg/l, the federal Maximum Contaminant Level (MCL) for total chromium (U.S. EPA, 1991). Data on?were collected in the target tissues after 7 and 90 days of exposure, and many of the

study results have been published (Kopec *et al.*, 2012a; Kopec *et al.*, 2012b; Proctor *et al.*, 2012; Thompson *et al.*, 2011b; Thompson *et al.*, 2012c).

The purpose of this article is to present a synthesis of the MOA study data, as well as other available data, to consider the weight of evidence for an hypothesized ~~determine the~~ MOA for mouse intestinal tumors ~~using a weight of evidence (WOE) approach~~. Findings relevant to the oral mucosal tumors observed in rats will be presented in a separate publication. reference? These results are also relevant to biological plausibility for Bradford Hill considerations for WOE for preferred hypothesis

2. SUMMARY OF INTESTINAL LESIONS IN THE RODENT 2-YEAR BIOASSAYS

~~A review of the intestinal anatomy and physiology as well as the findings of the NTP (2008a) bioassay is helpful for understanding the MOA for intestinal tumors.~~ The small intestine is compromised (proximal to distal) of the duodenum, jejunum, and ileum. Each segment is lined with a single layer of epithelial cells that is renewed every few days—making the intestinal mucosa one of the most proliferative tissues in the body (Berlanga-Acosta *et al.*, 2001). The small intestinal epithelium is extensively folded to maximize the absorptive surface area. Anatomically, the small intestinal epithelium is comprised of crypt and villus regions. The villi are multicellular finger-like projections that extend into the intestinal lumen (**Fig. 1A**); inside each villus is a capillary and lymphatic vessels (**Fig. 1B**). The crypts (also called glands of Lieberkühn) are invaginations of the epithelial surface at the base of the villi. Inside the crypts are actively dividing progenitors cell that differentiate into secretory (goblet cells), absorptive cells (enterocytes), and other cell types (e.g. Paneth cells) (Neal *et al.*, 2011; Potten *et al.*, 1997). Cell proliferation begins in the base of the intestinal crypts within a population of rapidly

dividing progenitor cells. Epithelial cells differentiate (mature) as they migrate toward the tips of the villi (**Fig. 1C**). The lifespan of the enterocyte, from progenitor cell through differentiation and migration to the tip of a villus, is 10-17 hours in rodents and ~24 hours in humans, and there is complete replacement of the entire epithelium within 2-3 days in rodents and 3-6 days in humans (Greaves, 2007). Upon migration from the crypt, cells differentiate and express proteins involved in nutrient absorption—which occurs primarily in the duodenum and proximal jejunum (DeSesso and Jacobson, 2001). At the tips of the villi, cells slough into the lumen and/or undergo apoptosis. Importantly, the absorptive villous enterocytes do not have proliferative potential, and thus it is unlikely that tumors originate in these cells (Barker *et al.*, 2009; Potten and Loeffler, 1990).

Results of the NTP 2-year drinking water bioassay for Cr(VI), administered as SDD what is this?, are described in detail in Stout *et al*. (2010). Findings in the small intestine and their statistical significance are summarized here in **Table 1.** Similar effects were observed in the small intestines of both male and female mice; however, for brevity only the results for females are summarized here~~in~~ The pattern of results for male mice is relevant to interpretation of the weight of evidence, since tumours were not observed (B/H consideration of biological plausibility). This is a critically important omission in the paper.. In female mice, the incidence of diffuse hyperplasia was significantly increased in all treatment groups in a dose-dependent manner and was characterized by short, broad and blunt villi and by elongated crypts that contained increased numbers of epithelial cells and mitotic figures (NTP, 2008a). These lesions were not observed in female or male rats. The NTP study authors considered the lesions in mice to be "*consistent with regenerative hyperplasia secondary to previous epithelial cell injury*" (NTP, 2008a).

The incidence of adenomas and carcinomas were statistically elevated relative to concurrent controls in the duodenum of female mice at ≥172 mg/l and 516 mg/l SDD, respectively. In the female mouse jejunum, diffuse hyperplasia and adenomas were significantly elevated relative to concurrent controls at 516 mg/l SDD. The overall combined incidence of intestinal tumors in female mice was significantly elevated relative to concurrent controls at ≥172 mg/l SDD (**Table 1** Suggest to compare & contrast this with regenerative hyperplasia upon introduction to facilitate MOA analysis. Consideration of relative incidence of tumours and early key events is important to address Bradford Hill consideration of consistency and specificity). Adenomas and carcinomas were not elevated in the small intestine in either male or female rats (NTP, 2008a).

3. APPLICATION OF THE MODE OF ACTION FRAMEWORK

U.S. EPA (2005) defines MOA as "*a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation.*" EPA defines a key event as "*an empirically observable and quantifiable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element.*" The EPA MOA Framework, like that proposed by the International Programme in Chemical Safety (IPCS) (Boobis *et al.*, 2006 Suggest to include the more recent Boobis et al., 2008 reference here
), stresses that the MOA for *each* tumor site should be evaluated separately. Moreover, toxicology data should ideally be evaluated (or generated if absent) in the target tissues of interest. Therefore, the MOA described below focuses on data obtained from the duodenal and jejunal intestinal mucosae of mice. Tumor incidence in the mouse small intestine provides the basis for several Cr(VI) risk assessments (McCarroll *et al.*, 2010; Stern, 2010; U.S. EPA, 2010), and thus understanding the MOA that is specific to tumors occurring in these tissues informs the low dose extrapolations Not exactly. It informs in the range of observation & potentially

identifies early key events that may be protective for tumours for risk assessments that rely on these data.

3.1. Summary of the Key Events in the MOA for Intestinal Tumors

The histological, biochemical, toxicogenomic, and pharmacokinetic data collected as a part of our MOA studies were evaluated along with other relevant data in the published literature as a basis to hypothesize potential key events, as characterized in ~~using the~~ MOA and human relevance frameworks developed by the U.S. EPA and other organizations. Based on this assessment, it was hypothesized concluded Shouldn't be concluding at this juncture. This is just the first step. What you're looking for here is the most likely of a range of options. What about support for a mutagenic MOA?

that the overall WOE supports a cytotoxic nonmutagenic MOA with the following key events (**Fig. 2**): **Key Event 1** - saturation of reduction and the absorption of Cr(VI) from the intestinal lumen; **Key Event 2** - villous cytotoxicity due, in part, to oxidative stress; **Key Event 3** - compensatory Sustained. It is sustained proliferative regeneration in the presence of continuing exposure which is critical; the work "sustained" should be added throughout crypt hyperplasia to repair/replace the damaged intestinal mucosa; and **Key Event 4** - expansion of spontaneous mutations in the crypt cells as a consequence of the constant proliferative pressure, ultimately leading to tumorigenesis. Each of these key events, and supporting data, are summarized in the sections that follow. Key events should be chosen carefully, and necessarily need to have been measured (sometimes through a surrogate) as a basis to consider weight of evidence for an hypothesized MOA. Absorption is rarely considered a key event. Key event 1 might more appropriately relate solely to saturation of reduction (as long as this has been measured)

3.1.1. I have never been particularly fond of this format of separation of evidence for key events - i.e., addressing them completely separately, since B/H considerations for weight of evidence and implications for dose-response analysis relate principally to their interdependence

Key Event 1: Saturation of Reduction and Absorption from the Lumen See comment above re reference to absorption as a key event

The absorption of Cr(VI) is thought to occur under very specific conditions. The chemistry of Cr(VI) dictates that below ~pH 6, Cr(VI) exists as dichromate ($Cr_2O_7^{2-}$) and chlorochromate (CrO_3Cl^-), whereas at ≥ pH 6, Cr(VI) primarily exists as chromate (CrO_4^{2-}) (Zhitkovich, 2005). Chromate is structurally similar to sulfate (SO_4^{2-}) and phosphate (PO_4^{2-}) ions and therefore enters cells through anion transporters (De Flora, 2000; Markovich, 2001; Salnikow and Zhitkovich, 2008; Zhitkovich, 2005). However, reduction of Cr(VI) to Cr(III) prevents entry through these transporters and is thus a critical kinetic process limiting toxicity (De Flora, 2000; De Flora *et al.*, 1997; Donaldson and Barreras, 1966; Febel *et al.*, 2001; Kerger *et al.*, 1996; U.S. EPA, 1998). The stoichiometry of Cr(VI) reduction to Cr(III) in the lumen of the stomach and small intestine is not fully known, but probably varies depending on stomach conditions (fasting/fed), pH, and dose. The most studied biological reducing agents for converting Cr(VI) to Cr(III) are GSH, ascorbate, and cysteine (Zhitkovich, 2005), and enzymatic and/or non-enzymatic reduction by proteins also likely plays a role in Cr(VI) reduction. Cr(VI) that escapes reduction in the stomach will be taken up into enterocytes, transited through the gastrointestinal tract where it will be reduced by microbiota (Shrivastava *et al.*, 2003) and intestinal secretions such as cysteine (Dahm and Jones, 2000), or excreted in feces. Decreasing luminal Cr(VI) concentrations, due to extracellular reduction of Cr(VI) to Cr(III), likely explains the decreasing level of tissue damage and tumor formation from the proximal to distal intestinal segments in the 90-day and 2-year studies. Contributes to consistency for B/H considerations for weight of evidence of the hypothesized MOA. There is no discussion of consistency and specificity in subsequent section in this paper (This is rather a critical omission).

In vivo and *ex vivo* pharmacokinetic data collected in the alimentary tract of rodents suggest species differences in the disposition of Cr(VI) (Proctor *et al.*, 2012; Thompson *et al.*,

2011b). Tissue chromium levels after 90 days of exposure to Cr(VI) were higher in mice than rats on a mg/kg bodyweight exposure basis (**Fig. 3A**), which may be explained, at least in part, by the higher levels of unreduced Cr(VI) predicted to enter the intestinal lumen (Proctor *et al.*, 2012). In mice exposed to Cr(VI) in drinking water for 90 days, a statistically significant increase in duodenal chromium levels was not observed at 0.3 or 4 mg/l SDD (**Figure 3B**)—suggesting that at these administered SDD concentrations, appreciable duodenal Cr(VI) absorption does not occur. As discussed above, reduction in the lumen of the stomach and intestine competes with kinetic processes of absorption and transit through the gastrointestinal tract (Proctor *et al.*, 2012), thus Cr(VI) transited to the small intestine is available for absorption even at concentrations that are lower than stomach reductive capacity. Nevertheless, as will be discussed in the following section, histopathological lesions were not observed in the mouse duodenum at (or below) 14 mg/l SDD following 90 days of exposure. As such lesions are critical for driving tumor formation Given that you haven't examined weight of evidence , currently, this conclusion is premature, small amounts of Cr(VI) that enter intestinal villi are likely inconsequential. PBPK models capable of accounting for the competing kinetic rates have been developed (Kirman *et al.*, in press) and will be useful for estimating the concentrations of Cr(VI) that would need to be ingested to induce cytotoxicity in humans. Why does this statement appear here. This relates to human relevance or concordance analysis which is generally addressed subsequently to consideration of WOE of hypothesized MOAs in animals.

3.1.2. Key Event 2: Villous Cytotoxicity

The non-neoplastic lesions observed in the mouse small intestine were characterized as being secondary to previous epithelial cell injury (NTP, 2008a). The villi of treated animals were short, broad, and blunt relative to unexposed animals (NTP, 2007; NTP, 2008a). Such effects are a nonspecific response to various types of injury, and can be associated with either hyperproliferation or hypoproliferation of crypt enterocytes (Greaves, 2007; Serra and Jani, 2006). Chemicals that are cytotoxic to cells of the villi can, after removal, stimulate crypt proliferation to repair the damaged mucosa. Alternatively, chemicals that are toxic to crypt cells can also result in blunting of villi due to hypoproliferation in the crypt and inability to replenish the mucosa. Because cytotoxicity can result from epigenetic and genotoxic effects, and the intestinal stem cells reside in the crypt, it is critical to determine a) whether toxicity is originating in the crypt or villus, b) whether there is any evidence of cytogenetic damage in the crypts, and c) whether there is evidence for epigenetic mechanisms associated with intestinal carcinogenesis

such as oxidative stress and inflammation. Each of these issues are discussed in the following subsections; and as a whole, indicate that Cr(VI) toxicity occurs in the villus. Again, this presupposes the outcome with sweeping generalization. Essential to drawing this conclusion is fulsome consideration of weight of evidence.

3.1.2.1. Histological evidence of cytotoxicity A table where data on temporal & dose response concordance is summarized would be much preferred, here, as a basis to illustrate suport (or not) for the hypothesized MOA (see template in comments). This provides visual communication of a large amount of text here which is very difficult to follow. It would also be much more informative to include in such a table benchmark doses for the various hypothesized key events. See Meek, M.E. and Klaunig, J. (2010) Interpreting available data and identifying critical data gaps for benzene risk assessment. Evolution and Contribution of the IPCS/ILSI framework for "Mode of Action/Human Relevance". Chemico-Biological Interactions 184: 279–285.

The term "diffuse hyperplasia" used in the NTP studies includes the blunting effects on the villi, as well as elongation of the crypts (see Plate 21 in NTP, 2008a). This terminology was not used in Thompson *et al*. (2011b); instead, villus damage and crypt hyperplasia were noted separately in order to provide greater resolution with respect to the location of the lesions. As shown in **Table 2** Seem to be missing some details of experimental design here. Can these be included in the legend to the table. Was the strain the same as that in the NTP study? Were conditions of administration the same?
, cytoplasmic vacuolization in the duodenal villi was the most sensitive endpoint—occurring at 170 and 60 mg/l SDD at day 8 and 91, respectively; while atrophy of the villi and crypt hyperplasia were first evident at higher concentrations (i.e., 520 and 170 mg/l on day 8 and 91, respectively). Vacuolization can be an indication of reversible injury to the cells (Henics and Wheatley, 1999), and can arise from altered lipid metabolism, sequestration of absorbed material, autophagy, endoplasmic reticulum (ER) stress, or proteasome dysfunction (Franco and Cidlowski, 2009; Henics and Wheatley, 1999; Mimnaugh *et al.*, 2006). Although the exact cause of the SDD-induced vacuolization is unclear, it is often regarded as a sign of cytotoxicity (Mimnaugh *et al.*, 2006).

The presence of cytoplasmic vacuolization in villi at lower concentrations than crypt hyperplasia suggests a mechanism whereby toxicity to cells in the villi triggers compensatory cell proliferation of crypt enterocytes. Area measurements of the crypt of the mouse duodenum at day 8 were increased ~45% at ≥170 mg/l SDD (O'Brien *et al.*, in prep). At day 91, the crypt area was increased ~45% at 60 mg/l, and was significantly increased ≥2-fold of control at ≥ 170 mg/l (**Fig. 4A and D**). Consistent with the increase in crypt area, the ratio of the villus to crypt area was decreased at ≥170 mg/l SDD (**Fig. 4B**). If SDD were toxic to crypt enterocytes, then one would expect a dose-dependent decrease in crypt area. In fact, the number of enterocytes per crypt increased significantly following exposure to ≥170 mg/l SDD (**Fig. 4C and E**). Despite the increase in the number of crypt enterocytes, the intestinal villi were damaged and blunted. This implies that the increased supply of crypt enterocytes was not sufficient to maintain the normal length and structure of healthy duodenal villi. Hence, the damage to the villi was caused by exposure to cytotoxic concentrations of Cr(VI).

As further evidence that the cytotoxicity of SDD is limited to the intestinal villi, the number of mitotic and apoptotic cells were counted in fully intact crypts in order to compute both a mitotic index (MI) and apoptotic index (AI). As shown in **Table 3**, there were no significant or dose-dependent effects on either endpoint—suggesting that Cr(VI) did not cause cell cycle arrest or increases in the percentage of crypt enterocytes undergoing programmed cell death. Previous studies have suggested that increased crypt hyperplasia without change in the MI indicates that the number of active crypt stem cells has increased (Snow and Altmann, 1983). Given that crypt stem cells are the most likely clonal sources of tumor formation (Barker *et al.*, 2009; Potten and Loeffler, 1990), increases in the numbers of crypt stem cells may increase the chance of spontaneous tumor formation (Feng *et al.*, 2011).

3.1.2.2. Assessment of cytogenetic damage in crypts Ok but need really to address in the context of potential mutagenic mode of action. This is strong counterfactual evidence against the likelihood of a mutagenic mode of action.

Data in the previous section indicate that Cr(VI)-induced damage to the intestinal mucosa originates in the villus. However, because Cr(VI) can be genotoxic—especially in *in vitro* systems, crypt enterocytes were also examined for aberrant nuclei such as karyorrhectic nuclei and micronuclei (MN). The former indicates a type of cell death that can be either a result of programmed death or necrosis (Kumar *et al.*, 2005), while the latter can arise from either DNA breakage or chromosomal disjunction (Fenech *et al.*, 2011; Vanhauwaert *et al.*, 2001). There were no treatment-related effects on the number of karyorrhectic nuclei or MN in duodenal crypts of exposed mice (**Table 3**). MN were also counted in three entire tissues sections, regardless of whether the crypts were fully intact (**Fig. 4F**). This analysis has the advantage of increasing the overall amount of crypt tissue that was analyzed for aberrant nuclei. There were no treatment-related increases in MN or karyorrhectic nuclei in crypts across any of the tissue sections examined in animals exposed to Cr(VI) for 7 or 90 days; however, both forms of aberrant nuclei were observed in villi (**Table 4**). Specifically, karyorrhectic nuclei were significantly increased in duodenal villi at ≥60 mg/l SDD at day 91 and primarily occurred in the tips, whereas MN were statistically increased in villi at ≥ 170 mg/l (**Table 4**). The concentrations where aberrant nuclei were observed in duodenal villi correspond to the concentrations where cytoplasmic vacuolization was observed (**Table 2**). This pattern of toxicity, specifically limited to the villi, supports that toxicity to the villi caused the regenerative response in the crypt in the absence of any direct genotoxicity or cytotoxicity in the crypt.

3.1.2.3. Oxidative stress

Cr(VI) that is not reduced in the lumen can be absorbed by villous enterocytes, and reduced to Cr(III) through binding to low molecular weight thiols (e.g. GSH) and antioxidants (e.g. ascorbate). Furthermore, Cr(VI) reduction can result in the production of Cr(V) and Cr(IV) reactive intermediate species and the generation of reactive oxygen species (ROS) (Liu and Shi, 2001; Nickens *et al.*, 2010). Thus, the chemistry and biochemistry of Cr(VI) reduction predicts that Cr(VI) can alter the redox status of enterocytes.

Cr(VI) significantly decreased the GSH/GSSG ratio, a key indicator of cellular redox status (Meister and Anderson, 1983; Moriarty-Craige and Jones, 2004; Schafer and Buettner, 2001), in the mouse small intestine in a concentration-dependent manner Doses? Strains? (compared to NTP studies)
(**Fig. 5**; (Thompson *et al.*, 2011b). Despite the change in redox status, the duodenum appeared to synthesize GSH as evidenced by dose-dependent increases in duodenal GSH levels (Thompson *et al.*, 2011b). The increase in GSH was accompanied by significant increases in expression of genes involved in GSH synthesis, and there were clear signs of oxidative stress responses in the transcriptome (see **Section 3.2**). These data indicate tissue oxidative stress that could not be mitigated even after relatively long durations of exposure (7-90 days), implying that the mice exposed to Cr(VI) in the 2-year NTP bioassay (NTP, 2008a) experienced increased intestinal oxidative stress nearly their entire lifespan. As per comment above, need comparison with NTP doses

Intestinal mucosal health is influenced by redox status (Circu and Aw, 2011; John *et al.*, 2011). For example, the intestinal mucosa regulates luminal redox status by secretion of thiols such as cysteine (Dahm and Jones, 2000; Hagen *et al.*, 1990). Chemical-mediated inhibition of GSH synthesis results in loss of intestinal epithelial cell height, desquamation of microvilli, mitochondrial swelling, and vacuolization in the tips of jejunal villi, all of which could be mitigated by GSH supplementation (Martensson *et al.*, 1990). These lesions are similar to those induced by Cr(VI). Although Cr(VI) caused a dose-dependent increase in GSH tissue levels, the GSH/GSSG ratio was nonetheless significantly decreased (Thompson *et al.*, 2011b). Given the

influence of redox status on intestinal health, it is highly likely that Cr(VI)-induced changes in oxidative status contribute to cytotoxicity in the intestinal villi.

3.1.2.4. Inflammation

Oxidative stress and inflammation are typically linked physiologically. For example, oxidative stress leads to activation of nuclear factor-□B (NF-□B) and subsequent downstream pathways resulting in the release of cytokines; this is well documented in the scientific literature for airway inflammation, intestinal inflammation, and certain cancers (Kruidenier and Verspaget, 2002; Rahman and MacNee, 2000; Roberts *et al.*, 2009). Increases in histiocytic infiltration of macrophages in the duodena of rats and mice were observed in the 90-day and 2-year NTP studies (NTP, 2007; NTP, 2008a), as well as in the subsequent 90-day studies (Thompson *et al.*, 2011b; Thompson *et al.*, 2012c) Doses?
; however, clear signs of chronic inflammation were not apparent in these studies. Although histiocytic infiltration can be associated with mild, chronic inflammation, ~~its meaning is unclear.~~ Not too sure what this is saying - suggest to delete
~~In fact,~~ the NTP study authors concluded that the biological significance of histiocytic infiltration was uncertain (NTP, 2008a).

Thompson *et al.* (2011b, 2012c) measured changes in about twenty cytokines and chemokines in both serum and duodenal mucosa of both mice and rats following 90 days of exposure to Cr(VI). In the serum, there were no clear treatment-related effects in mice, while IP-10 and IL-12(p70) were decreased in rats (**Supplemental Table 1**). In the duodenum, several cytokines were significantly decreased in treated mice relative to untreated mice Doses?
. TNFα and IL-1β both showed clear dose-dependent decreases following 90 days of exposure (Thompson *et al.*, 2011b). Other cytokines reduced were IL-7, 9, 12, 13, 17, IFNγ, and RANTES (**Supplemental Table 1**). In rats, only IL-1α was clearly altered by SDD, and was increased at ≥60 mg/l. Broadly, these data support a pro-inflammatory response in rats and a decrease in cytokines and chemokine production in mice Doses?. Toxicogenomic analyses lend additional support to this conclusion (see **Section 3.2)**.

3.1.3 Key Event 3: Crypt Cell Proliferation

Chronic cell proliferation is a well-known risk factor for carcinogenesis (Ames *et al.*, 1993; Boobis *et al.*, 2009; Cohen, 2010; Gaylor, 2005). Diffuse hyperplasia occurred in the duodenum of mice at all SDD concentrations examined in the 2-year bioassay, while there was no evidence

of diffuse hyperplasia in the rat duodenum at any dose level (NTP, 2008a). The same pattern was observed in the NTP 90-day study (NTP, 2007). The two 90-day drinking water studies conducted by Thompson *et al*. (2011b, 2012c), included evaluations at both day 8 and 91 of exposure. At day 8, duodenal crypt cell hyperplasia was present in 3 of 5 mice Small group sizes for dose-response exposed to 520 mg/l SDD (**Table 2**; (Thompson *et al.*, 2011b)). By day 91, crypt hyperplasia was present in almost all animals at ≥170 mg/l SDD (**Table 2**). At both time points, crypt cell hyperplasia was preceded on a dose-basis by cytoplasmic vacuolization in the villi. Together, these data suggest that Cr(VI) increased crypt hyperplasia as early as one week of exposure to very high concentrations in drinking water This pattern consistent with other cytotoxic MOAs - early appearance of late key events at high concentrations; this is best illustrated in dose-response/temporal concordance table. By day 91, there was a significant increase in the number of crypt enterocytes at the higher SDD concentrations (**Fig. 4C**). Not surprisingly, whole genome microarray analysis also indicated cell proliferation (see **Section 3.2**).

Importantly, focal hyperplasia, which NTP considers a preneoplastic lesion (NTP, 2008a), was not observed in any animals in the 90-day drinking water studies (NTP, 2007; Thompson *et al.*, 2011b; Thompson *et al.*, 2012c). Altogether, 260 rodents from the aforementioned three 90-day drinking water studies were exposed to ≥60 mg/l SDD yet focal hyperplasias (or other preneoplastic lesions) were not observed. Although focal hyperplasia was observed in a few animals in the 2-year NTP study (NTP, 2008a), the incidence was not statistically different from controls and was not observed in any female mice in the 520 mg/l SDD treatment group (**Table 1**). The presence of diffuse hyperplasia without focal hyperplasia is consistent with proliferation that is secondary to mucosal injury. The presence of a nearly 2-fold higher incidence of diffuse

hyperplasia in the duodenum at carcinogenic Cr(VI) concentrations, and the presence of significant diffuse hyperplasia in the jejunum at 520 mg/l SDD (the only carcinogenic dose in the jejunum) suggest that crypt hyperplasia is a critical key event in the MOA for intestinal carcinogenesis. The absence of tumors and hyperplasia in rats further indicates that increased crypt proliferation drives intestinal carcinogenesis. Speaks to biological plausibility - B/H considerations

It should be noted that other authors have suggested a sequence of key events whereby Cr(VI)-induced hyperplasia occurs *subsequent* to DNA mutation (McCarroll *et al.*, 2010) Present & consider as alternative hypothesis; see relevant template in Meek & Klaunig (2010)

. According to U.S. EPA (2005), a sequence of key events implies that prevention of a given key event stops the progression of subsequent key events Counterfactual evidence that is heavily weighted in WOE - addressed in consistency & specificity - B/H considerations; consitency & specificity is not addressed in this manuscript. Thus for a mutagenic MOA, prevention of DNA mutation would prevent hyperplasia. Consistent with this idea, it has recently been shown that transgenic mice expressing mutant *Kras* alleles exhibit increased cell numbers in intestinal crypts (Feng *et al.*, 2011), but without the villous damage that is induced by Cr(VI). As discussed in the following section, increases in *Kras* mutation frequency were not observed in the intestines of mice exposed to ≤520 mg/l SDD for 90 days. Moreover, our data indicate that crypt cell proliferation is increased as early as 7 days of exposure (**Table 2**), a period of time that would appear too short to increase the incidence of crypt hyperplasia due to mutagenesis Unless mutation is an early influential key event. Thus, data collected from the target tissues of mice exposed to Cr(VI) in drinking water indicate that crypt hyperplasia is not preceded/caused by DNA mutation (see additional discussion in **Section 3.6**).

3.1.4. Key Event 4: Expansion of Spontaneous Mutations

Target tissue mutation data provide the strongest evidence for a mutagenic MOA (U.S. EPA, 2007). Thus, to assess the potential for Cr(VI) to induce mutations in the small intestine following oral exposure to Cr(VI), we measured *Kras* codon 12 GAT mutation frequency in the duodenal epithelium of mice exposed to Cr(VI) for 90 days (O'Brien *et al.*, in prep). *Kras* was selected because it is often mutated early in human intestinal tumors (Wicki *et al.*, 2010), and codon 12 GGT to GAT mutation is one of the most commonly reported mutations in human duodenal tumors (Nishiyama *et al.*, 2002; Sutter *et al.*, 1996; Wicki *et al.*, 2010; Younes *et al.*, 1997), and accounts for 12.6% of colon tumors in the COSMIC database (Forbes *et al.*, 2010). Further, *Kras* codon 12 GAT mutation frequency was increased in colon tissue of rats exposed to azoxymethane within one week of exposure (McKinzie and Parsons, 2011) and in rat lung 28 days after exposure to benzo(a)pyrene (BaP) (Meng *et al.*, 2010). Meng *et al.* (2010) also found that *Kras* codon 12 GAT mutation frequency increased with dose and with BaP-DNA adduct formation. Thus, *Kras* codon 12 is a likely candidate oncogene for early mutations in intestinal carcinogenesis following exposure to mutagenic carcinogens.

To ensure detection of increased mutation frequency at very low levels (~10^{-6}), allele-specific competitive blocker (ACB) PCR analysis was used to measure mutation frequency in scraped duodenal epithelium From? (O'Brien *et al.*, in prep). Seems that this should be published prior to the current manuscript on MOA

No treatment-related affect on *Kras* codon 12 GAT mutation frequency was observed even at the high Cr(VI) doses that were carcinogenic in the 2-year bioassay and induced a proliferative responses at 8 and 90 days (**Fig. 6**). These findings are particularly interesting when contrasted with Cr-DNA binding data from genomic DNA isolated from scraped duodenal epithelia From?. Although increases in Cr-DNA binding were observed with increased SDD dose, there was no correlation with *Kras* mutation frequency (O'Brien *et al.*, in prep). Moreover, we determined that the Cr-DNA isolation technique, while similar to that used in other published

studies (Zhitkovich, 2005), was likely confounded by *ex vivo* chromium binding. Specifically, the addition of chromium chloride to scraped intestinal tissue from untreated animals prior to DNA isolation resulted in Cr-DNA levels that were comparable to those in treated animals—indicating that chromium present in (or on) scraped duodenal cells from treated animals was likely binding to genomic DNA *ex vivo* during the DNA isolation assay (O'Brien *et al.*, in prep).

This observation, as well as high Cr-DNA binding levels in the non-target tissue of the mouse liver following drinking water exposure to SDD (O'Brien *et al.*, in prep), calls into question the reliability of Cr-DNA binding data, and furthermore, the data do not support the role of Cr-DNA adducts as pre-mutagenic lesions relevant in the MOA for small intestinal tumors O'Brien paper needs to be available. Meng *et al.* (2010) reported that *Kras* mutation frequency in lung tissue increased with increasing numbers of DNA adducts after a single i.p. administration of BaP. BaP metabolites bind to DNA and are converted into mutations during DNA replication (Meng et al. 2010). This acute mutation response and the known DNA reactivity of BaP metabolites are suggestive of a mutagenic MOA. In fact, in the case of mutagenicity caused by BaP, *Kras* mutation frequency is considered a more sensitive endpoint than DNA adducts due to amplification of and the growth advantage of mutated cells. Furthermore, *Kras* Codon 12 GAT mutation frequency has been postulated to represent a functional reporter (i.e. reporter of tumor initiation and/or progression) which is amplified with mutational loading that is not necessary specific to a chemically induced DNA lesion of *Kras* Codon 12 (Meng *et al.*, 2010; Parsons *et al.*, 2010). Thus, although *Kras* Codon 12 GAT is a single mutation in a single oncogene, DNA mutations in other oncogenes, if they occurred with Cr(VI) exposure, may be expected to amplify *Kras* mutations and be detected in the ACB-PCR assay. However, no such increases were observed—even at carcinogenic concentrations—and there was no concordance between Cr-DNA binding and *Kras* codon 12 mutation frequency. Moreover, the increase in crypt cells (**Fig. 4C**), if preceded/induced by mutations, would likely increase the number of target *Kras* mutations thereby ensuring detection if present. Together, these data indicate that Cr-DNA binding is not representative of a pre-mutagenic lesion in the mouse small intestine. Or is that critical evaluation of available data do not support a mutagenic mode of action for the tumours observed in the mouse small intestine

A relatively high background *Kras* codon 12 GAT mutation frequency was observed in the duodenal mucosa of B6C3F1 mice (10^{-2} to 10^{-3}) (O'Brien *et al.*, in prep), which is approximately 100-times higher that that measured in rat colon tissue (McKinzie and Parsons, 2011) and rat liver (McKinzie *et al.*, 2006). Because *Kras* codon 12 GAT mutation frequency is known to accumulate with age in the rat colon (McKinzie and Parsons, 2011), and there appears

to be a relatively high background *Kras* mutation frequency in the mouse duodenum (O'Brien *et al.*, in prep), spontaneous *Kras* codon 12 mutations would likely accumulate with time, especially under proliferative pressure.

The apparent absence of an effect on *Kras* codon 12 GAT mutation frequency is consistent with the fact that early tumors, metastases, and mortality were not observed in the NTP 2-year bioassay (NTP, 2008a), as well as the absence of preneoplastic (e.g. focal hyperplasia) or neoplastic lesions in any of the 90-day Cr(VI) drinking water studies (NTP, 2007; Thompson *et al.*, 2011b; Thompson *et al.*, 2012c) Addresses consistency in the B/H considerations . It is also consistent with the absence of cytogenetic damage in the duodenal crypts (see **Section 3.1.2.2**). These facts support that Cr(VI) is not acting via a mutagenic MOA, specifically where mutation is an early key event, in the mouse small intestine.

3.2. Additional Supporting Information

3.2.1. Toxicogenomic responses to Cr(VI) Having supportive data at several levels of biological organization supports WOE

Toxicogenomic data were collected in the duodenum and jejunum of rats and mice after 7 and 90 days of exposure to Cr(VI) in drinking water Dose, strains? . Data analysis included examination of individual differentially expressed genes, quantitative dose-response modeling, functional enrichment analysis, Transcription Factor Analysis, and Downstream Effects Analysis. The latter two analyses are relatively new Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com/) functions that predict activation or inactivation of transcription factors and biological functions based on the direction (up or down) of genes changes in a dataset of interest.

As described in **Section 3.1.1**, toxicokinetic data indicated that very little chromium was detected in the small intestinal tissue below 14 mg/l SDD (see **Fig. 3B**). At 14 mg/l SDD, the GSH/GSSG ratio was significantly decreased, but there were no apparent histopathological lesions (see **Table 2**). Consistent with these data, the number of gene changes observed in each treatment group correlated well with tissue dosimetry. At day 91, the number of transcripts significantly altered ±1.5-fold at 0.3, 4, and 14 mg/l SDD were each ≤76 genes, whereas at 60,

170 and 520 mg/l more than 1,800 genes were altered in each group. These findings lend additional support to Key Event 1, i.e. that SDD concentrations ≥60 mg/l exceeded reductive capacity of the gastrointestinal fluids. Below these concentrations, very few mRNA transcripts were altered by Cr(VI) exposure.

With respect to Key Event 2 (cytotoxicity), the toxicogenomics data clearly support alterations in redox signaling. As described in Kopec *et al*. (2012a), Nrf2 oxidative stress signaling and GSH metabolism were among the top five enriched canonical pathways at the lowest treatment concentrations at day 8. Transcription factor analysis in IPA also indicated activation of Nrf2 signaling pathways at ≥170 mg/l SDD day 91 (Kopec *et al.*, 2012b). As shown in **Fig. 5**, Gsr, Gpx1 and Gpx2 were increased significantly in the higher treatment groups. Expression of levels of *Gclc*, the rate-limiting enzyme in GSH synthesis, were also elevated in response to Cr(VI) exposure (Kopec *et al.*, 2012a), further indicating response to changes in redox. Interestingly, Nrf2 null mice demonstrate increased colonic mucosal injury and oxidative stress following exposure to? dextran sulfate sodium (Osburn *et al.*, 2007). Thus, the presence of mucosal injury and Nrf2 signaling following Cr(VI) exposure suggests chronic induction of oxidative stress.

As described in Kopec *et al*. (2012a), significant enrichment of canonical pathways related to DNA repair was not observed at day 91. However, there was enrichment for pathways related to BRCA1 and mismatch repair at 520 mg/l SDD at day 8, as well as enrichment for pathways related to nucleotide excision repair (NER) at ≥170 mg/l. Base excision repair (BER) is a major repair pathway of oxidative DNA damage and the heatmap in **Fig. 7A** clearly shows induction of genes involved in BER at day 8 (and less so at day 91). Both BER and NER repair oxidative DNA damage (Go and Jones, 2010; Klaunig *et al.*, 2010; Sedelnikova *et al.*, 2010). Although significant increases in 8-OHdG were not evident in duodenal tissues from mice exposed to Cr(VI) for 90 days (Thompson *et al.*, 2011b), it is conceivable that changes in genes related to redox signaling and DNA repair prevented 8-OHdG formation/detection. Other possibilities?

Oxidative stress and inflammation often occur concomitantly as the result of common mechanisms. Specifically, ROS can activate NF-κB signaling that results in cytokine formation and immune cell infiltration, while inflammatory responses in turn generate ROS (Klaunig *et al.*, 2010; Rahman and MacNee, 2000; Roberts *et al.*, 2009). Given the observed oxidative responses and presence of histiocytes (which can release TNFα) following Cr(VI) exposure, inflammatory responses were expected. However, as described in **Section 3.1.2.4**, cytokine levels were lower in Cr(VI)-treated mice than untreated mice. Transcript analysis corroborated the decrease in IL-1β and TNFα cytokine levels in the mouse duodenum (reported in Thompson *et al.*, 2011b), as evidenced by decreased expression at ≥ 60 mg/l SDD (Kopec *et al.*, 2012a).

Inflammatory responses intentionally increase ROS production in order to fight infections. Nrf2 signaling can inhibit inflammatory responses (Jung and Kwak, 2010; Kim *et al.*, 2010), perhaps as feedback control to limit oxidative damage to the host. Osburn et al. (2007) showed that TNFα and IL-1β were elevated in Nrf2 null mice (relative to wild type mice) following exposure to dextran sulfate sodium. Thus, the decrease in TNFα and IL-1β following Cr(VI) exposure is likely due to Nrf2 activation. Indeed, Nrf2 regulation of inflammation is mediated by increasing heme oxygenase-1 (HO-1) expression (Jung and Kwak, 2010; Kim *et al.*, 2010), and HO-1 transcript levels were increased following Cr(VI) exposure (Kopec *et al.*, 2012a). *In vitro* studies have also shown that Cr(VI) increases HO-1 mRNA levels, as well as inhibit NF-κB signaling (Joseph *et al.*, 2008; Shumilla *et al.*, 1999). Thus, Nrf2 activation is likely due to Cr(VI)-induced oxidative stress that also results in suppression of inflammatory signals, which likely explains the absence of histological evidence of chronic inflammation in the mouse small intestine.

With respect to Key Event 3 (cell proliferation), transcription factor analysis indicated increased MYC signaling (based on expression levels of *Myc* downstream targets) at ≥ 60 mg/l SDD at day 91 (Kopec *et al.*, 2012b), despite the fact that *Myc* itself was not significantly induced at these concentrations (Kopec *et al.*, 2012a). At day 8, *Myc* was increased greater than 4-fold at 520 mg/l (Kopec *et al.*, 2012a). Moreover, transcript levels of *Ki67*, a common marker of crypt cell proliferation (Itzkovitz *et al.*, 2012; Potten *et al.*, 1997), were significantly elevated at ≥170 mg/l SDD at both day 8 and 91. These findings are consistent with the observed increase in the numbers of crypt enterocytes (see **Fig. 4C**) and increased incidence of crypt hyperplasia (see **Table 2**).

Finally, Cr(VI)-induced gene changes in the mouse duodenum were compared to gene changes produced by known mutagenic and nonmutagenic carcinogens (Thompson *et al.*, 2012b). The genomic response induced by Cr(VI) in the mouse intestinal tissue at doses that induce pathology and were carcinogenic following prolonged exposure were more similar to that induced by nonmutagenic than by mutagenic carcinogens (**Fig. 7B**; (Thompson *et al.*, 2012b)). Although transcriptional changes do not directly reflect changes to DNA (i.e. Key Event 4 - expansion of spontaneous mutations), these genomic signature data together with the occurrence of oxidative stress, cell proliferation, and lack of *Kras* mutations are consistent with a MOA for Cr(VI) carcinogenesis in the mouse small intestine that involves prolonged increases in cell turnover in an oxidized environment rather than a mutagenic MOA.

3.2.2. *In vitro* micronucleus formation Requires fuller consideration of option for mutagenic MOA; see comments & template

Previous studies have examined the ability of Cr(VI) to induce MN formation *in vitro*. For example, Cr(VI) induced MN in human bronchial epithelial cells (Reynolds *et al.*, 2006), but

only after pre-incubation with ascorbate. Other studies have reported that Cr(VI) induces MN in human fibroblasts through an aneuploidic rather than clastogenic mechanism (Seoane and Dulout, 2001; Seoane *et al.*, 2002). As part of our Cr(VI) MOA research program, Cr(VI) genotoxicity was assessed by high content screening methods in the CHO-K1 cell line (Thompson *et al.*, 2012a), which is a cell model recommended by OECD (OECD, 2010).

Exposure to Cr(VI) reduced viability and the number of bi-nucleated cells at ≥32 μM Cr(VI)—suggesting treatment-induced cell cycle arrest; at these concentrations Cr(VI) increased MN frequency (**Fig. 8A**). In contrast, the positive control mitomycin-C (MMC) significantly ($p < 0.001$) increased MN frequency at concentrations that did not greatly reduce cell numbers or the percentage of bi-nucleated cells Data not shown?
. Assays in A549 (a human lung adenocarcinoma epithelial cell line) caused relatively small but statistically significant ($p < 0.05$) increases in cytotoxicity and decreases in the percentage of bi-nucleated cells at 3.2 μM. At this concentration, the frequency of MN in bi-nucleated cells was slightly increased from 1.47±0.50 to 2.12±0.41% (**Fig. 8B**). At higher Cr(VI) concentrations, cell death was extensive. The positive control MMC significantly increased MN frequency from 1.47±0.50 to 6.89±2.24%. These data indicate that Cr(VI) has very weak (or negligible) genotoxic potential at noncytotoxic doses, and are consistent with the negative *in vivo* MN results discussed in **Section 3.1.2.2**.

3.2.3. In vitro intestinal Caco-2 cell model

Caco-2 cells are a well-accepted model for studying intestinal absorption, metabolism and cytotoxicity (Meunier *et al.*, 1995; Natoli *et al.*, 2011; Rawlinson *et al.*, 2010; Sambruy *et al.*, 2001; Shah *et al.*, 2006; Smetanova *et al.*, 2011). When initially plated, Caco-2 cells are undifferentiated and proliferating—similar to crypt enterocytes. However, when grown to confluency (~21 days), the cells spontaneously differentiate and exhibit structural and molecular characteristics more similar to villous enterocytes. This *in vitro* model allows for differentiation

of responses between villous and crypt enterocytes of the small intestine, which is the target tissue for carcinogenicity.

Caco-2 cells were treated with Cr(VI) for 24 hours and DNA damage was assessed by nuclear staining intensity of 8-OHdG and phosphorylated histone variant H2AX (γ-H2AX) using high content imaging methods (Thompson *et al.*, 2012a). In undifferentiated Caco-2, Cr(VI) increased 8-OHdG staining at non-cytotoxic concentrations, but increased both 8-OHdG and γ-H2AX staining at cytotoxic concentrations (*viz.* ≥3 μM). In contrast, differentiated Caco-2 cells were more resistant to Cr(VI)-induced DNA damage, and cytotoxicity was observed at 100 μM (but not at ≤30 μM). These *in vitro* results predict that Cr(VI) exposure *in vivo* might induce more adverse effects in crypt enterocytes than villous enterocytes. However, the *in vivo* Cr(VI) studies described herein show that Cr(VI) causes toxicity to intestinal villi without apparent treatment related effects on the crypt enterocyte—specifically, no change in mitotic and apoptotic indexes, or formation of micronuclei within the crypt. In fact, the higher Cr(VI) exposures resulted in more (not fewer) enterocytes in the crypts (**Fig. 4C**). Taken together, the *in vitro* and *in vivo* findings suggest that crypt enterocytes were minimally (or not at all) exposed to Cr(VI) *in vivo*. In fact, the intestinal mucosa is covered by a protective mucus layer that serves as a barrier to bacteria, viruses, particles, and other toxins (DeSesso and Jacobson, 2001; Greaves, 2007; Talley, 2010). Coupled with the fact that crypt enterocytes do not function to absorb dietary nutrients, the lack of toxicity in the crypts is consistent with basic intestinal biology Biological plausibility - B/H considerations
. Thus, the tumors that arose following chronic Cr(VI) ingestion were, as suggested by the other data summarized herein, due to prolonged increased cell turnover in an oxidized environment rather than direct DNA reactivity.

3.3. Concordance of Dose-Response As per previous comments, dose-response and temporal concordance need to be considered together; data should be assimilated in template format in Meek & Klaunig, 2010; if hypothesis is supported, pattern of results make this clearly evident

Following 90 days of exposure, chromium levels in duodenal tissue significantly increased at ≥14 mg/l SDD, and GSH/GSSG ratio significantly decreased at this dose. Cytoplasmic vacuolization and other signs of villous toxicity significantly increased at ≥60 mg/l SDD, and crypt cell proliferation significantly increased at ≥170 mg/l SDD (Thompson *et al.*, 2011b). This dose concordance of key events can also be seen when plotted on a full dose-response scale (**Fig. 9**). For simplicity, Key event 1 in the MOA (i.e. absorption As per previous comments, key events are those for which there is evidence to support or refute weight for the hypothesis; absorption is rarely a key event) is not shown on the plot because it is an important pharmacokinetic event that also serves as a potential dose metric (e.g. μg Cr/g duodenum) on which to plot the subsequent key events. Instead, the change in GSH/GSSG ratio serves as both an indicator of uptake as well as an early (and reversible) biochemical component of intestinal

cytotoxicity. Cytoplasmic vacuolization, which may also be reversible, is another indicator of cytotoxicity and can be clearly seen to precede crypt cell proliferation (Key Event 3). **Fig. 9A-B** demonstrate the dose concordance among these key events in the duodenum at both day 8 and 91. The dotted line in the **Fig 9B** represents the duodenal adenoma incidence in female mice at termination of the NTP 2-year bioassay. The plot clearly supports a sequence of events beginning with decreased GSH/GSSG ratio, followed by cytoplasmic vacuolization and crypt cell proliferation, and ultimately resulting in adenoma formation (which typically precedes carcinoma formation). A similar pattern of key events occurs in both the duodenum and jejunum at day 91 (**Fig 9C**), which is consistent with finding that gene responses to Cr(VI) in the duodenum and proximal jejunum were generally quite similar (Kopec *et al.*, 2012a). Preliminary benchmark dose modeling of these endpoints support the sequence of key events (data not shown This needs to be included in the dose-response/temporal concordance table. Benchmark doses are much more illustrative of the point.
).

3.4. Temporal Association Again, best Illustrated by dose-response temporal concordance template

Key events that occur at the same time as tumors are not likely to contribute to tumor development (Boobis *et al.*, 2006). In this regard, oxidative stress, toxicity to intestinal villi and crypt hyperplasia can be seen as early as 7 days of exposure (**Table** Figurative representation here might be helpful - time line for key events, though table makes the same point **5**). After 90 days of exposure, damage to villi and crypt hyperplasia can be seen across multiple doses without tumors or preneoplastic lesions such as focal hyperplasia. Similar intestinal pathology (i.e. “diffuse hyperplasia”) was reported in the NTP 90-day bioassay (NTP, 2007). After two years of exposure, diffuse hyperplasia was observed in all treatment groups (i.e. ≥14 mg/l SDD) and tumors were observed at ≥172 mg/l SDD relative to concurrent controls (≥57 mg/l relative to historical controls). These findings suggest that in the drinking water studies conducted to date, ~~mice experienced~~ there was sustained increased cell proliferation and redox changes ~~that began~~ in mice within the first week of exposure. The fact that neither preneoplastic lesions nor tumors were observed in the 90-day studies and that tumors did not occur until 450 days or later (NTP, 2007; Thompson *et al.*, 2011b) suggests that it takes the majority of the two-year mouse lifespan

for these events to contribute to tumor formation. A highly proliferative tissue experiencing oxidative stress is expected to provide the ideal environment for ~~an~~ early mutation—yet no increase in *Kras* codon 12 GAT mutation frequency was observed with Cr(VI) administration. Observations were s~~S~~imilar ~~conclusions can be gained from~~in Cr(VI) bioassays of the rat lung, where tumor formation is nonlinear and dependent on tissue damage and inflammation Might be helpful to add some additional detail here on nature of these bioassays. Should be cited more in the context of biological plausibility (i.e., consistency across strains, sexes, tissues, etc.) (Beaver *et al.*, 2009; Glaser *et al.*, 1986; Steinhoff *et al.*, 1986).

3.5. Plausibility In addition to dose-response and temporal concordance and biological plausibility, consistency and specificity need to be considered - this includes counterfactual evidence - i.e., preventing a key event and consistency of dose-response among early and late key events (i..e, incidence of latter is necessarily less than that of former)

Cytotoxicity and subsequent sustained regenerative hyperplasia is a well known MOA for cancer (Ames *et al.*, 1993; Boobis *et al.*, 2009; Meek *et al.*, 2003; U.S. EPA, 2005); in fact it has been suggested that cytotoxic and proliferative effects observable by 13 weeks of exposure can be predictive of effects in 2-year bioassays (Cohen, 2010; Gaylor, 2005; Slikker *et al.*, 2004). The small intestine is one of the most proliferative tissues in the body and thus the additional proliferative pressure due to chronic high dose Cr(VI) exposure is likely to result in more spontaneous mutations.

Considering that Cr(VI) exposure did not increase mortality and the tumors observed in mice did not appear to metastasize (NTP, 2008a), the WOE supports that the increase in tumors at the termination of the NTP 2-year bioassay was the result of increased lifetime crypt proliferation as opposed to direct mutagenesis. The lack of preneoplastic focal hyperplasia further suggests that pockets of clonal expansion that might arise from a mutagen did not occur. This is consistent with the lack of aberrant nuclei formation or change in *Kras* mutation frequency in the small intestine (O'Brien *et al.*, in prep As per previous comments, this needs to be published before the MOA analysis
).

The Content of this paragraph is more appropriate to the human relevance analysis role of spontaneous mutation and the relatively long time-to-tumors in mice is roughly similar to the likely roles of these events in humans. In sporadic colon cancer in humans, tumor initiation occurs after the third decade of life, progresses for 10-20 years, and results in carcinoma at a mean age of ~68 years (Grady and Carethers, 2008). In the context of historically shorter human lifespans, the intestine has likely evolved to withstand carcinogenesis over the normal human lifespan What does this mean?
. Genetic disorders, however, that result in either accelerated initiation or accelerated progression can lead to earlier tumor development (Grady and Carethers, 2008). In this colon cancer paradigm, increasing the number of cell divisions within the intestinal crypt over a lifetime due to chronic intestinal wounding would shift the normal progression of spontaneous tumors leftward resulting in earlier tumor formation. In this regard, the tumors observed in the NTP 2-year bioassay were the same as those that occur spontaneously, but only rarely in untreated animals (NTP, 2008a).

3.5.1. Similarities with other chemicals that have caused small intestinal tumors in rodents in two-year bioassays

NTP (2008a) noted that captan (NCI, 1977) was the only other chemical found to induce both benign and malignant intestinal neoplasms of epithelial origin in B6C3F1 mice in carcinogenicity studies conducted by NTP. Like Cr(VI), captan, and the structurally-similar chemical folpet, ~~have been shown to exhibit~~are mutagenic ~~activity~~ *in vitro*, but evidence for *in vivo* mutagenicity is equivocal or negative (Arce *et al.*, 2010; Bernard and Gordon, 2000). Captan and folpet induce similar phenotypes as Cr(VI) including cytotoxicity to villi, blunting of villi, regenerative crypt cell proliferation, and adenomas and carcinomas in the mouse duodenum and, to a lesser extent, jejunum (Cohen *et al.*, 2010). Furthermore, captan and folpet also do not induce intestinal tumors in rats. Mechanistically, captan and folpet induce toxicity by binding to thiols in GSH and proteins; and folpet ~~has been shown to~~ significantly depletes duodenal GSH within the first few hours of exposure and then significantly increases GSH by 24 hours relative to untreated animals (Arce *et al.*, 2010; Cohen *et al.*, 2010). Although GSH levels were not measured in the intestine within 24 hrs of exposure to Cr(VI), Cr(VI) elicited dose-dependent increases in intestinal GSH at both days 8 and ~~day~~ 91. Thus it seems likely Seemingly, this would have been pretty easy to ascertain. Unclear why days 8 and 91 were selected as those for examination in Thompson et al. (2011b) since at high doses, early key events often occur within one day.

that Cr(VI) would also acutely deplete GSH levels before cells have sufficient time to adapt and synthesize more GSH.

In This paragraph has little to do with biological plausibility of the hypothesized MOA. It should be deleted here & could, perhaps, be included in the discussion.

2004, the U.S. EPA changed their cancer classification of captan from "a probable human carcinogen" (Category B) to "not likely" after an independent peer-review concluded that captan acted through a non-mutagenic MOA that "required prolonged irritation of the duodenal villi as the initial key event" (Gordon, 2007; U.S. EPA, 2004). The similar effects induced by captan, folpet, and Cr(VI) make it plausible that they share a common non-mutagenic MOA that likely involves point of contact cytotoxicity in the villi followed by sustained proliferative pressure on crypt cells to regenerate the mucosa. Over a lifetime of exposure to such cytotoxic concentrations, there is an increased risk of carcinogenesis.

3.6. Alternative MOAs This really should be addressed at the outset, rather than as an afterthought. This has been noted in evolution of the MOA/HR framework (see Meek and Klaunig, 2010).

Alternative MOAs include mitogenic and mutagenic MOAs Weight of evidence for a mutagenic mode of action should be much more fulsomely considered. This would need to include consideration of the pattern of genotoxicity of chromium VI in in vitro and in vivo studies. Format of presentation of information that is extremely helpful in a mode of action context is that offered by GAP profiles. These contrast positive and negative results in phylogenetic order, taking into account dose-response, both in vitro and in vivo. The pattern of these results is critical in determining whether Cr VI is mutagenic principally at doses which are also cytotoxic. This would lend support to the preferred hypothesized MOA and would also contribute extensively to more meaningful consideration of weight of evidence for a mutagenic mode of action.

. With regard to the former, there is no evidence to suggest that Cr(VI) stimulates crypt hyperplasia in the absence of cytotoxicity, as hyperplasia occurs subsequent to villous cytotoxicity in both dose and time. With regard to the latter, prior to the conduct of the studies described herein, some investigators hypothesized that the intestinal tumors observed in mice arose by a mutagenic MOA—based primarily on data from non-target tissues and *in vitro* systems (McCarroll *et al.*, 2010). The U.S. EPA has proposed a general framework for assessing a mutagenic MOA in their draft guidance, *Framework for Detecting a Mutagenic Mode of Action for Carcinogenicity* (U.S. EPA, 2007). Although still in draft form, the framework nevertheless provides insightful approaches for determining whether a chemical acts through a mutagenic MOA. First and foremost, the framework explicitly states the critical question, "*Is mutation an early key event in the chemical's induction of cancer?*" As part of determining a mutagenic

MOA, one must a) establish whether a chemical has mutagenic properties; b) establish whether mutagenicity is relevant to the MOA in the tissue of interest by considering dose-response concordance, temporal concordance, and plausibility; and c) consider alternative MOAs.

As shown in **Table 6**, data from the small intestine do not support dose-response concordance, temporal concordance, or plausibility associated with a mutagenic MOA. With exposure to increasing concentrations of Cr(VI), there was no evidence of increased cytogenetic damage in crypt enterocytes or *Kras* mutations; in contrast, cytotoxicity, oxidative stress, and crypt hyperplasia were all increased in a dose-dependent manner. With increased duration of exposure to Cr(VI), there was no evidence of increased cytogenetic damage in crypt enterocytes or incidence of preneoplastic lesions, whereas oxidative stress, cytotoxicity, and crypt hyperplasia were all worse (or more prevalent) at day 91 relative to day 8. Thus, the hypothesis that mutation is an early initiating key event in the intestinal carcinogenesis is inconsistent with the absence of cytogenetic damage, *Kras* mutations, or preneoplastic lesions (NTP, 2007; NTP, 2008a; Thompson *et al.*, 2011b), as well as the late tumor onset and lack of increased mortality (NTP, 2008a) While this is extremely helpful information in supporting the hypothesized MOA, given the degree of controversy around CrVI, it seems important to also fulsomely integrate all of the genetox data on Cr into the analysis, as mentioned above. If mutagenic only at cytotoxic concentrations, this information actually supports the hypothesized MOA.

Note also that in the draft EPA guidance for a mutagenic mode of action, evidence or not of mutagenicity in the target tissue is given highest weight in a hierarchical approach. There was a very nice figure in the draft guidance that illustrated this.

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In addition, most *in vivo* micronucleus studies are conducted in proliferative tissues like bone marrow, skin, and intestine because proliferation facilitates the detection of genotoxicity (Morita *et al.*, 2011). Because the small intestine is a highly proliferative tissue, evidence of DNA damage and mutation would be readily apparent if Cr(VI) was acting via genotoxicity or mutagenicity.

U.S. EPA (2007) provides a list of characteristics typical of mutagens; as shown in **Table 7**, data from the small intestines indicate that Cr(VI) does not exhibit these characteristics. For example, mutations found in genes that are associated with carcinogenesis (e.g. p53) and in the presence of low cytotoxicity increase the WOE for a mutagenic MOA; however, mutations in *Kras* were not observed, despite the presence of tissue damage. It is also stated that tumor responses generally occur early in chronic studies (e.g. within 52 weeks Argues against biological plausibility

), yet the tumors in the NTP study were observed late in the study and did not increase mortality (NTP, 2008a).

Boobis *et al.* (2009) have also outlined general key events for chemicals with a mutagenic MOA (**Table 7** See also Preston & Williams, 2005; J.R. Preston, G.M. Williams, DNA-reactive Carcinogens: Mode of Action and Human Cancer Hazard, Crit. Rev.Toxicol, 35 (2005) 673-683.

). Notably, clonal expansion of mutated cells often increases mutations in other key genes and leads to preneoplastic lesions. Thus, even if Cr(VI) did not specifically "target" *Kras*, a general increase in mutations would likely lead to increases in additional mutations in *Kras* codon 12 GAT which was measured using the highly sensitive ACB-PCR assay. Furthermore, the lack of preneoplastic lesions suggests that clonal expansion of cells with growth advantages (such as can be conferred by *Kras* mutations) was~~ere~~ not present. Importantly, Boobis *et al.* state that, "...carcinogens are considered in two clearly distinguished groups based upon their MOA—DNA-reactive (mutagenic) and non-DNA-reactive (e.g. receptor-mediated, mitogenic, cytotoxic, oxidative stress)." The evidence herein supports involvement of both cytotoxicity and oxidative stress as key events in the MOA.

In summary, the DNA reactivity of chromium is well established (Chiu *et al.*, 2010; Nickens *et al.*, 2010; O'Brien *et al.*, 2003; Zhitkovich, 2011; Zhitkovich, 2005). Known and/or suspected forms of DNA damage associated with Cr(VI) exposure include DNA adducts, single and double strand DNA breaks, inter- and intra-strand crosslinks, oxidative DNA damage, and replication blockage. However, as discussed in the Eastern Research Group peer-review panel comments to the Draft EPA Toxicological Review for Hexavalent Chromium (ERG, 2011), most of these many genotoxic lesions are not likely to be mutagenic, and the WOE supports that Cr(VI) is, in fact, a very weak mutagen. Further, it is important to note that much of this evidence comes from *in vitro* systems, often comprising acellular *in vitro* reactions and possibly *ex vivo* Cr-DNA adduct formation This doesn't address the weight of evidence for genotoxicity in a form that is helpful for mode of action analysis. See comments above regarding need to consider "pattern" of results including types of responses, phylogenetic order and in particular, dose response

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3.7. Potentially Susceptible Subpopulations This is best addressed in the human relevance or concordance analysis including explicit consideration of not only qualitative aspects but quantitative implications.

See concordance tables in any of the MOA/HR papers

In fasting conditions, gastric reduction of Cr(VI) to Cr(III) is more rapid at low pH (Kirman et al. in press). This suggests that individuals with higher gastric pH might reduce Cr(VI) less efficiently, and as a result, have a higher tissue dose of Cr(VI) in the small intestine upon ingestion, relative to normal humans. This potential susceptibility is due to pharmacokinetic differences, rather than dynamic differences. Individuals with altered gastric secretion (e.g. achlorhydria or hypochlorhydria) have increased Cr(VI) absorption, presumably due to impaired gastric reduction of Cr(VI) to Cr(III) (Donaldson and Barreras, 1966). Individuals taking proton pump inhibitors (PPIs) have an intragastric pH range of 4 to 10. This range is higher than that in healthy adults, who have fasting gastric pH of around 1.5 and postprandial gastric pH of around 5 (Amendola *et al.*, 2007; Dressman *et al.*, 1990; Gardner *et al.*, 2004). Neonates, defined as infants less than 4 weeks old, have fasting gastric pH values of 3-4 and a postprandial gastric pH of 7 (Nagita *et al.*, 1996). Thus, neonates and individuals taking PPIs are likely to have higher gastric pH than the average adult. Notably, gastric pH of neonates will decrease to a range of 1 to 3 within 1 day post-birth, but their gastric acid production is poorly maintained and low compared to that of adults (H+ per hour, 0.15 mmol/10 kg bodyweight in neonates vs. 2 mmol/10 kg in adults) (Koren, 1997). However, by age at 2 to 3 years, gastric production reaches to that of adult capacity, and the gastric pH consistent to that of adults (Koren, 1997; Nagita *et al.*, 1996). It is important to consider, however, that ~~the pH of the stomach contents of~~ rats and mice of the same strain and in fed conditions, on the same chow as the animals in the NTP study, have a pH of 4.25 to 4.5 (Proctor *et al.*, 2012). This suggests that individuals who have a higher pH stomach

than healthy adults, have stomach conditions of similar pH to that of the rodents in the 2-year bioassay. Furthermore, the capacity of human stomach fluid to reduce Cr(VI) to Cr(III) increases substantially in fed conditions when gastric pH is higher relative to fasted conditions because of the excretion of gastric acid and enzymes associated with anticipation of and consumption of food. In fact, De Flora et al. (1987) evaluated 1 healthy volunteer and 16 duodenal ulcer patients on acid blockers and found maximum peaks in stomach reduction capacity post-prandially of 40-60 μg/l, whereas Cr(VI) reduction during the fasted state was less pronounced (< 20 μg/L) for all monitored subjects.

Individuals with conditions that include inflammation of the bowl are at increased risk of gastrointestinal cancer, and may be more sensitive to Cr(VI) exposure at doses are sufficient to cause cytotoxicity if villi. For example, people with celiac disease are at risk for adenocarcinomas (Green and Cellier, 2007), in part due to increased oxidative stress (Stojiljkovic *et al.*, 2012). Chronic infection with *H. pylori* can reduce gastric acid and enzyme secretions, which promotes bacterial growth, resulting in inflammation, epithelial cell proliferation, as well as oxidative stress (Kumar *et al.*, 2005). Individuals with *H. pylori* infection are at a 3- to 6-fold increased risk of stomach cancer as compared to non-infected individuals (EUROGAST, 1993). Prevalence of *H. pylori* infection in under developed countries is greatest, and rates of infection among older Japanese populations, as well as the rates of gastric cancer, in Japan have been elevated as compared to western countries (EUROGAST, 1993). Thus, if Cr(VI) increases the risk of stomach and intestinal cancer in individuals infected with *H. pylori*, it might be observable in Japanese cohorts of workers exposed to Cr(VI). However, a recent meta analysis of Cr(VI) exposed workers that assessed gastrointestinal tract cancers included subgroup analyses of studies by region including Japanese cohorts (Gatto *et al.*, 2010). In the meta-analysis, no

increase in stomach cancer (meta-SMR= 0.89 for stomach cancer) was found for the Japanese workers, and none of the studies of Japanese workers reported on small intestinal cancers What was the power of the analysis?. Although workers are exposed primarily by inhalation, a fraction of the inhaled dose is swallowed resulting in low-level exposure that may be comparable to drinking water exposures around the MCL (Gatto *et al.*, 2010). As the MOA for Cr(VI)-induced intestinal carcinogenesis requires long-term cytotoxicity and a regenerative pressure, enhanced susceptibility by *H. pylori* infection, and other inflammatory bowl conditions, is not expected to increase risk of cancer at non-cytotoxic exposures.

As discussed for Key Event 1, the gastrointestinal tract is a dynamic system, and the rate of Cr(VI) loading to the small intestine is dependent on the rate of Cr(VI) reduction in the lumen, stomach emptying, gastric acid production, production of other reducing agents such as cysteine in the small intestine, food consumption and tissue absorption. Hence, PBPK models are needed to extrapolate between species, across dose, and may also be useful for assessing the toxicokinetics of potentially susceptible populations for use in risk assessment This should be addressed in the human relevance/concordance analysis.

. Such an approach is a considerable improvement over the use of body-weight scaling to extrapolate between species and application of default uncertainty factors to account for sensitive subpopulations.

3.8. Data Gaps In MOA/HR analysis, the objective is not to identify data gaps, but those considered critically important to the analysis. What about the absence of counterfactual evidence? What about data for earlier time points?

As with all datasets, there are some gaps in our knowledge of the sequence of key events. However, these gaps represent more of a deficiency in the detailed mechanism of action as opposed to the more general key events (i.e. MOA) necessary for human health risk assessment (U.S. EPA, 2005). The ability to distinguish between Cr(III) and Cr(VI) in biological samples could greatly enhance detailed understanding of Cr(VI) toxicity. To date, however, these forms

cannot be readily distinguished. In addition to measures of the GSH/GSSG ratio, alternative measures of oxidative status, such as cysteine/cystine and NAD^+/NADH ratios and lipid oxidation, could be informative. Further, measures of Cr-DNA adducts that can be reliably measured *in vivo* and differentiated between crypt and villi enterocytes could be helpful. With regard to *in vivo* mutation analysis, access to paraffin embedded tumor samples from the NTP 2-year bioassay would allow for DNA extraction and determination of the specific mutations, if any, that might be more prevalent in Cr(VI)-induced intestinal tumors. Such knowledge would provide a means for more refined focus in the mutation analysis conducted in shorter-term assays; however, attempts to obtain this information and/or access these tissues for analysis have not been successful. Further, some studies have shown epigenetic changes in DNA methylation following chromium exposure (Klein *et al.*, 2002; Sun *et al.*, 2009; Takahashi *et al.*, 2005) and thus assessment of genomic methylation status might also provide additional useful information on chromium toxicity.

One of the goals of the 90-day MOA studies was to collect similar data in both mice and rats to better understand the species differences in tumor development. The previous NTP studies did not observe diffuse hyperplasia or tumors in the intestines of rats (NTP, 2007; NTP, 2008a). In contrast, Thompson *et al.* (2012c Strains & conditions of exposure still unclear.) reported that rats exhibited similar intestinal lesions as mice, *viz*. apoptosis in the villi and crypt hyperplasia at ≥170 mg/l SDD. The reason for these differences in the rat studies likely relates to water intake, which was nearly 2-fold higher in Thompson *et al.* (2012c), but consistent with published intake values (U.S.EPA, 1988). These findings indicate that with increased Cr(VI) exposure, rats begin to exhibit non-neoplastic lesions similar to those observed in mice, and suggest that dosimetry is a major factor in the intestinal carcinogenesis of Cr(VI).

4. HUMAN RELEVANCE

Human relevance addresses three fundamental questions: 1) is the WOE sufficient to establish the MOA in animals, 2) are the key events plausible in humans, and 3) are the key events

plausible in humans after accounting for pharmacokinetics and pharmacodynamics (Boobis *et al.*, 2006; Meek *et al.*, 2003). In our initial MOA assessment it was concluded that the WOE to establish the MOA in animals was not sufficient and that additional data were needed, in particular from the target tissues (Thompson *et al.*, 2011a). As such, an effort was undertaken to gather data necessary to fill the data gaps identified and the results of this research effort have been described in detail in the preceding sections of this manuscript. Based on findings summarized herein, we conclude that the MOA in mice likely involves the key events described in **Fig. 2**. These key events are potentially relevant to humans (provided that dose is sufficient), as the basic principles of Cr(VI) chemistry and reduction and intestinal structure are similar in humans and rodents. As shown in the human relevance framework diagram in **Fig. 10**, the remaining question is whether kinetic differences between humans and rodents indicate that the MOA is either likely or unlikely. The answer to this question is likely yes; however, given the lack of epidemiological evidence for increase in the risk of intestinal cancers in humans (Gatto *et al.*, 2010), it is critical that human pharmacokinetics be considered as well as typical environmental exposure levels I strongly suggest to conduct the tabular concordance analysis; it would very clearly indicate the nature of the information which would be informative in extrapolating to humans and would undoubtedly lead to additional development of this section.
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To address whether the pharmacokinetics of Cr(VI) differ sufficiently between mice and humans, physiologically based pharmacokinetic models have been developed to assess the disposition of Cr(VI) in the gastrointestinal tracts of mice, rats and humans (Kirman *et al.*, in press). The Cr(VI) concentrations that induced tumors in mice (≥ 20 mg/l Cr(VI)) were orders of magnitude higher than typical drinking water exposures, which average 0.001 to 0.005 mg Cr(VI)/l. Thus, human pharmacokinetics (or dynamics) would have to differ substantially from mice in order for typical exposures to pose a cancer risk. In this regard, populations that have been exposed to Cr(VI) do not appear to have elevated risks of intestinal cancer or elevated cancer rates overall (Fryzek *et al.*, 2001; Gatto *et al.*, 2010; Morgan, 2011).

5. DISCUSSION

MOA analysis is a key element in human health risk assessment (Boobis *et al.*, 2006; Boobis *et al.*, 2009; Meek *et al.*, 2003; Seed *et al.*, 2005; U.S. EPA, 2005). When a general MOA has been well-established, it "lowers the barrier" of acceptance when such a MOA is proposed for another chemical/tumor site of interest (Boobis *et al.*, 2006). In this regard, Cr(VI) shares similar toxicological and carcinogenic characteristics as captan and folpet,

which have previously been determined to act via a cytotoxic nonmutagenic MOA (Arce *et al.*, 2010; Cohen *et al.*, 2010; Gordon, 2007; U.S. EPA, 2004). The relative rarity of intestinal tumors means that risk assessors have had comparatively less experience analyzing MOAs for carcinogenesis of the small intestine. In fact, 80-90% of cancers observed in rodent bioassays occur in eight tissues/systems, and the small intestine is not among these (Gold *et al.*, 2001). Even though Cr(VI) and captan/folpet do not share structural similarity, their toxicological and carcinogenic phenotypes begin to establish a general MOA for intestinal neoplasms induced by environmental chemicals that involves chronic wounding and healing of the intestinal mucosa.

The newly developed target tissue-specific MOA data provides a substantially stronger and more scientifically robust basis for assessing the MOA for Cr(VI)-induced intestinal carcinogenesis than has been available previously. The WOE from these studies strongly support that Cr(VI) acts by a cytotoxic MOA in the development of rodent small intestinal tumors. Need to qualify this conclusion, highlighting uncertainties & then weighting them against WOE based on the Bradford Hill considerations

It is important to recognize that this MOA is specific to the small intestine, and although may be informative to other tissues, it is not necessarily specific to all tumors caused by Cr(VI). Chemicals may act by multiple MOAs This term is misleading. There is one mode of action leading to a particular (adverse) effect in a single tissue at relevant dose. While there may be competing pathways in early stages, they converge at later stages to produce the effect. , at different tumor sites and potentially at different doses; however the MOA that is specific to the tumor-response used for risk assessment is critical for low-dose extrapolation. Because mouse small intestinal cancers have been used as the basis of a number of quantitative risk assessments for ingested Cr(VI) (McCarroll *et al.*, 2010; OEHHA, 2009; Stern, 2010; U.S. EPA, 2010), these MOA findings specifically inform the most appropriate approach for low-dose extrapolations for these and other new risk assessments on oral exposure to Cr(VI) As per previous comment, need to be precise about how it might inform low dose extrapolation - ie, principally in the context of preventing an earlier key event which should be protective for tumours.

. To further improve the information available for risk assessment, newly developed rodent and human PBPK models will allow for extrapolation of dose across species and wide dose ranges, which will be critical for any human health risk assessment conducted for oral exposure to Cr(VI Again, this should be addressed in the concordance analysis for human relevance

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Tables

Table 1. Incidence of Intestinal Lesions in Female Rodents of NTP (2008a) Bioassay[a]

mg/l SDD:	0	14	57	172	516
mg/l Cr(VI):	0	5	20	60	180
Duodenum					
histiocytic infiltration	0/50	0/50	4/50	33/50**	40/50**
(rat)[b]	(0/46)	(0/49)	(1/48)	(30/46)**	(47/50)**
focal hyperplasia	0/50	0/50	1/50	2/50	0/50
diffuse hyperplasia	0/50	16/50**	35/50**	31/50**	42/50**
adenoma	0/50	0/50	2/50[e]	13/50***	12/50***
carcinoma	0/50	0/50	0/50	1/50[f]	6/50*
Jejunum[c]					
histiocytic infiltration	0/50	0/50	0/50	2/50	8/50*
diffuse hyperplasia	0/50	2/50	1/50	0/50	8/50**
adenoma	0/50	1/50[e]	0/50	2/50[e]	5/50*
carcinoma	1/50	0/50	2/50[e]	2/50[e]	1/50
Combined Tumors in Small Intestine[d]	1/50	1/50	4/50[e]	17/50***	22/50***

[a]Results for male mice are not shown (but were similar to female mice).

[b]Histiocytic infiltration was the only histopathological response reported in female (shown) and male (not shown) rat duodenum

[c]No lesions were reported in the female or male rat jejunum

[d]No intestinal tumors were observed in female or male rats

[e]Exceeded historical control range for all routes of exposure

[f]Exceeded historical control range for drinking water studies

*$p \leq 0.05$, **$p \leq 0.01$, or ***$p \leq 0.001$ compared to concurrent control by poly-3 test

Source: detailed results and statistical analyses can be found in NTP (2008a) and Stout *et al.* (2009).

Table 2: Summary of Mouse (Strain?) Intestinal Histopathology (This type of presentation not so helpful. Need combined consideration of dose-response and temporal concordance with BMDs, wherever possible)

	Duodenum[a]			Jejunum[a]		
mg/l SDD:	**60**	**170**	**520**	**60**	**170**	**520**
Day 8						
Villous cytoplasmic vacuolization	0/5	3/5	5/5	0/5	1/5	1/5
Villous Atrophy	0/5	0/5	3/5	0/5	0/5	0/5
Crypt Hyperplasia	0/5	0/5	3/5	0/5	0/5	0/5
Day 91						
Villous cytoplasmic vacuolization	5/10	10/10	7/10	4/10	8/10	5/10
Villous Atrophy	0/10	1/10	5/10	0/10	3/10	4/10
Apoptosis	0/10	3/10	4/10	0/10	0/10	2/10
Crypt Hyperplasia	0/10	9/10	9/10	0/10	5/10	7/10
Histiocytic infiltration[*]	1/10	10/10	10/10	0/10	8/10	9/10

[a]Intestinal lesions were not observed at 0.3, 4, or 14 mg/l SDD.
[*]Not observed at day 8.
Source: Thompson *et al.* (2011b)

Table 3. Total Number of Aberrant Nuclei in Fully Intact Duodenal Crypts (Ok, but need to consider this in the context of the totality of the data supporting a mutagenic MOA)

Dose	Mitotic Index (%)[a]			Apoptotic Index (%)[b]			Karryorhectic Nuclei	Micronuclei	Cells Evaluated[c]
0	1.43	±	1.17	0.47	±	0.22	0	0	1921
0.3	2.28	±	1.07	1	±	0.47	4	0	1707
4	2.36	±	0.684	0.5	±	0.41	0	0	1825
14	3.08	±	0.46	0.7	±	0.32	0	0	1420[d]
60	2.43	±	0.76	0.5	±	0.36	0	0	2386
170	2.72	±	0.97	0.84	±	0.96	0	0	2746
520	2.11	±	1.09	0.67	±	0.33	0	0	3194

[a]AI = total apoptotic nuclei ÷ total nuclei (in 10 crypts)

[b]MI = total apoptotic nuclei ÷ total nuclei (in 10 crypts)

[c]total number of cells evaluated (10 crypts per animal; n=5 animals per treatment group)

[d]n=4 animals

note: there were no statistically significant differences between treatment and control groups (ANOVA followed by Dunnett's test)

Table 4. Total Number of Aberrant Nuclei in Duodenal Mucosal Sections (Reference?)

	SDD, mg/L						
Pathology	0	0.3	4	14	60	170	520
Day 8							
Karyorrhectic							
crypt	0	0	0	0	0	0	1
villi	0	0	0	0	2	3[a]	9[a]
Micronuclei							
crypt	1	0	0	0	0	0	0
villi	1	3	5	2	1	6	11[a]
Day 91							
Karyorrhectic							
crypt	0	1	0	0	1	1	0
villi	0	1	0	0	5[a]	6[a]	25[a]
Micronuclei							
crypt	2	2	1	1	0	0	0
villi	1	1	2	0	2	9[a]	9[a]

Values represent total number of aberrant nuclei in 15 sections (3 slides per animal; 5 animals per treatment group, except only 4 animals for 14 mg/L SDD treatment group at day 91).

[a]Significantly different ($p < 0.05$) from control group by Poisson regression

Table 5. Temporal Sequence (As per comment above. Need to assimilate temporal and D/R concordance in tabular format)

Time	Effect
7 Days	Decreased GSH/GSSG ratio (Thompson *et al.,* 2011b) Nrf2 activation/oxidative stress (Kopec *et al.,* 2012) Cytoplasmic vacuolization (Thompson *et al.,* 2011b) Crypt hyperplasia (Thompson *et al.,* 2011b) Absence of aberrant nuclei in duodenal crypts (O'Brien *et al.*, in prep) Transcript changes consistent with nonmutagenic MOA (Thompson *et al.*, 2012b)
90 days	Diffuse hyperplasia (NTP, 2007) Decreased GSH/GSSG ratio (Thompson *et al.,* 2011b) Nrf2 activation (Kopec *et al.,* 2012) Cytoplasmic vacuolization (Thompson *et al.,* 2011b) Crypt hyperplasia (Thompson *et al.,* 2011b) Absence of aberrant nuclei in duodenal crypts (O'Brien *et al.*, in prep) No change in *Kras* mutation (O'Brien *et al.*, in prep)
2 years	Diffuse hyperplasia (NTP, 2008a) Adenomas and carcinomas (NTP, 2008a)

Table 6. U.S. EPA Mutagenic Framework (U.S. EPA, 2007) and Application to Cr(VI) in the Small Intestine

Steps	Evidence
1) Establish whether chemical has mutagenic activity	Cr(VI) is a weak mutagen
2) Establish whether mutagenicity is relevant to MOA in target tissue (follow MOA framework in U.S. EPA (2005) Cancer Guidelines)	
• Dose-response concordance	No evidence of increased crypt cytogenetic damage or *Kras* mutations with increased dose
• Temporal concordance	Villous cytotoxicity and crypt hyperplasia evident as early as day 8, without evidence crypt cytogenetic damage (at day 8 or day 91) or *Kras* mutations at day 91
• Plausibility	Early mutation in highly proliferative intestinal mucosa is not consistent with absence of cytogenetic damage, absence of *Kras* mutations, absence of preneoplastic lesions (e.g. focal hyperplasia) (NTP, 2007, 2008a; Thompson *et al.*, 2011b), late tumor onset (NTP, 2008a), and lack of affect("effect") on survival (relevance of lack of impact on survival not well described) (NTP, 2008a).
3) Consider alternative MOAs	Described herein

Table 7. Select Properties of Mutagenicity as Key Events and Application to the Cr(VI) in the Small Intestine

U.S. EPA (2007)	Cr(VI) Study Results
Mutations seen in the presence of low cytotoxicity increase WOE	Mutations not seen, even in the presence of toxicity.
Mutations in genes that affect carcinogenesis increase WOE.	No mutations in *Kras* observed.
Direct DNA reactivity	Equivocal.
The target cell/tissue is exposed to the ultimate DNA reactive chemical.	Data suggest that intestinal crypts were not directly exposed.
DNA of target cell is damaged	No evidence of cytogenetic damage.
Tumors observed at multiple sites, in multiple species.	Only one tumor location in each species, despite presence of Cr in multiple tissues.
Tumor response generally occur early in chronic study (e.g. within 52 weeks).	Tumors in small intestine or mice and oral mucosa of rats did not occur early, and were not associated with lethality or even metastases.
Boobis *et al.* (2009)	Cr(VI) Study Results
Exposure of target cells to ultimate DNA reactive and mutagenic species—with or without metabolism	Evidence does not support that Cr(VI) is reaching the target cells (i.e. crypts)
Reaction with DNA in target cells to produce DNA damage	No evidence of DNA damage, as evidenced by negative results for MN, KN, AI, and MI in crypts.
Misreplication on a damaged DNA template or misrepair of DNA damage	No evidence of DNA damage, as evidenced by negative results for MN, KN, AI, and MI in crypts.

Mutations in critical genes in replicating target cells	No evidence of increased *Kras* mutation frequency.
Clonal expansion leads to further mutations in critical genes.	No evidence of increased *Kras* mutation frequency due to Cr or clonal expansion.
Imbalanced and uncontrolled clonal growth of mutant cells may lead to preneoplastic lesions.	No evidence of preneoplastic lesions.

Figures

Fig. 1

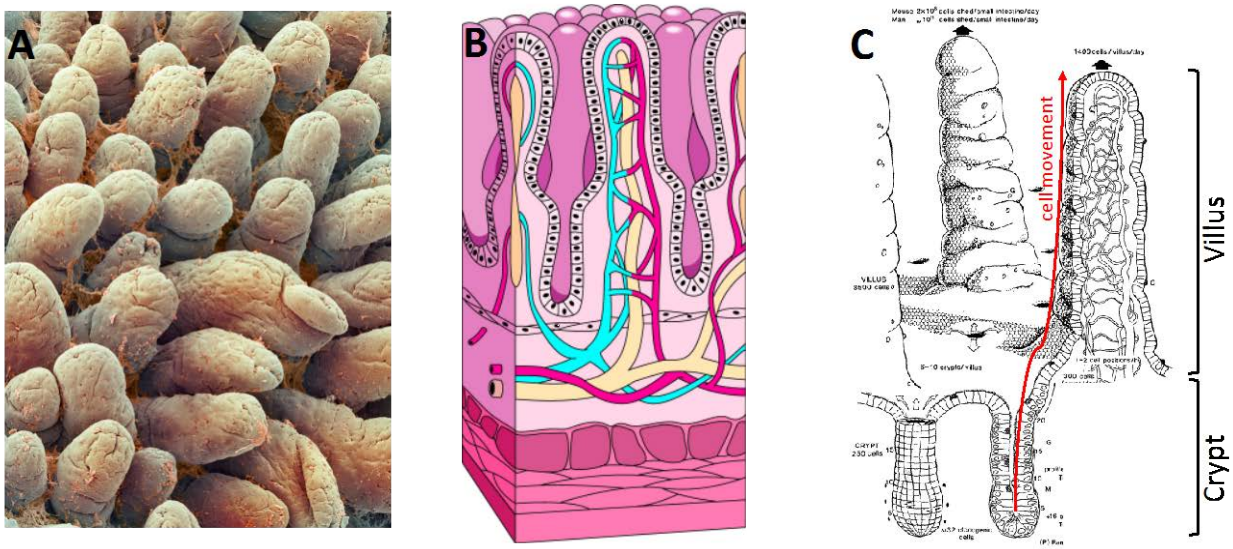

Figure 1. Intestinal structure. (A) TEM of human duodenum showing villi. (B) Diagram of small intestine showing crypts, villi, and vasculature. (C) Diagram showing migration of enterocytes from the crypts below the luminal surface to the tips of villi. Need permissions.

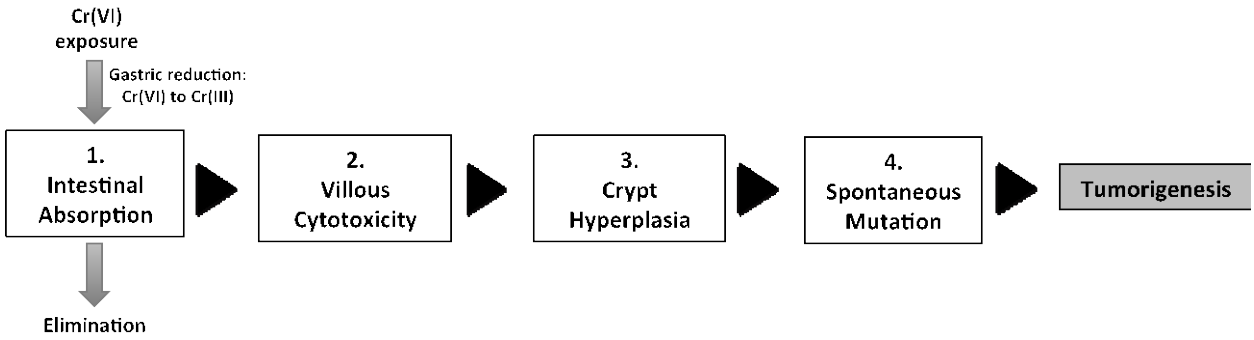

Figure 2. Proposed MOA for Cr(VI) carcinogenesis in the small intestine.

Fig. 3.

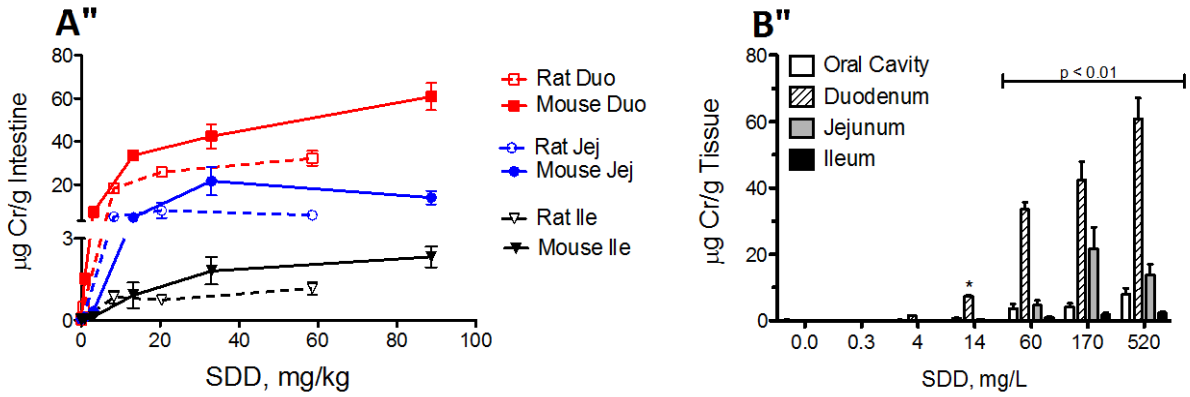

Figure 3. Total chromium (Cr) tissue concentration in alimentary canal. (A) Comparison of intestinal levels of total Cr in mice and rats on a mg/kg basis of at day 91. Data plotted are mean and SEM. (B) Total Cr levels in mice at day 91; data plotted are mean and SEM. Total Cr was significantly ($p < 0.01$) elevated in all four tissues at ≥ 60 mg/l SDD by Shirley's test, and in the duodenum at ≥ 14 mg/l SDD by Shirley's test.

Fig 4

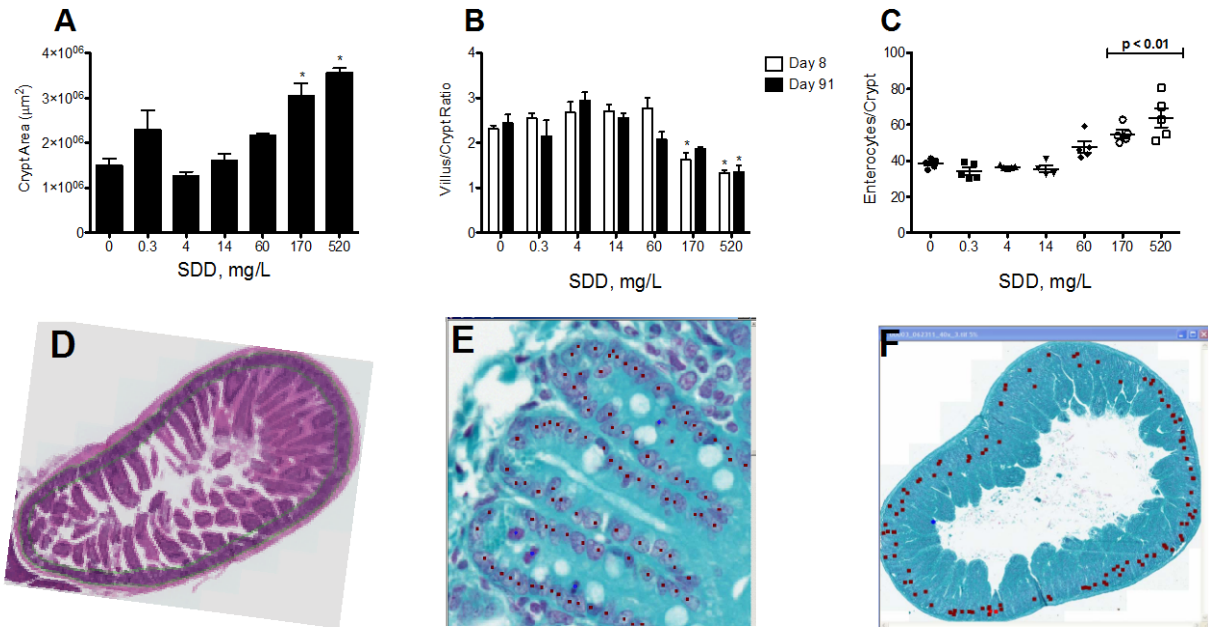

Figure 4. Quantitative assessment of intestinal structure in mice. (A) Measures of crypt area in 3 contiguous tissue sections at day 91 (*, $p < 0.05$ by ANOVA/Dunn's). (B) Villus/crypt area ratios at days 8 and 91 (*, $p < 0.05$ by ANOVA/Dunnett's). (C) Number of enterocytes per crypt at day 91 (10 full crypts assessed per animal; $p < 0.01$ by ANOVA/Dunnett's). (D) Representative image of crypt area analysis in 4A. (E) Representative image of crypt enterocyte analysis in 4C and Table 3. (F) Representative image of aberrant nuclei analysis in Table 4.

Fig 5

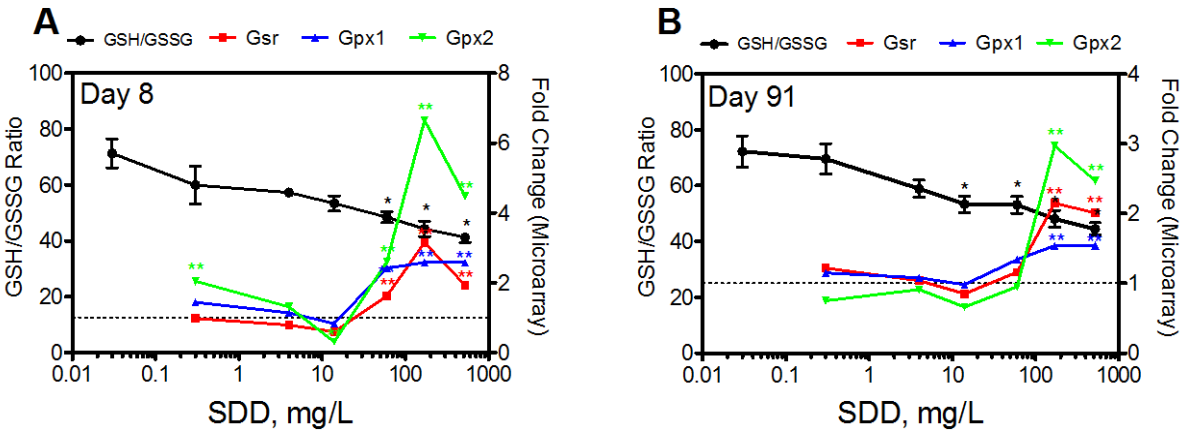

Figure 5. Redox changes in the intestine. The left axes represent the GSH/GSSG ratio and the right axes show the fold-change in gene expression at day 8 (A) and day 91 (B). Gsr, glutathione reductase; Gpx, glutathione peroxidase. *statistically significant decrease in GSH/GSSG ratio by Shirly's test; **statistically significant increase (>1.5-fold, P1(*t*)>0.999) relative to control by empirical Bayes method for posterior probabilities.

Fig. 6

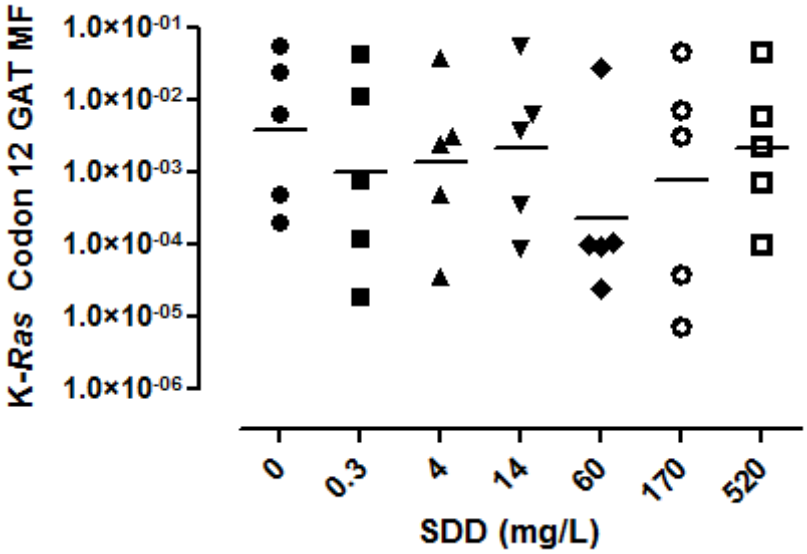

Figure 6. *Kras* mutation frequency (MF). ACB-PCR measurement of *Kras* codon 12 GGT to GAT MF in mouse duodenum. DNA was isolated from duodenal epithelium from mice exposed to Cr(VI), as SDD, at the indicated concentrations for 90 days and used as a template for ACB-PCR. Plot showing the average MF (based on triplicate analyses) from each animal. The line (—) line is the geometric mean for each dose group (n = 5).

Fig.7

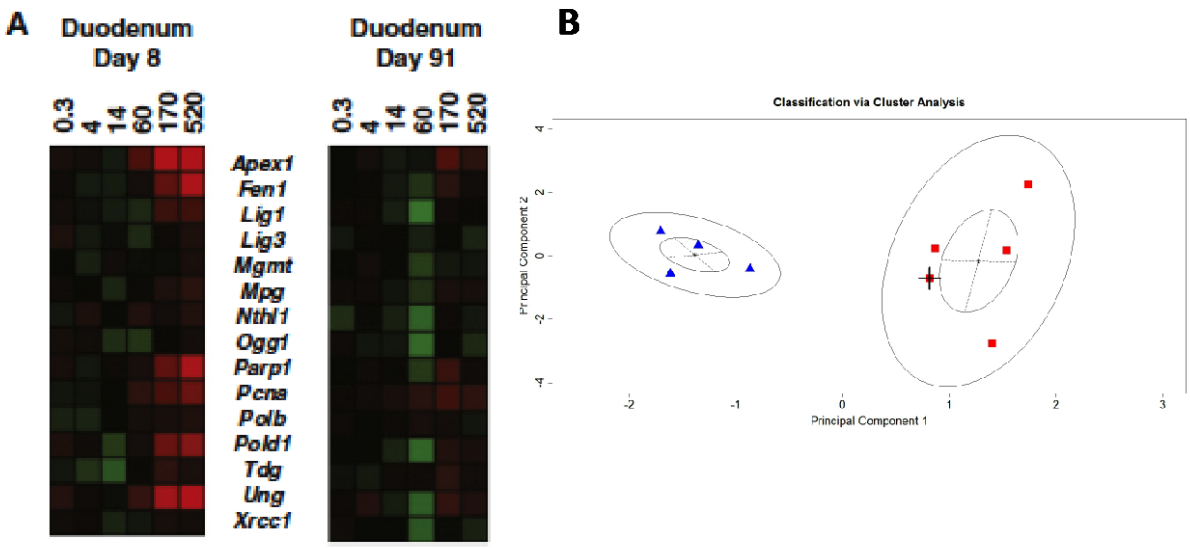

Figure 7. Assessment of genotoxicity. (A) Heatmap of DNA repair genes in mouse duodenum at day 8 and 91. Adapted from Kopec *et al.* 2012a. (B) Cluster analysis of expression values of 116 genes from 4 mutagenic (triangles) and 4 non-mutagenic (squares) carcinogens. The inner ellipse is the standard variance ellipse, and the outer ellipse is the 95% confidence boundary. The square with crosshair represents Cr(VI), indicating that the

expression profile for Cr(VI) clusters more closely with non-mutagenic compounds (see Thompson *et al.*, 2012a) for further details.

Fig 8.

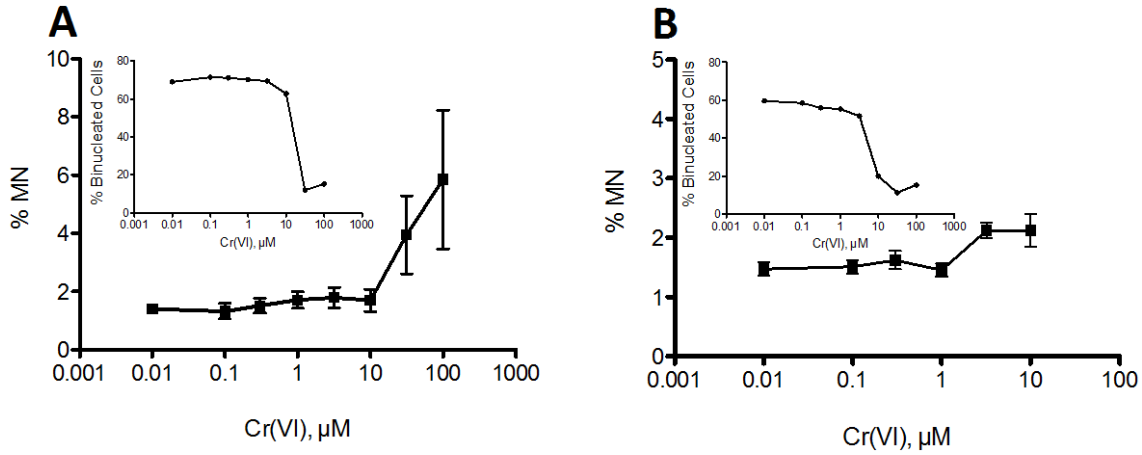

Figure 8. Micronucleus formation in CHO-K1 (A) and A549 (B) cells. Cells were treated with the indicated concentrations of Cr(VI), and MN formation was assessed in bi-nucleated cells (insets). Plots indicate the percentage of MN in bi-nucleated cells. Data represent mean ± sem, n = 9.

Fig. 9

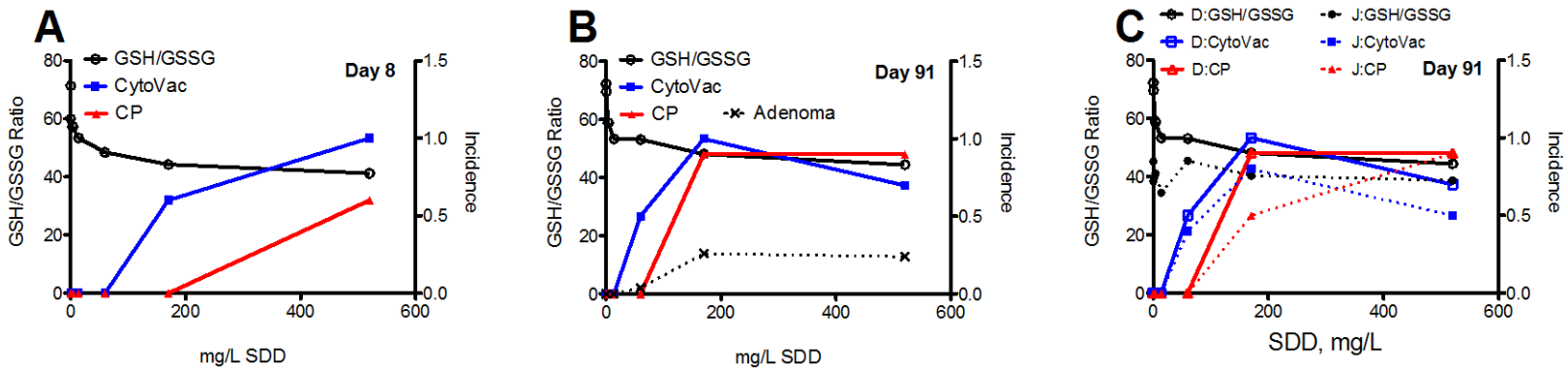

Figure 9. Dose-concordance. (A) Dose-response of key events in the mouse duodenum at day 8. (B) Dose-response of key events in the mouse duodenum at day 91. The dotted line represents the incidence of duodenal adenomas in female mice in the NTP 2-year bioassay. (C) Dose-response of key events in the mouse duodenum (solid lines) and jejunum (dotted lines) at day 91. Note: CP = crypt proliferation; CytoVac = cytoplasmic vacuolization

Fig. 10.

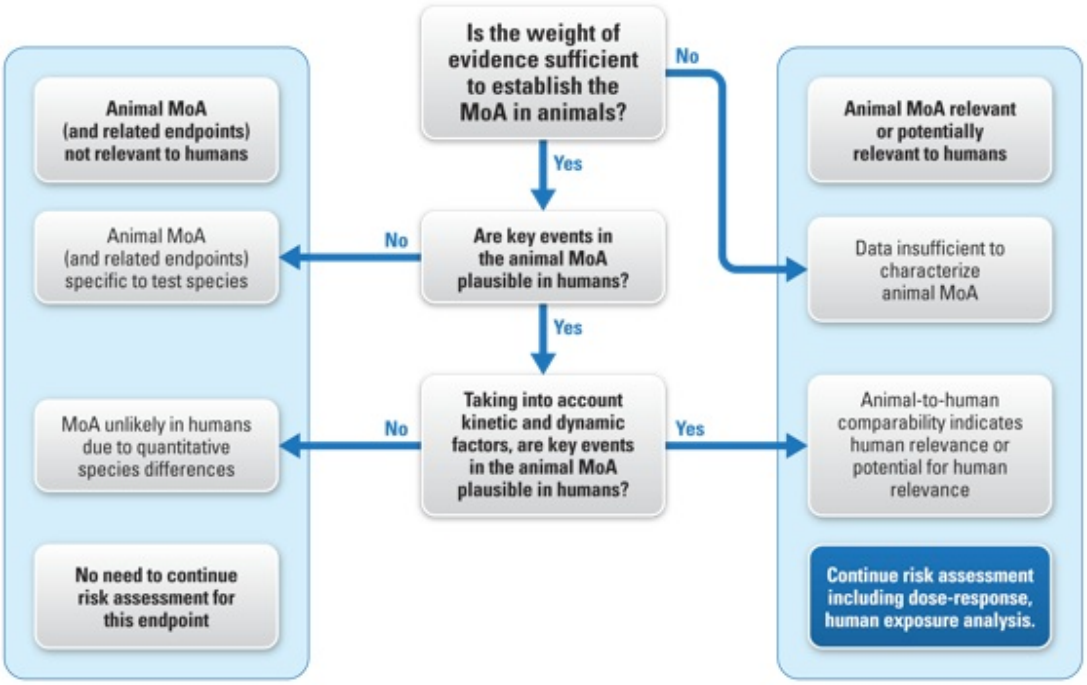

Figure 10. General schematic of the human relevance framework as developed by Meek *et al.* (2003) and revised by Seed *et al.* (2005). The box highlighted in blue indicates that risk assessment should proceed with dose response and exposure analysis. Adapted from Seed *et al.* (2005).(The framework was additionally considered in Boobis et al. (2006,2008) though essential content remains the same. A manuscript to update the framework is also currently in final stages of preparation)

Appendix D

Reviewer 5 Comments in Manuscript

Assessment of the Mode of Action Underlying Development of Rodent Small Intestinal Tumors Following Oral Exposure to Hexavalent Chromium and Relevance to Humans

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Short title: MOA for Cr(VI) in Mouse Small Intestines

Abstract (248 of 250)

Although chemicals may act by multiple modes of action (MOAs) at different tumor sites and at different doses, knowledge of the MOA that is specific to the tumor-response used for risk assessment is critical for low-dose extrapolation. For a mutagenic MOA, a chemical or its metabolites interacts directly with DNA to induce heritable mutations, whereas nonmutagenic carcinogens display a wider range of mechanisms that may involve genotoxicity and/or nongenotoxicity (e.g. cytotoxicity- or hormonally-induced cell proliferation) that result in tumorigenesis after prolonged exposure. Chronic exposure to high concentrations of hexavalent chromium (Cr(VI)) in drinking water was shown to cause intestinal adenomas and carcinomas in mice, but not rats, and the MOA underlying these tumors has been the subject of recent research. Although Cr(VI) is known to be genotoxic, subchronic studies indicate that Cr(VI) causes blunting of intestinal villi and crypt hyperplasia in mice as early as 8 days of exposure—suggesting that the tumors may have arisen from chronic mucosal injury. To better understand the MOA of Cr(VI) in the intestine, a 90-day drinking water study was conducted to collect histological, biochemical, toxicogenomic, and pharmacokinetic data in target tissues. Using MOA analyses and human relevance frameworks proposed by national and international regulatory agencies, the weight-of-evidence supports a nonmutagenic cytotoxic MOA with the following key events: a) saturation of luminal reductive capacity and elevated intestinal absorption, b) toxicity to villi, c) crypt hyperplasia and d) clonal expansion of spontaneous mutations within the crypt stem cells, resulting in late onset tumorigenesis.

Key Words: Risk Assessment, Cancer, hexavalent chromium, Cr(VI), Mode of Action

Table of Contents

1. INTRODUCTION (742 of 750)

Chromium is a naturally occurring element that primarily exists in two oxidation states: hexavalent chromium (Cr(VI)) and trivalent chromium (Cr(III)). Cr(III) is a putative micronutrient that may have a role in insulin sensitivity (Anderson, 2000; Di Bona *et al.*, 2011). Oral toxicity studies indicate that Cr(III) has only limited acute and chronic toxicity and is not carcinogenic (IARC, 1990; NTP, 2008b; Stout *et al.*, 2009b). In contrast, occupational exposure to high concentrations of Cr(VI) has been shown to cause lung cancer in workers in certain industries (IARC, 1990), and lung tumors in rodents when administered by inhalation or intratracheal instillation (Glaser *et al.*, 1986; Steinhoff *et al.*, 1986). Differences in toxicity between Cr(III) and Cr(VI) are primarily due to the lower cell permeability of Cr(III). However, Cr(VI) is reduced to Cr(III) in many bodily fluids thereby mitigating the effects of Cr(VI) exposures, especially via ingestion due to acidic reducing conditions of the stomach (De Flora *et al.*, 1987; De Flora *et al.*, 1997; Febel *et al.*, 2001). Recognizing differences in kinetics, biochemistry, and toxicology of Cr(III) and Cr(VI), health risk assessment of chromium compounds has historically been both route- and valence-specific (U.S. EPA, 1998).

In 2008, the National Toxicology Program (NTP) reported findings from 2-year cancer bioassays for both Cr(III) and Cr(VI) in drinking water (NTP, 2008b; NTP, 2008a). Consistent with the low bioavailability, NTP found that Cr(III) (administered as chromium picolinate) demonstrated “no evidence of carcinogenicity in mice or female rats”, and only “equivocal evidence for preputial gland adenomas in male rats” (NTP, 2008b; Stout *et al.*, 2009b). By contrast, Cr(VI) (administered as sodium dichromate dihydrate; SDD) produced a dose-related increase in tumors in the small intestine of mice and oral mucosa of rats (NTP, 2008a; Stout *et al.*, 2009a). A significant question arising from this bioassay is whether the high Cr(VI) concentrations employed exceeded the reductive capacity of the mouse stomach and intestinal

lumen (Collins *et al.*, 2010; De Flora *et al.*, 2008; Proctor *et al.*, 2011; Stern, 2010). Recently published studies on species-specific elements of Cr(VI) toxicokinetics suggest that the carcinogenic concentrations in the 2-year bioassay indeed exceeded reductive capacity (Kirman *et al.*, in press; Proctor *et al.*, 2012).

Hexavalent chromium can induce DNA adducts, DNA damage, mutation, and transformation (Chiu *et al.*, 2010; Holmes *et al.*, 2008; Nickens *et al.*, 2010; Zhitkovich, 2011). It has therefore been argued that the tumors observed following chronic oral exposure to Cr(VI) arose by a mutagenic mode of action (MOA) involving DNA mutation as an early initiating key event in the carcinogenic process (McCarroll *et al.*, 2010; U.S. EPA, 2010; Zhitkovich, 2011). However, the NTP study authors described the non-neoplastic lesions in the mouse intestine (*viz*. diffuse hyperplasia) as secondary to previous epithelial injury (NTP, 2008a). In contrast to mice, diffuse hyperplasia and tumors were not observed in rats (NTP, 2008a), suggesting that the intestinal tumors in mice may have occurred via a cytotoxic MOA. Until recently, there was insufficient information to determine the MOA underlying the development of intestinal tumors in mice with any degree of certainty because data describing the dose-response and sequence of key events in the target tissue did not exist.

To investigate the MOA underlying the intestinal tumors in mice, a MOA analysis was conducted by applying the MOA framework outlined in the U.S. EPA *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005) to: a) hypothesize a plausible MOA for the mouse intestinal tumors; b) identify data gaps; and c) design and conduct a 90-day drinking water study to acquire critical data to fill these gaps (Thompson *et al.*, 2011a). The study design, described in detail in Thompson *et al*. (2011b and 2012c), employed the same drinking water concentrations from the NTP 2-year bioassay (NTP, 2008a) as well as two lower drinking water concentrations; one of

which was 100 μg/l, the federal Maximum Contaminant Level (MCL) for total chromium (U.S. EPA, 1991). Data were collected in the target tissues after 7 and 90 days of exposure, and many of the study results have been published (Kopec *et al.*, 2012a; Kopec *et al.*, 2012b; Proctor *et al.*, 2012; Thompson *et al.*, 2011b; Thompson *et al.*, 2012c).

The purpose of this article is to present a synthesis of the MOA study data, as well as other available data, to determine the MOA for mouse intestinal tumors using a weight of evidence (WOE) approach. Findings relevant to the oral mucosal tumors observed in rats will be presented in a separate publication.

2. SUMMARY OF INTESTINAL LESIONS IN THE RODENT 2-YEAR BIOASSAYS

A review of the intestinal anatomy and physiology as well as the findings of the NTP (2008a) bioassay is helpful for understanding the MOA for intestinal tumors. The small intestine is compromised (proximal to distal) of the duodenum, jejunum, and ileum. Each segment is lined with a single layer of epithelial cells that is renewed every few days—making the intestinal mucosa one of the most proliferative tissues in the body (Berlanga-Acosta *et al.*, 2001). The small intestinal epithelium is extensively folded to maximize the absorptive surface area. Anatomically, the small intestinal epithelium is comprised of crypt and villus regions. The villi are multicellular finger-like projections that extend into the intestinal lumen (**Fig. 1A**); inside each villus is a capillary and lymphatic vessels (**Fig. 1B**). The crypts (also called glands of Lieberkühn) are invaginations of the epithelial surface at the base of the villi. Inside the crypts are actively dividing progenitors cells that differentiate into secretory (goblet cells), absorptive cells (enterocytes), and other cell types (e.g. Paneth cells) (Neal *et al.*, 2011; Potten *et al.*, 1997). Cell proliferation begins in the base of the intestinal crypts within a population of rapidly

dividing progenitor cells. Epithelial cells differentiate (mature) as they migrate toward the tips of the villi (**Fig. 1C**). The lifespan of the enterocyte, from progenitor cell through differentiation and migration to the tip of a villus, is 10-17 hours in rodents and ~24 hours in humans, and there is complete replacement of the entire epithelium within 2-3 days in rodents and 3-6 days in humans (Greaves, 2007). Upon migration from the crypt, cells differentiate and express proteins involved in nutrient absorption—which occurs primarily in the duodenum and proximal jejunum (DeSesso and Jacobson, 2001). At the tips of the villi, cells slough into the lumen and/or undergo apoptosis. Importantly, the absorptive villous enterocytes do not have proliferative potential, and thus it is unlikely that tumors originate in these cells (Barker *et al.*, 2009; Potten and Loeffler, 1990).

Results of the NTP 2-year drinking water bioassay for Cr(VI), administered as SDD, are described in detail in Stout *et al*. (2010). Findings in the small intestine and their statistical significance are summarized here in **Table 1.** Similar effects were observed in the small intestines of both male and female mice; however, for brevity only the results for females are summarized herein. In female mice, the incidence of diffuse hyperplasia was significantly increased in all treatment groups in a dose-dependent manner and was characterized by short, broad and blunt villi and by elongated crypts that contained increased numbers of epithelial cells and mitotic figures (NTP, 2008a). These lesions were not observed in female or male rats. The NTP study authors considered the lesions in mice to be "*consistent with regenerative hyperplasia secondary to previous epithelial cell injury*" (NTP, 2008a).

The incidence of adenomas and carcinomas were statistically elevated relative to concurrent controls in the duodenum of female mice at ≥172 mg/l and 516 mg/l SDD, respectively. In the female mouse jejunum, diffuse hyperplasia and adenomas were significantly

elevated relative to concurrent controls at 516 mg/l SDD. The overall combined incidence of intestinal tumors in female mice was significantly elevated relative to concurrent controls at ≥172 mg/l SDD (**Table 1**). Adenomas and carcinomas were not elevated in the small intestine in either male or female rats (NTP, 2008a).

3. APPLICATION OF THE MODE OF ACTION FRAMEWORK

U.S. EPA (2005) defines MOA as "*a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation.*" EPA defines a key event as "*an empirically observable and quantifiable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element.*" The EPA MOA Framework, like that proposed by the International Programme in Chemical Safety (IPCS) (Boobis *et al.*, 2006), stresses that the MOA for *each* tumor site should be evaluated separately. Moreover, toxicology data should ideally be evaluated (or generated if absent) in the target tissues of interest. Therefore, the MOA described below focuses on data obtained from the duodenal and jejunal intestinal mucosae of mice. Tumor incidence in the mouse small intestine provides the basis for several Cr(VI) risk assessments (McCarroll *et al.*, 2010; Stern, 2010; U.S. EPA, 2010), and thus understanding the MOA that is specific to tumors occurring in these tissues informs the low dose extrapolations for risk assessments that rely on these data.

3.1. Summary of the Key Events in the MOA for Intestinal Tumors

The histological, biochemical, toxicogenomic, and pharmacokinetic data collected as a part of our MOA studies were evaluated along with other relevant data in the published literature using the MOA and human relevance frameworks developed by the U.S. EPA and other organizations.

Based on this assessment, it was concluded that the overall WOE supports a cytotoxic nonmutagenic MOA with the following key events (**Fig. 2**): **Key Event 1** - saturation of reduction and the absorption of Cr(VI) from the intestinal lumen; **Key Event 2** - villous cytotoxicity due, in part, to oxidative stress; **Key Event 3** - compensatory crypt hyperplasia to repair/replace the damaged intestinal mucosa; and **Key Event 4** - expansion of spontaneous mutations in the crypt cells as a consequence of the constant proliferative pressure, ultimately leading to tumorigenesis. Each of these key events, and supporting data, are summarized in the sections that follow.

3.1.1. Key Event 1: Saturation of Reduction and Absorption from the Lumen

The absorption of Cr(VI) is thought to occur under very specific conditions. The chemistry of Cr(VI) dictates that below ~pH 6, Cr(VI) exists as dichromate ($Cr_2O_7^{2-}$) and chlorochromate (CrO_3Cl^-), whereas at ≥ pH 6, Cr(VI) primarily exists as chromate (CrO_4^{2-}) (Zhitkovich, 2005). Chromate is structurally similar to sulfate (SO_4^{2-}) and phosphate (PO_4^{2-}) ions and therefore enters cells through anion transporters (De Flora, 2000; Markovich, 2001; Salnikow and Zhitkovich, 2008; Zhitkovich, 2005). However, reduction of Cr(VI) to Cr(III) prevents entry through these transporters and is thus a critical kinetic process limiting toxicity (De Flora, 2000; De Flora *et al.*, 1997; Donaldson and Barreras, 1966; Febel *et al.*, 2001; Kerger *et al.*, 1996; U.S. EPA, 1998). The stoichiometry of Cr(VI) reduction to Cr(III) in the lumen of the stomach and small intestine is not fully known, but probably varies depending on stomach conditions (fasting/fed), pH, and dose. The most studied biological reducing agents for converting Cr(VI) to Cr(III) are GSH, ascorbate, and cysteine (Zhitkovich, 2005), and enzymatic and/or non-enzymatic reduction by proteins also likely plays a role in Cr(VI) reduction. Cr(VI) that escapes reduction in the stomach will be taken up into enterocytes, transited through the gastrointestinal tract where it

will be reduced by microbiota (Shrivastava *et al.*, 2003) and intestinal secretions such as cysteine (Dahm and Jones, 2000), or excreted in feces. Decreasing luminal Cr(VI) concentrations, due to extracellular reduction of Cr(VI) to Cr(III), likely explains the decreasing level of tissue damage and tumor formation from the proximal to distal intestinal segments in the 90-day and 2-year studies.

In vivo and *ex vivo* pharmacokinetic data collected in the alimentary tract of rodents suggest species differences in the disposition of Cr(VI) (Proctor *et al.*, 2012; Thompson *et al.*, 2011b). Tissue chromium levels after 90 days of exposure to Cr(VI) were higher in mice than rats on a mg/kg bodyweight exposure basis (**Fig. 3A**), which may be explained, at least in part, by the higher levels of unreduced Cr(VI) predicted to enter the intestinal lumen (Proctor *et al.*, 2012). In mice exposed to Cr(VI) in drinking water for 90 days, a statistically significant increase in duodenal chromium levels was not observed at 0.3 or 4 mg/l SDD (**Figure 3B**)—suggesting that at these administered SDD concentrations, appreciable duodenal Cr(VI) absorption does not occur. As discussed above, reduction in the lumen of the stomach and intestine competes with kinetic processes of absorption and transit through the gastrointestinal tract (Proctor *et al.*, 2012), thus Cr(VI) transited to the small intestine is available for absorption even at concentrations that are lower than stomach reductive capacity. Nevertheless, as will be discussed in the following section, histopathological lesions were not observed in the mouse duodenum at (or below) 14 mg/l SDD following 90 days of exposure. As such lesions are critical for driving tumor formation within a regenerative hyperplasia MOA, small amounts of Cr(VI) that enter intestinal villi are likely inconsequential. PBPK models capable of accounting for the competing kinetic rates have been developed (Kirman *et al.*, in press) and will be useful for

estimating the concentrations of Cr(VI) that would need to be ingested to induce cytotoxicity in humans.

3.1.2. Key Event 2: Villous Cytotoxicity

The non-neoplastic lesions observed in the mouse small intestine were characterized as being secondary to previous epithelial cell injury (NTP, 2008a). The villi of treated animals were short, broad, and blunt relative to unexposed animals (NTP, 2007; NTP, 2008a). Such effects are a nonspecific response to various types of injury, and can be associated with either hyperproliferation or hypoproliferation of crypt enterocytes (Greaves, 2007; Serra and Jani, 2006). Chemicals that are cytotoxic to cells of the villi can, after removal, stimulate crypt proliferation to repair the damaged mucosa. Alternatively, chemicals that are toxic to crypt cells can also result in blunting of villi due to hypoproliferation in the crypt and inability to replenish the mucosa. Because cytotoxicity can result from epigenetic and genotoxic effects, and the intestinal stem cells reside in the crypt, it is critical to determine a) whether toxicity is originating in the crypt or villus, b) whether there is any evidence of cytogenetic damage in the crypts, and c) whether there is evidence for epigenetic mechanisms associated with intestinal carcinogenesis such as oxidative stress and inflammation. Each of these issues are discussed in the following subsections; and as a whole, indicate that Cr(VI) toxicity occurs in the villus.

3.1.2.1. Histological evidence of cytotoxicity

The term “diffuse hyperplasia” used in the NTP studies includes the blunting effects on the villi, as well as elongation of the crypts (see Plate 21 in NTP, 2008a). This terminology was not used in Thompson *et al*. (2011b); instead, villus damage and crypt hyperplasia were noted separately in order to provide greater resolution with respect to the location of the lesions. As shown in **Table 2**, cytoplasmic vacuolization in the duodenal villi was the most sensitive endpoint—occurring at 170 and 60 mg/l SDD at day 8 and 91, respectively; while atrophy of the villi and

crypt hyperplasia were first evident at higher concentrations (i.e., 520 and 170 mg/l on day 8 and 91, respectively). Vacuolization can be an indication of reversible injury to the cells (Henics and Wheatley, 1999), and can arise from altered lipid metabolism, sequestration of absorbed material, autophagy, endoplasmic reticulum (ER) stress, or proteasome dysfunction (Franco and Cidlowski, 2009; Henics and Wheatley, 1999; Mimnaugh *et al.*, 2006). Although the exact cause of the SDD-induced vacuolization is unclear, it is often regarded as a sign of cytotoxicity (Mimnaugh *et al.*, 2006).

The presence of cytoplasmic vacuolization in villi at lower concentrations than crypt hyperplasia suggests a mechanism whereby toxicity to cells in the villi triggers compensatory cell proliferation of crypt enterocytes. Area measurements of the crypt of the mouse duodenum at day 8 were increased ~45% at ≥170 mg/l SDD (O'Brien *et al.*, in prep). At day 91, the crypt area was increased ~45% at 60 mg/l, and was significantly increased ≥2-fold of control at ≥ 170 mg/l (**Fig. 4A and D**). Consistent with the increase in crypt area, the ratio of the villus to crypt area was decreased at ≥170 mg/l SDD (**Fig. 4B**). If SDD were toxic to crypt enterocytes, then one would expect a dose-dependent decrease in crypt area. In fact, the number of enterocytes per crypt increased significantly following exposure to ≥170 mg/l SDD (**Fig. 4C and E**). Despite the increase in the number of crypt enterocytes, the intestinal villi were damaged and blunted. This implies that the increased supply of crypt enterocytes was not sufficient to maintain the normal length and structure of healthy duodenal villi. Hence, the damage to the villi was caused by exposure to cytotoxic concentrations of Cr(VI).

As further evidence that the cytotoxicity of SDD is limited to the intestinal villi, the number of mitotic and apoptotic cells were counted in fully intact crypts in order to compute both a mitotic index (MI) and apoptotic index (AI). As shown in **Table 3**, there were no

significant or dose-dependent effects on either endpoint—suggesting that Cr(VI) did not cause cell cycle arrest or increases in the percentage of crypt enterocytes undergoing programmed cell death. Previous studies have suggested that increased crypt hyperplasia without change in the MI indicates that the number of active crypt stem cells has increased (Snow and Altmann, 1983). Given that crypt stem cells are the most likely clonal sources of tumor formation (Barker *et al.*, 2009; Potten and Loeffler, 1990), increases in the numbers of crypt stem cells may increase the chance of spontaneous tumor formation **(Feng *et al.*, 2011). [Note to authors: ok, but if the MI is not increasing in crypt cells, this is evidence against the regenerative hyperplasia MOA, correct?**

3.1.2.2. Assessment of cytogenetic damage in crypts

Data in the previous section indicate that Cr(VI)-induced damage to the intestinal mucosa originates in the villus. However, because Cr(VI) can be genotoxic—especially in *in vitro* systems, crypt enterocytes were also examined for aberrant nuclei such as karyorrhectic nuclei and micronuclei (MN). The former indicates a type of cell death that can be either a result of programmed death or necrosis (Kumar *et al.*, 2005), while the latter can arise from either DNA breakage or chromosomal disjunction (Fenech *et al.*, 2011; Vanhauwaert *et al.*, 2001). There were no treatment-related effects on the number of karyorrhectic nuclei or MN in duodenal crypts of exposed mice (**Table 3**). MN were also counted in three entire tissues sections, regardless of whether the crypts were fully intact (**Fig. 4F**). This analysis has the advantage of increasing the overall amount of crypt tissue that was analyzed for aberrant nuclei. There were no treatment-related increases in MN or karyorrhectic nuclei in crypts across any of the tissue sections examined in animals exposed to Cr(VI) for 7 or 90 days; however, both forms of aberrant nuclei were observed in villi (**Table 4**). Specifically, karyorrhectic nuclei were significantly increased in duodenal villi at ≥60 mg/l SDD at day 91 and primarily occurred in the

tips, whereas MN were statistically increased in villi at ≥ 170 mg/l (**Table 4**). The concentrations where aberrant nuclei were observed in duodenal villi correspond to the concentrations where cytoplasmic vacuolization was observed (**Table 2**). This pattern of toxicity, specifically limited to the villi, supports that toxicity to the villi caused the regenerative response in the crypt in the absence of any direct genotoxicity or cytotoxicity in the crypt**. [Note to authors: ok, but the MI does not increase in the crypt (Table 3)]**

3.1.2.3. Oxidative stress

Cr(VI) that is not reduced in the lumen can be absorbed by villous enterocytes, and reduced to Cr(III) through binding to low molecular weight thiols (e.g. GSH) and antioxidants (e.g. ascorbate). Furthermore, Cr(VI) reduction can result in the production of Cr(V) and Cr(IV) reactive intermediate species and the generation of reactive oxygen species (ROS) (Liu and Shi, 2001; Nickens *et al.*, 2010). Thus, the chemistry and biochemistry of Cr(VI) reduction predicts that Cr(VI) can alter the redox status of enterocytes.

Cr(VI) significantly decreased the GSH/GSSG ratio, a key indicator of cellular redox status (Meister and Anderson, 1983; Moriarty-Craige and Jones, 2004; Schafer and Buettner, 2001), in the mouse small intestine in a concentration-dependent manner (**Fig. 5**; (Thompson *et al.*, 2011b). Despite the change in redox status, the duodenum appeared to synthesize GSH as evidenced by dose-dependent increases in duodenal GSH levels (Thompson *et al.*, 2011b). The increase in GSH was accompanied by significant increases in expression of genes involved in GSH synthesis, and there were clear signs of oxidative stress responses in the transcriptome (see **Section 3.2**). These data indicate tissue oxidative stress that could not be mitigated even after relatively long durations of exposure (7-90 days), implying that the mice exposed to Cr(VI) in the 2-year NTP bioassay (NTP, 2008a) experienced increased intestinal oxidative stress nearly their entire lifespan.

Intestinal mucosal health is influenced by redox status (Circu and Aw, 2011; John *et al.*, 2011). For example, the intestinal mucosa regulates luminal redox status by secretion of thiols such as cysteine (Dahm and Jones, 2000; Hagen *et al.*, 1990). Chemical-mediated inhibition of GSH synthesis results in loss of intestinal epithelial cell height, desquamation of microvilli, mitochondrial swelling, and vacuolization in the tips of jejunal villi, all of which could be mitigated by GSH supplementation (Martensson *et al.*, 1990). These lesions are similar to those induced by Cr(VI). Although Cr(VI) caused a dose-dependent increase in GSH tissue levels, the GSH/GSSG ratio was nonetheless significantly decreased (Thompson *et al.*, 2011b). Given the influence of redox status on intestinal health, it is highly likely that Cr(VI)-induced changes in oxidative status contribute to cytotoxicity in the intestinal villi.

3.1.2.4. Inflammation

Oxidative stress and inflammation are typically linked physiologically. For example, oxidative stress leads to activation of nuclear factor-□B (NF-□B) and subsequent downstream pathways resulting in the release of cytokines; this is well documented in the scientific literature for airway inflammation, intestinal inflammation, and certain cancers (Kruidenier and Verspaget, 2002; Rahman and MacNee, 2000; Roberts *et al.*, 2009). Increases in histiocytic infiltration of macrophages in the duodena of rats and mice were observed in the 90-day and 2-year NTP studies (NTP, 2007; NTP, 2008a), as well as in the subsequent 90-day studies (Thompson *et al.*, 2011b; Thompson *et al.*, 2012c); however, clear signs of chronic inflammation were not apparent in these studies. Although histiocytic infiltration can be associated with mild, chronic inflammation, its meaning is unclear. In fact, the NTP study authors concluded that the biological significance of histiocytic infiltration was uncertain (NTP, 2008a).

Thompson *et al.* (2011b, 2012c) measured changes in about twenty cytokines and chemokines in both serum and duodenal mucosa of both mice and rats following 90 days of

exposure to Cr(VI). In the serum, there were no clear treatment-related effects in mice, while IP-10 and IL-12(p70) were decreased in rats (**Supplemental Table 1**). In the duodenum, several cytokines were significantly decreased in treated mice relative to untreated mice. TNFα and IL-1β both showed clear dose-dependent decreases following 90 days of exposure (Thompson *et al.*, 2011b). Other cytokines reduced were IL-7, 9, 12, 13, 17, IFNγ, and RANTES (**Supplemental Table 1**). In rats, only IL-1α was clearly altered by SDD, and was increased at ≥60 mg/l. Broadly, these data support a pro-inflammatory response in rats and a decrease in cytokines and chemokine production in mice. Toxicogenomic analyses lend additional support to this conclusion (see **Section 3.2)**.

3.1.3 Key Event 3: Crypt Cell Proliferation

Chronic cell proliferation is a well-known risk factor for carcinogenesis (Ames *et al.*, 1993; Boobis *et al.*, 2009; Cohen, 2010; Gaylor, 2005). Diffuse hyperplasia occurred in the duodenum of mice at all SDD concentrations examined in the 2-year bioassay, while there was no evidence of diffuse hyperplasia in the rat duodenum at any dose level (NTP, 2008a). The same pattern was observed in the NTP 90-day study (NTP, 2007). The two 90-day drinking water studies conducted by Thompson *et al*. (2011b, 2012c), included evaluations at both day 8 and 91 of exposure. At day 8, duodenal crypt cell hyperplasia was present in 3 of 5 mice exposed to 520 mg/l SDD (**Table 2**; (Thompson *et al.*, 2011b)). By day 91, crypt hyperplasia was present in almost all animals at ≥170 mg/l SDD (**Table 2**). At both time points, crypt cell hyperplasia was preceded on a dose-basis by cytoplasmic vacuolization in the villi. Together, these data suggest that Cr(VI) increased crypt hyperplasia as early as one week of exposure to very high concentrations in drinking water. By day 91, there was a significant increase in the number of

crypt enterocytes at the higher SDD concentrations (**Fig. 4C**). Not surprisingly, whole genome microarray analysis also indicated cell proliferation (see **Section 3.2**).

Importantly, focal hyperplasia, which NTP considers a preneoplastic lesion (NTP, 2008a), was not observed in any animals in the 90-day drinking water studies (NTP, 2007; Thompson *et al.*, 2011b; Thompson *et al.*, 2012c). Altogether, 260 rodents from the aforementioned three 90-day drinking water studies were exposed to ≥60 mg/l SDD yet focal hyperplasias (or other preneoplastic lesions) were not observed. Although focal hyperplasia was observed in a few animals in the 2-year NTP study (NTP, 2008a), the incidence was not statistically different from controls and was not observed in any female mice in the 520 mg/l SDD treatment group (**Table 1**). The presence of diffuse hyperplasia without focal hyperplasia is consistent with proliferation that is secondary to mucosal injury. The presence of a nearly 2-fold higher incidence of diffuse hyperplasia in the duodenum at carcinogenic Cr(VI) concentrations, and the presence of significant diffuse hyperplasia in the jejunum at 520 mg/l SDD (the only carcinogenic dose in the jejunum) suggest that crypt hyperplasia is a critical key event in the MOA for intestinal carcinogenesis. The absence of hyperplasia and tumors in rats is consistent with increased crypt proliferation driving intestinal carcinogenesis in mice.

It should be noted that other authors have suggested a sequence of key events whereby Cr(VI)-induced hyperplasia occurs *subsequent* to a DNA mutation MOA (McCarroll *et al.*, 2010). We evaluated this potential MOA according to U.S. EPA (2005), where a sequence of key events implies that prevention of a given key event stops the progression of subsequent key events. Thus for a mutagenic MOA, prevention of DNA mutation would prevent hyperplasia. Consistent with this idea **[Note to authors: from whom, McCarroll or EPA?],** it has recently been shown that transgenic mice expressing mutant *Kras* alleles exhibit increased cell numbers

in intestinal crypts (Feng *et al.*, 2011), but without the villous damage that is induced by Cr(VI). **[[Note to authors: did the Feng study administer CrVI?]** As discussed in the following section, increases in *Kras* mutation frequency were not observed in the intestines of mice exposed at all concentrations (≤520 mg/l) SDD for 90 days. In contrast, our data indicate that crypt cell proliferation is increased as early as 8 days of exposure at 520 mg/l SSD, and at 170 mg/l after 91 days (**Table 2**). Thus, hyperplasia appears to proceed mutations in both time and concentration, suggesting that the increase incidence of crypt hyperplasia is not due to mutagenesis (see additional discussion in **Section 3.6**).

3.1.4. Key Event 4: Expansion of Spontaneous Mutations

Target tissue mutation data provide the strongest evidence for a mutagenic MOA (U.S. EPA, 2007). Thus, to assess the potential for Cr(VI) to induce mutations in the small intestine following oral exposure to Cr(VI), we measured *Kras* codon 12 GAT mutation frequency in the duodenal epithelium of mice exposed to Cr(VI) for 90 days (O'Brien *et al.*, in prep). *Kras* was selected because it is often mutated early in human intestinal tumors (Wicki *et al.*, 2010), and codon 12 GGT to GAT mutation is one of the most commonly reported mutations in human duodenal tumors (Nishiyama *et al.*, 2002; Sutter *et al.*, 1996; Wicki *et al.*, 2010; Younes *et al.*, 1997), and accounts for 12.6% of colon tumors in the COSMIC database (Forbes *et al.*, 2010). Further, *Kras* codon 12 GAT mutation frequency was increased in colon tissue of rats exposed to azoxymethane within one week of exposure (McKinzie and Parsons, 2011) and in rat lung 28 days after exposure to benzo(a)pyrene (BaP) (Meng *et al.*, 2010). Meng *et al.* (2010) also found that *Kras* codon 12 GAT mutation frequency increased with dose and with BaP-DNA adduct formation. Thus, *Kras* codon 12 is a likely candidate oncogene for early mutations in intestinal

carcinogenesis following exposure to mutagenic carcinogens. **[Note to authors: any data in mice for *Kras* mutations?]**

To ensure detection of increased mutation frequency at very low levels (~10^{-6}), allele-specific competitive blocker (ACB) PCR analysis was used to measure mutation frequency in scraped duodenal epithelium (O'Brien *et al.*, in prep). No treatment-related affect on *Kras* codon 12 GAT mutation frequency was observed even at the high Cr(VI) doses that were carcinogenic in the 2-year bioassay and induced a proliferative responses at 8 and 90 days (**Fig. 6**). These findings are particularly interesting when contrasted with Cr-DNA binding data from genomic DNA isolated from scraped duodenal epithelia. Although increases in Cr-DNA binding were observed with increased SDD dose, there was no correlation with *Kras* mutation frequency (O'Brien *et al.*, in prep). Moreover, we determined that the Cr-DNA isolation technique, while similar to that used in other published studies (Zhitkovich, 2005), was likely confounded by *ex vivo* chromium binding. Specifically, the addition of chromium chloride to scraped intestinal tissue from untreated animals prior to DNA isolation resulted in Cr-DNA levels that were comparable to those in treated animals—indicating that chromium present in (or on) scraped duodenal cells from treated animals was likely binding to genomic DNA *ex vivo* during the DNA isolation assay (O'Brien *et al.*, in prep**). [Note to authors: ok, but why is the chromium binding important when mutations are not found?]**

This observation, as well as high Cr-DNA binding levels in the non-target tissue of the mouse liver following drinking water exposure to SDD (O'Brien *et al.*, in prep), calls into question the reliability of Cr-DNA binding data, and furthermore, the data do not support the role of Cr-DNA adducts as pre-mutagenic lesions relevant in the MOA for small intestinal tumors. **[Note to authors: ok, so now you explain why the chromium binding is important. I**

believe this is better handled as a footnote. The important thing to state is that mutations are not proceeding hyperplasia.] Meng *et al.* (2010) reported that *Kras* mutation frequency in lung tissue **[of what specie?]** increased with increasing numbers of DNA adducts after a single i.p. administration of BaP. BaP metabolites bind to DNA and are converted into mutations during DNA replication (Meng et al. 2010). This acute mutation response and the known DNA reactivity of BaP metabolites are suggestive of a mutagenic MOA. In fact, in the case of mutagenicity caused by BaP, *Kras* mutation frequency is considered a more sensitive endpoint than DNA adducts due to amplification of and the growth advantage of mutated cells. Furthermore, *Kras* Codon 12 GAT mutation frequency has been postulated to represent a functional reporter (i.e. reporter of tumor initiation and/or progression) which is amplified with mutational loading that is not necessary specific to a chemically induced DNA lesion of *Kras* Codon 12 (Meng *et al.*, 2010; Parsons *et al.*, 2010). Thus, although *Kras* Codon 12 GAT is a single mutation in a single oncogene, DNA mutations in other oncogenes, if they occurred with Cr(VI) exposure, may be expected to amplify *Kras* mutations and be detected in the ACB-PCR assay. However, no such increases were observed—even at carcinogenic concentrations—and there was no concordance between Cr-DNA binding and *Kras* codon 12 mutation frequency. Moreover, the increase in crypt cells (**Fig. 4C**), if preceded/induced by mutations, would likely increase the number of target *Kras* mutations thereby ensuring detection if present. Together, these data indicate that Cr-DNA binding is not representative of a pre-mutagenic lesion in the mouse small intestine.

A relatively high background *Kras* codon 12 GAT mutation frequency was observed in the duodenal mucosa of B6C3F1 mice (10^{-2} to 10^{-3}) (O'Brien *et al.*, in prep), which is approximately 100-times higher that that measured in rat colon tissue (McKinzie and Parsons,

2011) and rat liver (McKinzie *et al.*, 2006). Because *Kras* codon 12 GAT mutation frequency is known to accumulate with age in the rat colon (McKinzie and Parsons, 2011), and there appears to be a relatively high background *Kras* mutation frequency in the mouse duodenum (O'Brien *et al.*, in prep), spontaneous *Kras* codon 12 mutations would likely accumulate with time, especially under proliferative pressure.

The apparent absence of an effect on *Kras* codon 12 GAT mutation frequency is consistent with the fact that early tumors, metastases, and mortality were not observed in the NTP 2-year bioassay (NTP, 2008a), as well as the absence of preneoplastic (e.g. focal hyperplasia) or neoplastic lesions in any of the 90-day Cr(VI) drinking water studies (NTP, 2007; Thompson *et al.*, 2011b; Thompson *et al.*, 2012c). It is also consistent with the absence of cytogenetic damage in the duodenal crypts (see **Section 3.1.2.2**). These facts do not support a mutagenic MOA for Cr(VI), specifically where mutation is an early key event, in the mouse small intestine.

3.2. Additional Supporting Information

3.2.1. Toxicogenomic responses to Cr(VI)

Toxicogenomic data were collected in the duodenum and jejunum of rats and mice after 7 and 90 days of exposure to Cr(VI) in drinking water. Data analysis included examination of individual differentially expressed genes, quantitative dose-response modeling, functional enrichment analysis, Transcription Factor Analysis, and Downstream Effects Analysis. The latter two analyses are relatively new Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com/) functions that predict activation or inactivation of transcription factors and biological functions based on the direction (up or down) of genes changes in a dataset of interest.

As described in **Section 3.1.1**, toxicokinetic data indicated that very little chromium was detected in the small intestinal tissue below 14 mg/l SDD (see **Fig. 3B**). At 14 mg/l SDD, the

GSH/GSSG ratio was significantly decreased, but there were no apparent histopathological lesions (see **Table 2**). Consistent with these data, the number of gene changes observed in each treatment group correlated well with tissue dosimetry. At day 91, the number of transcripts significantly altered ±1.5-fold at 0.3, 4, and 14 mg/l SDD were each ≤76 genes, whereas at 60, 170 and 520 mg/l more than 1,800 genes were altered in each group. These findings lend additional support to Key Event 1, i.e. that SDD concentrations ≥60 mg/l exceeded reductive capacity of the gastrointestinal fluids. Below these concentrations, very few mRNA transcripts were altered by Cr(VI) exposure.

With respect to Key Event 2 (cytotoxicity), the toxicogenomics data clearly support alterations in redox signaling. As described in Kopec *et al.* (2012a), Nrf2 oxidative stress signaling and GSH metabolism were among the top five enriched canonical pathways at the lowest treatment concentrations at day 8. Transcription factor analysis in IPA also indicated activation of Nrf2 signaling pathways at ≥170 mg/l SDD day 91 (Kopec *et al.*, 2012b). As shown in **Fig. 5**, Gsr, Gpx1 and Gpx2 were increased significantly in the higher treatment groups. Expression of levels of *Gclc*, the rate-limiting enzyme in GSH synthesis, were also elevated in response to Cr(VI) exposure (Kopec *et al.*, 2012a), further indicating response to changes in redox. Interestingly, Nrf2 null mice demonstrate increased colonic mucosal injury and oxidative stress following dextran sulfate sodium (Osburn *et al.*, 2007). Thus, the presence of mucosal injury and Nrf2 signaling following Cr(VI) exposure suggests chronic induction of oxidative stress.

As described in Kopec *et al.* (2012a), significant enrichment of canonical pathways related to DNA repair was not observed at day 91. However, there was enrichment for pathways related to BRCA1 and mismatch repair at 520 mg/l SDD at day 8, as well as enrichment for

pathways related to nucleotide excision repair (NER) at ≥170 mg/l. Base excision repair (BER) is a major repair pathway of oxidative DNA damage and the heatmap in **Fig. 7A** clearly shows induction of genes involved in BER at day 8 (and less so at day 91). Both BER and NER repair oxidative DNA damage (Go and Jones, 2010; Klaunig *et al.*, 2010; Sedelnikova *et al.*, 2010). Although significant increases in 8-OHdG were not evident in duodenal tissues from mice exposed to Cr(VI) for 90 days (Thompson *et al.*, 2011b), it is conceivable that changes in genes related to redox signaling and DNA repair prevented 8-OHdG formation/detection.

Oxidative stress and inflammation often occur concomitantly as the result of common mechanisms. Specifically, ROS can activate NF-κB signaling that results in cytokine formation and immune cell infiltration, while inflammatory responses in turn generate ROS (Klaunig *et al.*, 2010; Rahman and MacNee, 2000; Roberts *et al.*, 2009). Given the observed oxidative responses and presence of histiocytes (which can release TNFα) following Cr(VI) exposure, inflammatory responses were expected. However, as described in **Section 3.1.2.4**, cytokine levels were lower in Cr(VI)-treated mice than untreated mice. Transcript analysis corroborated the decrease in IL-1β and TNFα cytokine levels in the mouse duodenum (reported in Thompson *et al.*, 2011b), as evidenced by decreased expression at ≥ 60 mg/l SDD (Kopec *et al.*, 2012a).

Inflammatory responses intentionally increase ROS production in order to fight infections. Nrf2 signaling can inhibit inflammatory responses (Jung and Kwak, 2010; Kim *et al.*, 2010), perhaps as feedback control to limit oxidative damage to the host. Osburn et al. (2007) showed that TNFα and IL-1β were elevated in Nrf2 null mice (relative to wild type mice) following exposure to dextran sulfate sodium. Thus, the decrease in TNFα and IL-1β following Cr(VI) exposure is likely due to Nrf2 activation. Indeed, Nrf2 regulation of inflammation is mediated by increasing heme oxygenase-1 (HO-1) expression (Jung and Kwak, 2010; Kim *et*

al., 2010), and HO-1 transcript levels were increased following Cr(VI) exposure (Kopec *et al.*, 2012a). *In vitro* studies have also shown that Cr(VI) increases HO-1 mRNA levels, as well as inhibit NF-κB signaling (Joseph *et al.*, 2008; Shumilla *et al.*, 1999). Thus, Nrf2 activation is likely due to Cr(VI)-induced oxidative stress that also results in suppression of inflammatory signals, which likely explains the absence of histological evidence of chronic inflammation in the mouse small intestine.

With respect to Key Event 3 (cell proliferation), transcription factor analysis indicated increased MYC signaling (based on expression levels of *Myc* downstream targets) at ≥ 60 mg/l SDD at day 91 (Kopec *et al.*, 2012b), despite the fact that *Myc* itself was not significantly induced at these concentrations (Kopec *et al.*, 2012a). At day 8, *Myc* was increased greater than 4-fold at 520 mg/l (Kopec *et al.*, 2012a). Moreover, transcript levels of *Ki67*, a common marker of crypt cell proliferation (Itzkovitz *et al.*, 2012; Potten *et al.*, 1997), were significantly elevated at ≥170 mg/l SDD at both day 8 and 91. These findings are consistent with the observed increase in the numbers of crypt enterocytes (see **Fig. 4C**) and increased incidence of crypt hyperplasia (see **Table 2**).

Finally, Cr(VI)-induced gene changes in the mouse duodenum were compared to gene changes produced by known mutagenic and nonmutagenic carcinogens (Thompson *et al.*, 2012b). The genomic response induced by Cr(VI) in the mouse intestinal tissue at doses that induce pathology and were carcinogenic following prolonged exposure were more similar to that induced by nonmutagenic than by mutagenic carcinogens (**Fig. 7B**; (Thompson *et al.*, 2012b)). Although transcriptional changes do not directly reflect changes to DNA (i.e. Key Event 4 - expansion of spontaneous mutations), these genomic signature data together with the occurrence of oxidative stress, cell proliferation, and lack of *Kras* mutations are consistent with a MOA for

Cr(VI) carcinogenesis in the mouse small intestine that involves prolonged increases in cell turnover in an oxidized environment rather than a mutagenic MOA.

3.2.2. In vitro micronucleus formation

Previous studies have examined the ability of Cr(VI) to induce MN formation *in vitro*. For example, Cr(VI) induced MN in human bronchial epithelial cells (Reynolds *et al.*, 2006), but only after pre-incubation with ascorbate. Other studies have reported that Cr(VI) induces MN in human fibroblasts through an aneuploidic rather than clastogenic mechanism (Seoane and Dulout, 2001; Seoane *et al.*, 2002). As part of our Cr(VI) MOA research program, Cr(VI) genotoxicity was assessed by high content screening methods in the CHO-K1 cell line (Thompson *et al.*, 2012a), which is a cell model recommended by OECD (OECD, 2010).

Exposure to Cr(VI) reduced viability and the number of bi-nucleated cells at ≥32 μM Cr(VI)—suggesting treatment-induced cell cycle arrest; at these concentrations Cr(VI) increased MN frequency (**Fig. 8A**). In contrast, the positive control mitomycin-C (MMC) significantly ($p < 0.001$) increased MN frequency at concentrations that did not greatly reduce cell numbers or the percentage of bi-nucleated cells. Assays in A549 (a human lung adenocarcinoma epithelial cell line) caused relatively small but statistically significant ($p < 0.05$) increases in cytotoxicity and decreases in the percentage of bi-nucleated cells at 3.2 μM. At this concentration, the frequency of MN in bi-nucleated cells was slightly increased from 1.47±0.50 to 2.12±0.41% (**Fig. 8B**). At higher Cr(VI) concentrations, cell death was extensive. The positive control MMC significantly increased MN frequency from 1.47±0.50 to 6.89±2.24%. These data indicate that Cr(VI) has very weak (or negligible) genotoxic potential at noncytotoxic doses, and are consistent with the negative *in vivo* MN results discussed in **Section 3.1.2.2**.

3.2.3. In vitro intestinal Caco-2 cell model

Caco-2 cells are a well-accepted model for studying intestinal absorption, metabolism and cytotoxicity (Meunier *et al.*, 1995; Natoli *et al.*, 2011; Rawlinson *et al.*, 2010; Sambruy *et al.*, 2001; Shah *et al.*, 2006; Smetanova *et al.*, 2011). When initially plated, Caco-2 cells are undifferentiated and proliferating—similar to crypt enterocytes. However, when grown to confluency (~21 days), the cells spontaneously differentiate and exhibit structural and molecular characteristics more similar to villous enterocytes. This *in vitro* model allows for differentiation of responses between villous and crypt enterocytes of the small intestine, which is the target tissue for carcinogenicity.

Caco-2 cells were treated with Cr(VI) for 24 hours and DNA damage was assessed by nuclear staining intensity of 8-OHdG and phosphorylated histone variant H2AX (γ-H2AX) using high content imaging methods (Thompson *et al.*, 2012a). In undifferentiated Caco-2, Cr(VI) increased 8-OHdG staining at non-cytotoxic concentrations, but increased both 8-OHdG and γ-H2AX staining at cytotoxic concentrations (*viz.* ≥3 μM). In contrast, differentiated Caco-2 cells were more resistant to Cr(VI)-induced DNA damage, and cytotoxicity was observed at 100 μM (but not at ≤30 μM). These *in vitro* results predict that Cr(VI) exposure *in vivo* might induce more adverse effects in crypt enterocytes than villous enterocytes. However, the *in vivo* Cr(VI) studies described herein show that Cr(VI) causes toxicity to intestinal villi without apparent treatment related effects on the crypt enterocyte—specifically, no change in mitotic and apoptotic indexes, or formation of micronuclei within the crypt. In fact, the higher Cr(VI) exposures resulted in more (not fewer) enterocytes in the crypts (**Fig. 4C**). Taken together, the *in vitro* and *in vivo* findings suggest that crypt enterocytes were minimally (or not at all) exposed to Cr(VI) *in vivo*. In fact, the intestinal mucosa is covered by a protective mucus layer that serves as a barrier to bacteria, viruses, particles, and other toxins (DeSesso and Jacobson, 2001; Greaves, 2007;

Talley, 2010). Coupled with the fact that crypt enterocytes do not function to absorb dietary nutrients, the lack of toxicity in the crypts is consistent with basic intestinal biology. Thus, the tumors that arose following chronic Cr(VI) ingestion were, as suggested by the other data summarized herein, due to prolonged increased cell turnover in an oxidized environment rather than direct DNA reactivity.

3.3. Concordance of Dose-Response

Following 90 days of exposure, chromium levels in duodenal tissue significantly increased at ≥14 mg/l SDD, and GSH/GSSG ratio significantly decreased at this dose. Cytoplasmic vacuolization and other signs of villous toxicity significantly increased at ≥60 mg/l SDD, and crypt cell proliferation significantly increased at ≥170 mg/l SDD (Thompson *et al.*, 2011b). This dose concordance of key events can also be seen when plotted on a full dose-response scale (**Fig. 9**). For simplicity, Key event 1 in the MOA (i.e. absorption) is not shown on the plot because it is an important pharmacokinetic event that also serves as a potential dose metric (e.g. μg Cr/g duodenum) on which to plot the subsequent key events. Instead, the change in GSH/GSSG ratio serves as both an indicator of uptake as well as an early (and reversible) biochemical component of intestinal cytotoxicity. Cytoplasmic vacuolization, which may also be reversible, is another indicator of cytotoxicity and can be clearly seen to precede crypt cell proliferation (Key Event 3). **Fig. 9A-B** demonstrate the dose concordance among these key events in the duodenum at both day 8 and 91. The dotted line in the **Fig 9B** represents the duodenal adenoma incidence in female mice at termination of the NTP 2-year bioassay. The plot clearly supports a sequence of events beginning with decreased GSH/GSSG ratio, followed by cytoplasmic vacuolization and crypt cell proliferation, and ultimately resulting in adenoma formation (which typically precedes carcinoma formation). A similar pattern of key events occurs in both the duodenum and jejunum

at day 91 (**Fig 9C**), which is consistent with finding that gene responses to Cr(VI) in the duodenum and proximal jejunum were generally quite similar (Kopec *et al.*, 2012a). Preliminary benchmark dose modeling of these endpoints support the sequence of key events (data not shown).

3.4. Temporal Association

Key events that occur at the same time as tumors are not likely to contribute to tumor development (Boobis *et al.*, 2006). In this regard, oxidative stress, toxicity to intestinal villi and crypt hyperplasia can be seen as early as 7 days of exposure (**Table 5**). After 90 days of exposure, damage to villi and crypt hyperplasia can be seen across multiple doses without tumors or preneoplastic lesions such as focal hyperplasia. Similar intestinal pathology (i.e. "diffuse hyperplasia") was reported in the NTP 90-day bioassay (NTP, 2007). After two years of exposure, diffuse hyperplasia was observed in all treatment groups (i.e. ≥14 mg/l SDD) and tumors at ≥172 mg/l SDD relative to concurrent controls (≥57 mg/l relative to historical controls). These findings suggest that in the drinking water studies conducted to date, mice experienced increased cell proliferation and redox changes that began within the first week of exposure. The fact that neither preneoplastic lesions nor tumors were observed in the 90-day studies and that tumors did not occur until 450 days or later (NTP, 2007; Thompson *et al.*, 2011b) suggests that it takes the majority of the two-year mouse lifespan for these events to contribute to tumor formation. A highly proliferative tissue experiencing oxidative stress is expected to provide the ideal environment of an early mutation—yet no increase in *Kras* codon 12 GAT mutation frequency was observed with Cr(VI) administration. Similar conclusions can be gained from Cr(VI) bioassays of the rat lung, where tumor formation is nonlinear and

dependent on tissue damage and inflammation (Beaver *et al.*, 2009; Glaser *et al.*, 1986; Steinhoff *et al.*, 1986).

3.5. Plausibility

Cytotoxicity and subsequent regenerative hyperplasia is a well known MOA (Ames *et al.*, 1993; Boobis *et al.*, 2009; Meek *et al.*, 2003; U.S. EPA, 2005); in fact it has been suggested that cytotoxic and proliferative effects observable by 13 weeks of exposure can be predictive of effects in 2-year bioassays (Cohen, 2010; Gaylor, 2005; Slikker *et al.*, 2004). The small intestine is one of the most proliferative tissues in the body and thus the additional proliferative pressure due to chronic high dose Cr(VI) exposure is likely to result in more spontaneous mutations.

Considering that Cr(VI) exposure did not increase mortality and the tumors observed in mice did not appear to metastasize (NTP, 2008a), the WOE supports that the increase in tumors at the termination of the NTP 2-year bioassay was the result of increased lifetime crypt proliferation as opposed to direct mutagenesis. The lack of preneoplastic focal hyperplasia further suggests that pockets of clonal expansion that might arise from a mutagen did not occur. This is consistent with the lack of aberrant nuclei formation or change in *Kras* mutation frequency in the small intestine (O'Brien *et al.*, in prep).

The role of spontaneous mutation and the relatively long time-to-tumors in mice is roughly similar to the likely roles of these events in humans. In sporadic colon cancer in humans, tumor initiation occurs after the third decade of life, progresses for 10-20 years, and results in carcinoma at a mean age of ~68 years (Grady and Carethers, 2008). In the context of historically shorter human lifespans, the intestine has likely evolved to withstand carcinogenesis over the normal human lifespan. Genetic disorders, however, that result in either accelerated initiation or

accelerated progression can lead to earlier tumor development (Grady and Carethers, 2008). In this colon cancer paradigm, increasing the number of cell divisions within the intestinal crypt over a lifetime due to chronic intestinal wounding would shift the normal progression of spontaneous tumors leftward resulting in earlier tumor formation. In this regard, the tumors observed in the NTP 2-year bioassay were the same as those that occur spontaneously, but only rarely in untreated animals (NTP, 2008a).

3.5.1. Similarities with other chemicals that have caused small intestinal tumors in rodents in two-year bioassays

NTP (2008a) noted that captan (NCI, 1977) was the only other chemical found to induce both benign and malignant intestinal neoplasms of epithelial origin in B6C3F1 mice in carcinogenicity studies conducted by NTP. Like Cr(VI), captan, and the structurally-similar chemical folpet, have been shown to exhibit mutagenic activity *in vitro*, but evidence for *in vivo* mutagenicity is equivocal or negative (Arce *et al.*, 2010; Bernard and Gordon, 2000). Captan and folpet induce similar phenotypes as Cr(VI) including cytotoxicity to villi, blunting of villi, regenerative crypt cell proliferation, and adenomas and carcinomas in the mouse duodenum and, to a lesser extent, jejunum (Cohen *et al.*, 2010). Furthermore, captan and folpet also do not induce intestinal tumors in rats. Mechanistically, captan and folpet induce toxicity by binding to thiols in GSH and proteins; and folpet has been shown to significantly deplete duodenal GSH within the first few hours of exposure and then significantly increase GSH by 24 hours relative to untreated animals (Arce *et al.*, 2010; Cohen *et al.*, 2010). Although GSH levels were not measured in the intestine within 24 hrs of exposure to Cr(VI), Cr(VI) elicited dose-dependent increases in intestinal GSH at both day 8 and day 91. Thus it seems likely that Cr(VI) would also acutely deplete GSH levels before cells have sufficient time to adapt and synthesize more GSH.

In 2004, the U.S. EPA changed their cancer classification of captan from “a probable human carcinogen” (Category B) to “not likely” after an independent peer-review concluded that captan acted through a non-mutagenic MOA that “required prolonged irritation of the duodenal villi as the initial key event” (Gordon, 2007; U.S. EPA, 2004). The similar effects induced by captan, folpet, and Cr(VI) make it plausible that they share a common non-mutagenic MOA that likely involves point of contact cytotoxicity in the villi followed by sustained proliferative pressure on crypt cells to regenerate the mucosa. Over a lifetime of exposure to such cytotoxic concentrations, there is an increased risk of carcinogenesis.

3.6. Alternative MOAs

Alternative MOAs include mitogenic and mutagenic MOAs. With regard to the former, there is no evidence to suggest that Cr(VI) stimulates crypt hyperplasia in the absence of cytotoxicity, as hyperplasia occurs subsequent to villous cytotoxicity in both dose and time. With regard to the latter, prior to the conduct of the studies described herein, some investigators hypothesized that the intestinal tumors observed in mice arose by a mutagenic MOA—based primarily on data from non-target tissues and *in vitro* systems (McCarroll *et al.*, 2010). The U.S. EPA has proposed a general framework for assessing a mutagenic MOA in their draft guidance, *Framework for Detecting a Mutagenic Mode of Action for Carcinogenicity* (U.S. EPA, 2007). Although still in draft form, the framework nevertheless provides insightful approaches for determining whether a chemical acts through a mutagenic MOA. First and foremost, the framework explicitly states the critical question, "*Is mutation an early key event in the chemical's induction of cancer?*" As part of determining a mutagenic MOA, one must a) establish whether a chemical has mutagenic properties; b) establish whether mutagenicity is relevant to the MOA in the tissue of interest by considering dose-response concordance, temporal concordance, and plausibility; and c) consider alternative MOAs.

As shown in **Table 6**, data from the small intestine do not support dose-response concordance, temporal concordance, or plausibility associated with a mutagenic MOA. With exposure to increasing concentrations of Cr(VI), there was no evidence of increased cytogenetic damage in crypt enterocytes or *Kras* mutations; in contrast, cytotoxicity, oxidative stress, and crypt hyperplasia were all increased in a dose-dependent manner. With increased duration of exposure to Cr(VI), there was no evidence of increased cytogenetic damage in crypt enterocytes or incidence of preneoplastic lesions, whereas oxidative stress, cytotoxicity, and crypt hyperplasia were all worse (or more prevalent) at day 91 relative to day 8. Thus, the hypothesis

that mutation is an early initiating key event in the intestinal carcinogenesis is inconsistent with the absence of cytogenetic damage, *Kras* mutations, or preneoplastic lesions (NTP, 2007; NTP, 2008a; Thompson *et al.*, 2011b), as well as the late tumor onset, lack of tumors in non-portal of entry tissues, and lack of increased mortality (NTP, 2008a).

In addition, most *in vivo* micronucleus studies are conducted in proliferative tissues like bone marrow, skin, and intestine because proliferation facilitates the detection of genotoxicity (Morita *et al.*, 2011). Because the small intestine is a highly proliferative tissue, evidence of DNA damage and mutation would be readily apparent if Cr(VI)'s tumorigenicity was acting via a genotoxicity or mutagenicity MOA.

U.S. EPA (2007) provides a list of characteristics typical of mutagens; as shown in **Table 7**, data from the small intestines indicate that Cr(VI) does not exhibit these characteristics. For example, mutations found in genes that are associated with carcinogenesis (e.g. p53) and in the presence of low cytotoxicity increase the WOE for a mutagenic MOA; however, mutations in *Kras* were not observed, despite the presence of tissue damage. It is also stated that tumor responses generally occur early in chronic studies (e.g. within 52 weeks), yet the tumors in the NTP study were observed late in the study and did not increase mortality (NTP, 2008a).

Boobis *et al.* (2009) have also outlined general key events for chemicals with a mutagenic MOA (**Table 7**). Notably, clonal expansion of mutated cells often increases mutations in other key genes and leads to preneoplastic lesions. Thus, even if Cr(VI) did not specifically "target" *Kras*, a general increase in mutations would likely lead to increases in additional mutations in *Kras* codon 12 GAT which was measured using the highly sensitive ACB-PCR assay. Furthermore, the lack of preneoplastic lesions suggests that clonal expansion of cells with growth advantages (such as can be conferred by *Kras* mutations) were not present. Importantly, Boobis

et al. state that, "...carcinogens are considered in two clearly distinguished groups based upon their MOA—DNA-reactive (mutagenic) and non-DNA-reactive (e.g. receptor-mediated, mitogenic, cytotoxic, oxidative stress)." The evidence herein supports involvement of both cytotoxicity and oxidative stress as key events in the MOA**. [Note to authors: I believe that this argument might be enhanced, if the gene array data do not suggest DNA replication due to regenerative hyperplasia rather than DNA repair. Do they?]**

In summary, the DNA reactivity of chromium is well established (Chiu *et al.*, 2010; Nickens *et al.*, 2010; O'Brien *et al.*, 2003; Zhitkovich, 2011; Zhitkovich, 2005). Known and/or suspected forms of DNA damage associated with Cr(VI) exposure include DNA adducts, single and double strand DNA breaks, inter- and intra-strand crosslinks, oxidative DNA damage, and replication blockage. However, as discussed in the Eastern Research Group peer-review panel comments to the Draft EPA Toxicological Review for Hexavalent Chromium (ERG, 2011), most of these many genotoxic lesions are not likely to be mutagenic, and the WOE supports that Cr(VI) is, in fact, a very weak mutagen. Further, it is important to note that much of this evidence does not come from *in vivo* data, as we show here, but rather from *in vitro* systems, often comprising acellular *in vitro* reactions and possibly *ex vivo* Cr-DNA adduct formation.

3.7. Potentially Susceptible Subpopulations

In fasting conditions, gastric reduction of Cr(VI) to Cr(III) is more rapid at low pH (Kirman et al. in press). This suggests that individuals with higher gastric pH might reduce Cr(VI) less efficiently, and as a result, have a higher tissue dose of Cr(VI) in the small intestine upon ingestion, relative to normal humans. This potential susceptibility is due to pharmacokinetic differences, rather than dynamic differences. Individuals with altered gastric secretion (e.g. achlorhydria or hypochlorhydria) have increased Cr(VI) absorption, presumably due to impaired

gastric reduction of Cr(VI) to Cr(III) (Donaldson and Barreras, 1966). Individuals taking proton pump inhibitors (PPIs) have an intragastric pH range of 4 to 10. This range is higher than that in healthy adults, who have fasting gastric pH of around 1.5 and postprandial gastric pH of around 5 (Amendola *et al.*, 2007; Dressman *et al.*, 1990; Gardner *et al.*, 2004). Neonates, defined as infants less than 4 weeks old, have fasting gastric pH values of 3-4 and a postprandial gastric pH of 7 (Nagita *et al.*, 1996). Thus, neonates and individuals taking PPIs are likely to have higher gastric pH than the average adult. Notably, gastric pH of neonates will decrease to a range of 1 to 3 within 1 day post-birth, but their gastric acid production is poorly maintained and low compared to that of adults (H+ per hour, 0.15 mmol/10 kg bodyweight in neonates vs. 2 mmol/10 kg in adults) (Koren, 1997). However, by age at 2 to 3 years, gastric production reaches to that of adult capacity, and the gastric pH is consistent to that of adults (Koren, 1997; Nagita *et al.*, 1996). It is important to consider, however, that the pH of the stomach contents of rats and mice of the same strain and in fed conditions, on the same chow as the animals in the NTP study, have a pH of 4.25 to 4.5 (Proctor *et al.*, 2012). This suggests that individuals who have a higher pH stomach than healthy adults, have stomach conditions of similar pH to that of the rodents in the 2-year bioassay. Furthermore, the capacity of human stomach fluid to reduce Cr(VI) to Cr(III) increases substantially in fed conditions when gastric pH is higher relative to fasted conditions because of the excretion of gastric acid and enzymes associated with anticipation of and consumption of food. In fact, De Flora et al. (1987) evaluated 1 healthy volunteer and 16 duodenal ulcer patients on acid blockers and found maximum peaks in stomach reduction capacity post-prandially of 40-60 μg/l, whereas Cr(VI) reduction during the fasted state was less pronounced (< 20 μg/L) for all monitored subjects.

Individuals with conditions that include inflammation of the bowl are at increased risk of gastrointestinal cancer, and may be more sensitive to Cr(VI) exposure at doses that are sufficient to cause cytotoxicity to villi. For example, people with celiac disease are at risk for adenocarcinomas (Green and Cellier, 2007), in part due to increased oxidative stress (Stojiljkovic *et al.*, 2012). Chronic infection with *H. pylori* can reduce gastric acid and enzyme secretions, which promotes bacterial growth, resulting in inflammation, epithelial cell proliferation, as well as oxidative stress (Kumar *et al.*, 2005). Individuals with *H. pylori* infection are at a 3- to 6-fold increased risk of stomach cancer as compared to non-infected individuals (EUROGAST, 1993). Prevalence of *H. pylori* infection in under developed countries is greatest, and rates of infection among older Japanese populations, as well as the rates of gastric cancer, in Japan have been elevated as compared to western countries (EUROGAST, 1993). Thus, if Cr(VI) increases the risk of stomach and intestinal cancer in individuals infected with *H. pylori*, it might be observable in Japanese cohorts of workers exposed to Cr(VI). However, a recent meta analysis of Cr(VI) exposed workers that assessed gastrointestinal tract cancers included subgroup analyses of studies by region including Japanese cohorts (Gatto *et al.*, 2010). In the meta-analysis, no increase in stomach cancer (meta-SMR= 0.89 for stomach cancer) was found for the Japanese workers, and none of the studies of Japanese workers reported on small intestinal cancers. Although workers are exposed primarily by inhalation, a fraction of the inhaled dose is swallowed resulting in low-level exposure that may be comparable to drinking water exposures around the MCL (Gatto *et al.*, 2010). As the MOA for Cr(VI)-induced intestinal carcinogenesis requires long-term cytotoxicity and a regenerative pressure, enhanced susceptibility by *H. pylori* infection, and other inflammatory bowl conditions, is not expected to increase risk of cancer at non-cytotoxic exposures.

As discussed for Key Event 1, the gastrointestinal tract is a dynamic system, and the rate of Cr(VI) loading to the small intestine is dependent on the rate of Cr(VI) reduction in the lumen, stomach emptying, gastric acid production, production of other reducing agents such as cysteine in the small intestine, food consumption and tissue absorption. Hence, PBPK models are needed to extrapolate between species, across dose, and may also be useful for assessing the toxicokinetics of potentially susceptible populations for use in risk assessment. Such an approach is a considerable improvement over the use of body-weight scaling to extrapolate between species and application of default uncertainty factors to account for sensitive subpopulations.

3.8. Data Gaps

As with all datasets, there are some gaps in our knowledge of the sequence of key events. However, these gaps represent more of a deficiency in the detailed mechanism of action as opposed to the more general key events (i.e. MOA) necessary for human health risk assessment (U.S. EPA, 2005). The ability to distinguish between Cr(III) and Cr(VI) in biological samples could greatly enhance detailed understanding of Cr(VI) toxicity. To date, however, these forms cannot be readily distinguished. In addition to measures of the GSH/GSSG ratio, alternative measures of oxidative status, such as cysteine/cystine and NAD^+/NADH ratios and lipid oxidation, could be informative. Further, measures of Cr-DNA adducts that can be reliably measured *in vivo* and differentiated between crypt and villi enterocytes could be helpful. With regard to *in vivo* mutation analysis, our requested access to paraffin embedded tumor samples from the NTP 2-year bioassay would allow for DNA extraction and determination of the specific mutations, if any, that might be more prevalent in Cr(VI)-induced intestinal tumors. Such knowledge would provide a means for more refined focus in the mutation analysis conducted in

shorter-term assays~~; however, attempts to obtain this information and/or access these tissues for analysis have not been successful~~. Further, some studies have shown epigenetic changes in DNA methylation following chromium exposure (Klein *et al.*, 2002; Sun *et al.*, 2009; Takahashi *et al.*, 2005) and thus assessment of genomic methylation status might also provide additional useful information on chromium toxicity.

One of the goals of the 90-day MOA studies was to collect similar data in both mice and rats to better understand the species differences in tumor development. The previous NTP studies did not observe diffuse hyperplasia or tumors in the intestines of rats (NTP, 2007; NTP, 2008a). In contrast, Thompson *et al.* (2012c) reported that rats exhibited similar intestinal lesions as mice, *viz*. apoptosis in the villi and crypt hyperplasia at ≥170 mg/l SDD. The reason for these differences in the rat studies likely relates to water intake, which was nearly 2-fold higher in Thompson *et al.* (2012c), but consistent with published intake values (U.S.EPA, 1988). These findings indicate that with increased Cr(VI) exposure, rats begin to exhibit non-neoplastic lesions similar to those observed in mice, and suggest that dosimetry is a major factor in the intestinal carcinogenesis of Cr(VI).

4. HUMAN RELEVANCE

Human relevance addresses three fundamental questions: 1) is the WOE sufficient to establish the MOA in animals, 2) are the key events plausible in humans, and 3) are the key events plausible in humans after accounting for pharmacokinetics and pharmacodynamics (Boobis *et al.*, 2006; Meek *et al.*, 2003). In our initial MOA assessment it was concluded that the WOE to establish the MOA in animals was not sufficient and that additional data were needed, in particular from the target tissues (Thompson *et al.*, 2011a). As such, an effort was undertaken to gather data necessary to fill the data gaps identified and the results of this research effort have

been described in detail in the preceding sections of this manuscript. Based on findings summarized herein, we conclude that the WOE is sufficient to establish the MOA in mice and that this involves the key events described in **Fig. 2**. Furthermore, these key events are relevant to humans (provided that dose is sufficient), as the basic principles of Cr(VI) chemistry and reduction and intestinal structure are similar in humans and rodents. As shown in the human relevance framework diagram in **Fig. 10**, the remaining question is whether kinetic differences between humans and rodents indicate that the MOA is either likely or unlikely. The answer to this question depends on whether the pharmacokinetics of Cr(VI) are sufficiently known between mice and humans, and physiologically based pharmacokinetic models have been developed to assess the disposition of Cr(VI) in the gastrointestinal tracts of mice, rats and humans (Kirman *et al.*, in press). The Cr(VI) concentrations that induced tumors in mice (≥ 20 mg/l Cr(VI)) are orders of magnitude higher than typical drinking water exposures, which average 0.001 to 0.005 mg Cr(VI)/l. Given the lack of epidemiological evidence for increase in the risk of intestinal cancers in humans at these environmental levels (Gatto *et al.*, 2010; Fryzek *et al.*, 2001; Gatto *et al.*, 2010; Morgan, 2011), it would be necessary for human pharmacokinetics (or dynamics) ~~would have~~ to differ substantially from mice in order for typical exposures to pose a cancer risk. In this regard, ~~populations that have been exposed to Cr(VI) do not appear to have elevated risks of intestinal cancer or elevated cancer rates overall).~~the physiologically based pharmacokinetic models**… [Note to authors: SHOW WHAT?]**

~~is likely yes; however, given the lack of epidemiological evidence for increase in the risk of intestinal cancers in humans (Gatto *et al.*, 2010), it is critical that human pharmacokinetics be considered as well as typical environmental exposure levels.~~

~~To address~~

5. CONCLUSION

MOA analysis is a key element in human health risk assessment (Boobis *et al.*, 2006; Boobis *et al.*, 2009; Meek *et al.*, 2003; Seed *et al.*, 2005; U.S. EPA, 2005). When a general MOA has been well-established, it "lowers the barrier" of acceptance when such a MOA is proposed for another chemical/tumor site of interest (Boobis *et al.*, 2006). In this regard, Cr(VI) shares similar toxicological and carcinogenic characteristics as captan and folpet, which have previously been determined to act via a cytotoxic nonmutagenic MOA (Arce *et al.*, 2010; Cohen *et al.*, 2010; Gordon, 2007; U.S. EPA, 2004). The relative rarity of intestinal tumors means that risk assessors have had comparatively less experience analyzing MOAs for carcinogenesis of the small intestine. In fact, 80-90% of cancers observed in rodent bioassays occur in eight tissues/systems, and the small intestine is not among these (Gold *et al.*, 2001). Even though Cr(VI) and captan/folpet do not share structural similarity, their toxicological and carcinogenic phenotypes ~~begin to~~ establish a general MOA for intestinal neoplasms induced by environmental chemicals that involves chronic wounding and healing of the intestinal mucosa.

The newly developed target and tissue-specific MOA data provides a substantially stronger and more scientifically robust basis for assessing the MOA for Cr(VI)-induced intestinal carcinogenesis than has been available previously. The WOE from these studies strongly support that Cr(VI) acts by a cytotoxic MOA in the development of rodent small intestinal tumors. It is important to recognize that this MOA is specific to the small intestine, and although may be informative to other tissues, it is not necessarily specific to all tumors caused by Cr(VI). Chemicals may act by multiple MOAs, at different tumor sites and potentially at different doses (EPA, 2005); however the MOA that is specific to the tumor-response used for risk assessment is critical for low-dose extrapolation. Because mouse small intestinal cancers have been used as the basis of a number of quantitative risk assessments for ingested Cr(VI) (McCarroll *et al.*, 2010; OEHHA, 2009; Stern, 2010; U.S. EPA, 2010), these MOA findings specifically inform the most appropriate approach for low-dose extrapolations for these and other new risk assessments on oral exposure to Cr(VI). To further improve the information available for risk assessment, newly developed rodent and human PBPK models will allow for extrapolation of dose across species and wide dose ranges, which will be critical for any human health risk assessment conducted for oral exposure to Cr(VI).

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Table 6. U.S. EPA Mutagenic Framework (U.S. EPA, 2007) and Application to Cr(VI) in the Small Intestine

Steps	Evidence
1) Establish whether chemical has mutagenic activity	Cr(VI) is a weak mutagen
2) Establish whether mutagenicity is relevant to MOA in target tissue (follow MOA framework in U.S. EPA (2005) Cancer Guidelines)	
• Dose-response concordance	No evidence of increased crypt cytogenetic damage or *Kras* mutations with increased dose
• Temporal concordance	Villous cytotoxicity and crypt hyperplasia evident as early as day 8, without evidence crypt cytogenetic damage (at day 8 or day 91) or *Kras* mutations at day 91
• Plausibility	Early mutation in highly proliferative intestinal mucosa is not consistent with absence of cytogenetic damage, absence of *Kras* mutations, absence of preneoplastic lesions (e.g. focal hyperplasia) (NTP, 2007, 2008a; Thompson *et al.*, 2011b), late tumor onset (NTP, 2008a), lack of tumors in non-portal of entry tissues, and lack of affect on survival (NTP, 2008a).
3) Consider alternative MOAs	Described herein

Table 7. Select Properties of Mutagenicity as Key Events and Application to the Cr(VI) in the Small Intestine

U.S. EPA (2007)	Cr(VI) Study Results
Mutations seen in the presence of low cytotoxicity increase WOE	Mutations not seen, even in the presence of toxicity.
Mutations in genes that affect carcinogenesis increase WOE.	No mutations in *Kras* observed.
Direct DNA reactivity	Evidence for this is equivocal.
The target cell/tissue is exposed to the ultimate DNA reactive chemical.	Data suggest that intestinal crypts were not directly exposed.
DNA of target cell is damaged	No evidence of cytogenetic damage.
Tumors observed at multiple sites, in multiple species.	Only one tumor location in each species, despite presence of Cr in multiple tissues.
Tumor response generally occur early in chronic study (e.g. within 52 weeks).	Tumors in small intestine or mice and oral mucosa of rats did not occur early, and were not associated with lethality or even metastases.
Boobis *et al.* (2009)	Cr(VI) Study Results
Exposure of target cells to ultimate DNA reactive and mutagenic species—with or without metabolism	Evidence does not support that Cr(VI) is reaching the target cells (i.e. crypts)
Reaction with DNA in target cells to produce DNA damage	No evidence of DNA damage, as evidenced by negative results for MN, KN, AI, and MI in crypts.
Misreplication on a damaged DNA template or misrepair of DNA damage	No evidence of DNA damage, as evidenced by negative results for MN, KN, AI, and MI in crypts.

Mutations in critical genes in replicating target cells	No evidence of increased *Kras* mutation frequency.
Clonal expansion leads to further mutations in critical genes.	No evidence of increased *Kras* mutation frequency due to Cr or clonal expansion.
Imbalanced and uncontrolled clonal growth of mutant cells may lead to preneoplastic lesions.	No evidence of preneoplastic lesions.