Research Framework for Evaluating the Potential Mode(s) of Action Underlying the Carcinogenicity of Hexavalent Chromium Following Exposure in Drinking Water

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Executive Summary: Hexavalent chromium (hereafter referred to as Cr(VI)) is a strong oxidizing agent. A two year cancer bioassay was completed for Cr(VI) (i.e., sodium dichromate dihydrate) in drinking water by the US National Toxicology Program (NTP, 2008; Stout et al., 2008). The NTP study design resulted in the development of intestinal tumors in mice and oral mucosal tumors in rats at daily concentrations in excess of 85 mg/L. Cr(VI) has been found to exhibit mutagenic activity in various *in vitro* tests and has been recognized to be an inhalation carcinogen in occupationally exposed humans. In the absence of data concerning the mode(s) of action underlying the carcinogenic response, the positive genotoxicity findings will likely lead state and federal regulatory agencies to develop an oral cancer slope factor for Cr(VI) that is based on a low-dose linear risk assessment.

Cr(VI) is readily absorbed into cells. However, ingested Cr(VI) is rapidly reduced to Cr(III) by saliva, gastric juices, and intestinal bacteria before it reaches the intestinal cell membranes through which it might be absorbed. The reduction of Cr(VI) to Cr(III) is an important defense mechanism, since Cr(III) complexes with other extracellular components and does not readily permeate the cell membrane. Studies have shown that the reduction of Cr(VI) to Cr(III) is through saturable kinetic processes (Snow 1992). Therefore, if these processes were saturated at the high doses used in the NTP study, a greater proportion of Cr(VI) would be available to cross the cell membranes and enter the target cells. Thus, results from the NTP high dose drinking water study in rats and mice are unlikely by themselves to provide accurate information estimating the carcinogenic potential of a strong oxidant like Cr(VI) at lower levels of exposures.

Once Cr(VI) crosses the cell membrane, a rapid reduction takes place (Snow 1992), due to intracellular reductants within the cytosol, endoplasmic reticulum, and mitochondria, including NAD(P)H, cytochrome P-450, ascorbate, and thiols (i.e., glutathione (GSH) and cysteine), with ascorbate and GSH being the most significant reductants. At the high doses used in the NTP study it is possible that the capacity of intracellular reduction could also be saturated, leading to greater penetration of Cr(VI) and the unstable reactive intermediates, Cr(V) and Cr(IV) to the nucleus.

Overall, low dose tissue responses to metals will be affected by non-linear absorptive processes in epithelial cells and exposures to oxidants will be countered by adaptive anti-oxidant stress pathways. Default low-dose linear, cancer risk assessments do not consider these other aspects of the biology of Cr(VI). Dose response relationships for various precursor responses to Cr(VI) need to be carefully evaluated in rodent tissues and cells *in vitro* to evaluate whether a linear risk assessment approach is appropriate for Cr(VI).

In order to develop a research framework to examine the potential mode(s) of action

underlying the carcinogenic response observed in rodents exposed to Cr(VI) in drinking water, we have developed a hypothesized mode of action (MOA). This hypothesized mode of action is based on the premise that high concentration drinking water exposure to Cr(VI) results in excessive oxidative stress that occurs when epithelial tissue burdens of Cr(VI), and even more reactive intermediate Cr valence states such as Cr(V) and Cr(IV), exceed the ability of the cells to counter oxidant stress. In the face of these higher levels of oxidant stressors, cytotoxicity ensues, leading to oxidative DNA damage, mutation and cancer at high exposure levels. In addition, high concentrations of Cr(VI) saturate reductive capabilities in rodent alimentary tract epithelial cells, resulting in non-linear accumulation of Cr(VI). This accumulation of Cr(VI) results in chronic irritation and inflammation, which in turn accentuate the tumor process at higher exposures. While this hypothesis is consistent with the reactivity of Cr(VI) and knowledge of homeostatic compensation processes in the face of normal oxidative stressors, these precursor responses have not been carefully evaluated over a broad range of Cr(VI) concentrations in buccal and intestinal epithelial cells *in vivo* or with *in vitro* cell models. An alternative mode of action has also been proposed in which the carcinogenic activity is attributed to interactions of Cr(III) with DNA. Under this hypothesis, the role of Cr(VI) would be to facilitate penetration of Cr(III) into the target cells. If Cr(III) interaction with DNA is the key element in the mode of action for Cr(VI) carcinogenicity, the potentially important sources of nonlinearity would be saturation of extracellular reduction and homeostatic control of Cr(III) associated with its essentiality. To distinguish between these alternative mode-of-action hypotheses, our proposed research plan will provide detailed data on the dose-response relationships for key events that lead to tumor development in rodents following exposure to Cr(VI) drinking water. The *in vivo* studies in rodents will be augmented with *in vitro* analysis of key precursor events in rodent and human cell lines. Collectively, these studies will build the scientific data needed to better understand the mode(s) of action underlying the carcinogenic response observed in rodents and will allow for development of a more robust cancer risk assessment for Cr(VI) ingestion in drinking water.

This proposal focuses on a series of studies associated with five key areas that affect evaluation of MOA for Cr(VI): (1) a 90-day in-life study to assess histological responses of the buccal and intestinal epithelium over six drinking water concentrations; (2) genomic studies on tissues from the 90-day study to assess dose response for alterations in gene families over the broad dose range; (3) pharmacokinetic modeling to evaluate expected non-linearities in epithelial tissue dose of Cr(VI); (4) *in vivo* mutation analysis from the exposed animals; and, (5) high data content *in vitro* studies to differentially assess the relative dose response for oxidative stress and DNA damage in relevant epithelial cells from rodents and humans. A sixth part of the proposal integrates these *in vivo* and *in vitro* studies into a MOA-based risk assessment for Cr(VI). This integrated body of information will provide quantitative relationships between dose and response for multiple endpoints and the differential dose-response relationships for these endpoints will support development of a more robust cancer risk assessments for Cr(VI) based on our understanding of dose-dependent transitions in the MOA over broad ranges of dose. The PK models will evaluate expected tissue concentrations at the site of contact (i.e., at the epithelial cells within the alimentary tract for Cr(VI)). Cumulatively these data are

expected to lead to a non-linear cancer risk assessment based on a point-of-departure calculation from the 2-year or 90-day exposure and estimation of the margin of exposure between the point-of-departure tissue Cr(VI) levels and current Cr(VI) tissue levels associated with ambient exposures in the general population.

Introduction:

Cr(III) is an essential trace element. The recommended daily intake of Cr for adults ranges between 25 and 45 μg (IOM, 2001). Cr(III) deficiency contributes to glucose intolerance and diabetes mellitus (Type 2). Cr(III), which is poorly soluble, displays little to no toxicity in humans and animals (ATSDR, 2000). Cr(VI), in contrast, is highly soluble and a strong oxidant.

Ingestion of high concentrations of Cr(VI) in drinking water for two years caused intestinal tumors in mice and oral mucosal tumors in rats (NTP, 2008; Stout et al., 2008). Even though the relevance of cancer at very high concentrations of Cr(VI) in drinking water is highly uncertain for humans exposed at environmental levels, regulatory agencies will likely rely on default, low-dose linear risk assessment modeling to derive an oral cancer slope factor for Cr(VI). More robust cancer risk assessments for Cr(VI) based on an understanding of the underlying mode(s) of action require more detailed evaluations of the dose response relationships for cell and tissue responses over a broader range of exposure concentration than those used in the NTP cancer bioassay. Because of its strong oxidant potential, a variety of studies are required to assess the carcinogenic MOA of Cr(VI) across various dose ranges. These targeted studies should be able to delineate regions of exposure where there is no significant response to Cr(VI), regions in which there is activation of adaptive, anti-oxidant response pathways, and, finally, regions where adaptive pathways are overwhelmed leading to cytotoxicity, DNA-reactivity, mutation and carcinogenicity. We hypothesize that only in the presence of overt cellular toxicity would there be a significant probability of cancer. Dose-dependent transitions in MOA are likely with many compounds (Slikker et al., 2004a, 2004b) and were highlighted as a key component in recent NAS reports addressing toxicity testing in the 21st century (NRC, 2006; 2007; Andersen and Krewski, 2009).

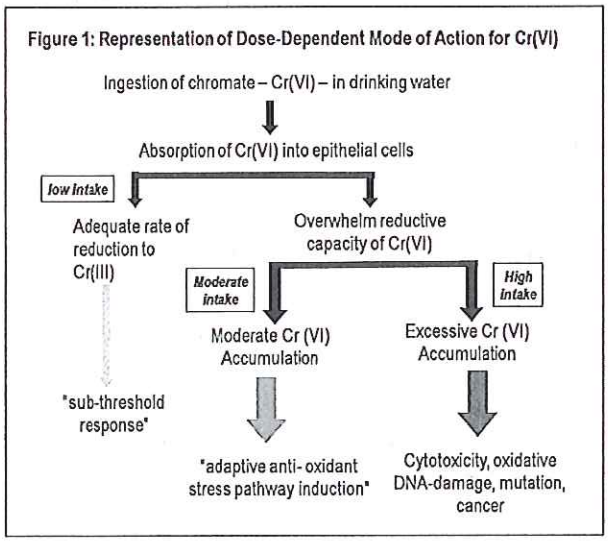


Figure 1. Representation of Dose-Dependent Mode of Action for Cr(VI)

A representation of our hypothesized MOA for Cr(VI) has differential responses for low, moderate and high intake (Figure 1). At high doses, reactive carbon-based radical species resulting from Cr(VI) accumulation in epithelial cells could lead to DNA-changes, including DNA strand breaks. These

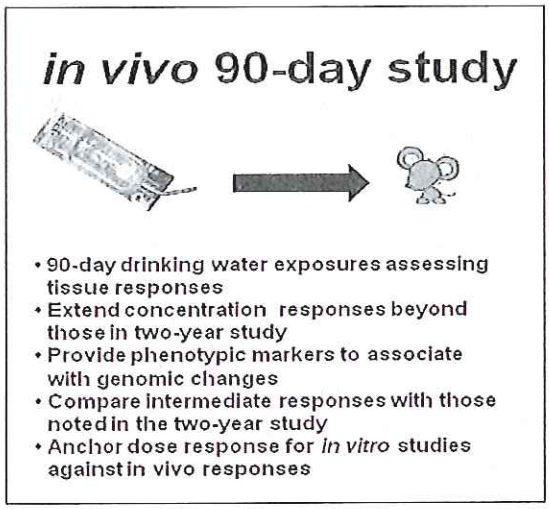
strand breaks attract specific binding proteins to participate in detection of the damage and initiate repair processes (See Attachment: BD Biosciences Application Note). With the reductive capacity overwhelmed the increasing Cr(VI) concentrations in the epithelial tissues would causes chronic irritancy and inflammation. Cytotoxicity would lead to apoptosis and cell proliferation allowing for increased mutational frequency. These key events only occur at high doses that overwhelm reduction to lead to accumulation, toxicity, and some probability of mutation. These key events do not occur at lower doses. Appendix A to this work plan provides additional information regarding what is known about the mode of action of hexavalent chromium.

There are no standard, off-the-shelf protocols or study designs that are available to investigate chemical MOA. Information from completed studies indicate a complex MOA for Cr(VI) including cellular stress, cytotoxicity, and genotoxicity. The DNA-reactivity is believed to arise from oxidative stress rather than adduction of DNA by Cr(VI). Experience with other compounds has demonstrated that the collection of data on the dose-response for genomic alterations following exposures of less than two years can provide the evidence for identifying the key components of the mode of action (Thomas et al., 2009) and for characterizing dose-dependent transitions (Thomas et al., 2007 (see attachment 2); Andersen et al., 2008). These genomic studies have assessed the alterations in gene families that indicate the changing biological processes affected at particular doses. For Cr(VI), the gene families of particular interest are those associated with oxidative stress, apoptosis, cell-replication, and DNA damage. The dose-response relationships for these alterations in gene families can be compared to the highly non-linear dose response curve for tumors, for *in vivo* mutation rates, or for histological tissue responses to provide evidence for dose-dependent transitions in the MOA for Cr(VI).

New technologies have also become available to simultaneously assess multiple cellular responses with high content imaging using cells from both rodents and humans (See Attachment 4: BD Biosciences Application Note). These approaches have great potential for assessing the cellular MOA of Cr(VI) at different concentrations and to evaluate species differences in cellular response through comparison of responses in rodent and human cells. High data content analysis (HCA) is particularly suited for assessing general cellular toxicity, oxidative stress and DNA-damage pathways over very broad dose ranges. The examination of broad dose ranges using sensitive assay procedures with low background can produce much more definitive evidence for thresholds and dose-dependent transitions for Cr(VI). *In vivo* assays are restricted in the number of dose groups and in variability in PK and PD processes within the test population. The dose-dependent transitions that need to be evaluated are associated with non-linear alterations in tissue response with increasing tissue dose (i.e., PD transitions) and with pharmacokinetic non-linearity's that arise from saturation of various processes (i.e., PK transitions). With Cr(VI), various kinetic parameters regulate total Cr distribution to epithelial cells in the buccal cavity and intestinal tissues, including capacity-limited site-specific reduction rates of Cr(VI) to Cr(III). The interaction of these processes determine the biologically effective target tissue dose of Cr(VI) at differing intakes and the magnitude of the cellular concentration determines whether the response is sub-threshold, adaptive, or cytotoxic.

Our overall research program consists of five key components providing results to evaluate non-linear, dose-dependent transitions related to the cancer MOA of Cr(VI): (1) a 90-day, in-life drinking water study at six concentrations, (2) a genomic evaluation of target tissue responses to Cr(VI), (3) an in life mutation study assessing mutational dose response in cells from animals in the 90-day study, (4) pharmacokinetic modeling to assess factors regulating epithelial tissue dose and how they vary across species, and (5) HCA of target epithelial cells *in vitro* assessing toxicity, oxidative stress, and DNA-damage in target epithelial cells exposed *in vitro*. A sixth portion of the program integrates the results of these studies to conduct a cancer risk assessment based on more complete evaluation of the dose-dependent transitions occurring upon exposure to Cr(VI) in drinking water. This proposal is organized into six sections, each describing a specific part of the overall project. Each section begins with a statement of the goal of the activity and then provides a rationale for the proposed work and an outline of the study design.

Section 1 – Subchronic (90-Day) Drinking Water Study



Goal: Conduct a 90-day drinking water study of hexavalent chromium [Cr(VI)] in female rats and mice to collect target tissues for evaluation of histopathology, gene expression, and mutagenicity.

Rationale: A 2-year drinking water study of Cr(VI) in male and female F344 rats and B6C3F1 mice resulted in the development of neoplasms of the squamous epithelium of the oral mucosa and tongue in rats, and increased incidences of neoplasms of the epithelial lining of the small intestine in mice (Stout et al., 2008). The only endpoint evaluated in these target tumor sites was histopathology. To fully characterize the dose-response relationship of Cr(VI) in these target tissues, gene expression (section 2) and mutation (section 4) analyses will be conducted to identify potential mode of action pathways of Cr(VI).

The Cr(VI) drinking water concentrations selected for evaluation will include the concentration range used in the NTP 2-year drinking water study and extend below the lowest concentration studied (14.3 mg/L), including an extremely low "environmentally relevant" concentration.. Females only will be evaluated based on the results of the 2-year drinking water study where differences in the carcinogenic responses between sexes were not significant. A 7-day interim necropsy will be performed to study acute changes that may form due to initial exposure, but possibly resolve with increasing duration of treatment. Magnetic resonance imaging (MRI) will also be conducted in mice following 7 days of Cr(VI) treatment (section 5).

The 90-day toxicity study has been recognized by regulatory agencies as a landmark study designed to permit the determination of a no-observed-adverse-effect-level (NOAEL) and adverse effects associated with continuous or repeated exposure to a test substance for a period of 90 days. Cellular events that occur during the course of a 90-day study may be predictive of effects that require a long latency for development (e.g., carcinogenesis). Ninety-day studies of five NTP chemical carcinogens recently conducted at The Hamner Institutes resulted in dose-related transcriptional changes correlating with 2-year tumor incidence (Thomas, 2009).

Description: Groups of female F344 rats and female B6C3F1 mice will be exposed to sodium dichromate dihydrate (SDD; CAS #7789-12-0) in the drinking water for either 7 days (n = 15/group/species) or 90 days (n = 20/group/species). Six SDD concentration groups will be selected for study and will include the concentration range used in the NTP 2-year drinking water study and extend below the lowest concentration studied (14.3 mg/L). to an extremely low "environmentally relevant" concentration where no effects are expected.

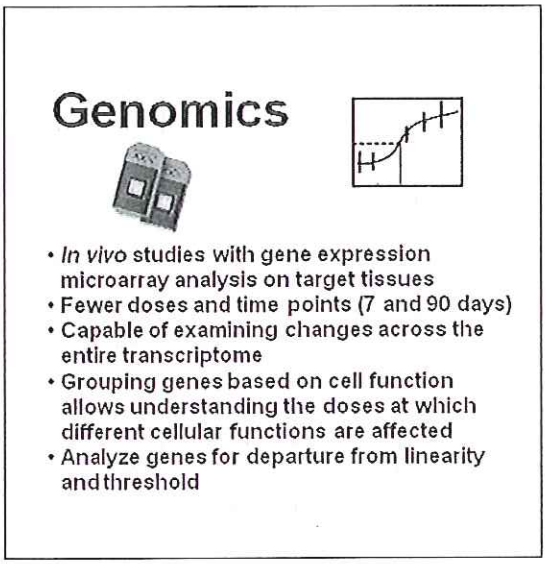
SDD Concentration	**Group Size**	**Animals for Histopathology**		**Animals for Gene Expression Analysis**		**Animals for Mutation Analysis**
		7 days	**90 days**	**7 days**	**90 days**	**90 days**
0 mg/L (control)	35 FR* 35 FM	5 FR 5 FM	5 FR 5 FM	10 FR 10 FM	10 FR 10 FM	5 FR 5 FM
X mg/L (environmentally-relevant)	35 FR 35 FM	5 FR 5 FM	5 FR 5 FM	10 FR 10 FM	10 FR 10 FM	5 FR 5 FM
X mg/L	35 FR 35 FM	5 FR 5 FM	5 FR 5 FM	10 FR 10 FM	10 FR 10 FM	5 FR 5 FM
X mg/L	35 FR 35 FM	5 FR 5 FM	5 FR 5 FM	10 FR 10 FM	10 FR 10 FM	5 FR 5 FM
X mg/L	35 FR 35 FM	5 FR 5 FM	5 FR 5 FM	10 FR 10 FM	10 FR 10 FM	5 FR 5 FM
X mg/L	35 FR 35 FM	5 FR 5 FM	5 FR 5 FM	10 FR 10 FM	10 FR 10 FM	5 FR 5 FM
X mg/L (high)	35 FR 35 FM	5 FR 5 FM	5 FR 5 FM	10 FR 10 FM	10 FR 10 FM	5 FR 5 FM
Total Animals	**245 FR** **245 FM**	**35 FR** **35 FM**	**35 FR** **35 FM**	**70 FR** **70 FM**	**70 FR** **70 FM**	**35 FR** **35 FM**

FR = female rats; FM = female mice

Quantitative analysis of drinking water solutions containing SDD will be conducted according to EPA method 218.6 "Determination of Hexavalent Chromium in Drinking Water, Groundwater, and Industrial Wastewater Effluents by Ion Chromatography". The

published detection limit for EPA method 218.6 is ~0.3 µg/L, making this an acceptable method to be used for this study. The Hamner's Research Quality Standards will govern the study. In-life evaluations include clinical observations, body weights, and food and water consumption. At conclusion of the exposure period, animals will be subjected to a full necropsy and observations recorded. Select tissues, including target tissues and gross lesions will be saved in 10% neutral buffered formalin. Target tissues (oral cavity and tongue in rats and small intestine in mice) will be embedded in paraffin wax, sectioned, deparaffinized and stained with hematoxylin and eosin (5/group/species/time point). Histopathologic examination of H&E stained slides of target tissues will be performed initially on the high dose and control groups. Histopathologic examination will continue on lower dose groups until a NOAEL is clearly recognized. In addition, target cell types will be collected for RNA isolation (10/group/species/time point) and DNA isolation (5/group/species at 90 days) for gene expression (section 2) and mutation (section 4) analyses, respectively.

Section 2 – Microarray Analysis



Goals:

- Analyze the dose-response changes in gene expression in the rat oral cavity and mouse duodenum following a 7 day and 90 day exposure to Cr(VI) in the drinking water.
- Perform benchmark dose analysis of the gene expression changes to identify potential modes-of-action for Cr(VI) and assess dose-dependent transitions in mechanism with a particular focus on genotoxicity.

Rationale: Over the past decade, the development and application of high-throughput, broad-coverage technologies have made it possible to comprehensively examine transcriptional changes following chemical exposure that can provide insights into potential key events that occur in the biological response. When these studies are performed in dose response, the analysis of the transcriptional alterations can be used to inform risk-based decisions on doses where biological effects are likely or unlikely to be observed and the potential modes-of-action of a chemical. Figure 2-1 shows a flowchart for analyzing gene expression data using gene ontology classification of families of genes rather than focusing on alterations of single transcripts in the toxicity studies.
An example of the application of gene expression dose-response analysis in examining modes-of-action can be found in the investigation of the transcriptional alternations following nasal formaldehyde exposure using microarray technology (Thomas *et al.*

2007). In this study, rats were exposed to different doses of formaldehyde and nasal epithelial tissue was isolated from the region with high tumor incidence. Gene expression microarray analysis was performed on the epithelial tissue. The gene expression data were analyzed using a novel method that combined benchmark dose methods with gene ontology analysis to provide reference dose estimates at which different cellular processes are altered (e.g., apoptosis, lipid metabolism, and DNA repair). The results with formaldehyde showed that transcriptional benchmark dose estimates for the gene ontology categories related to cell proliferation and DNA damage were similar to those measured in previous studies using cell labelling indices and DNA-protein cross-links and consistent with the benchmark dose estimated for rat nasal tumors. The combination of microarray technology with these analysis methods provided a broad survey of molecular changes in the nasal target tissues and showed that the majority of functional changes were occurring at a threshold above 1 ppm.

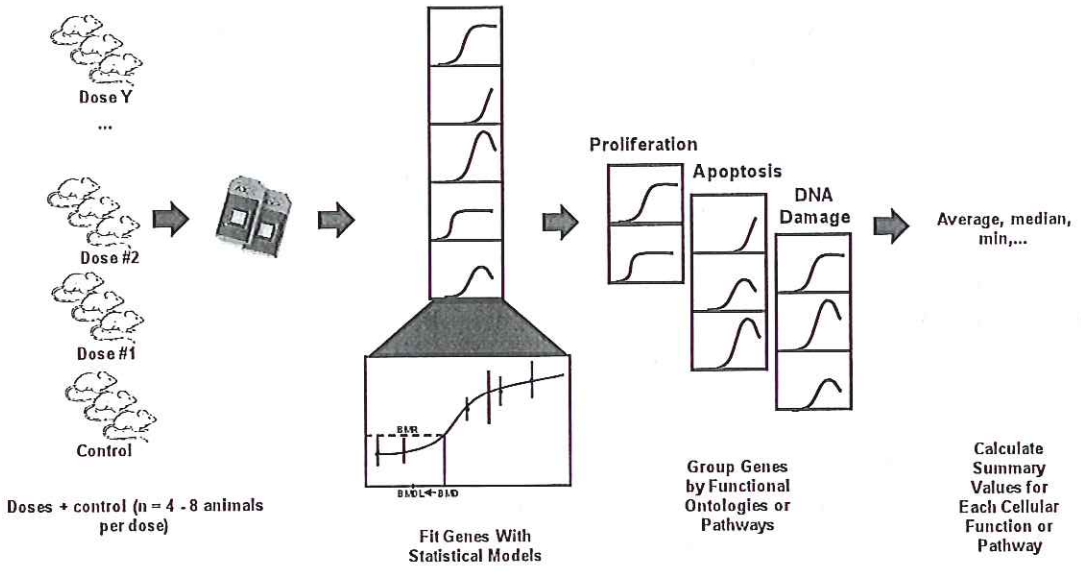


Figure 2-1: A flow chart outlining the stepwise analysis of dose-response microarray data using BMD calculations and GO classification analysis. The methodology provides dose estimates at which different cellular processes are altered at a defined increase in risk based on expression levels in the untreated controls.

The NTP rodent cancer bioassay for sodium dichromate dihydrate produced tumors in both rats and mice. In rats, squamous cell papillomas and carcinomas were observed in the oral mucosa at high doses in both males and females. In mice, adenomas and carcinomas were observed in the small intestine with a particularly high incidence in the duodenum. In this section, dose-response changes in gene expression will be analyzed in the target cells using benchmark doses methods with gene ontology (GO) categories to identify reference doses at which particular cellular processes are altered. As with other high dose carcinogens, we hypothesize that different groups of gene families will be altered at various exposure levels. These families would progress from mild markers of oxidative stress and general cell stress responses through apoptosis, inflammation, cell replication, and DNA damage responses pathways. The results from this study will used

to establish the presence of dose-dependent transitions in the mode-of-action in the intact rodent.

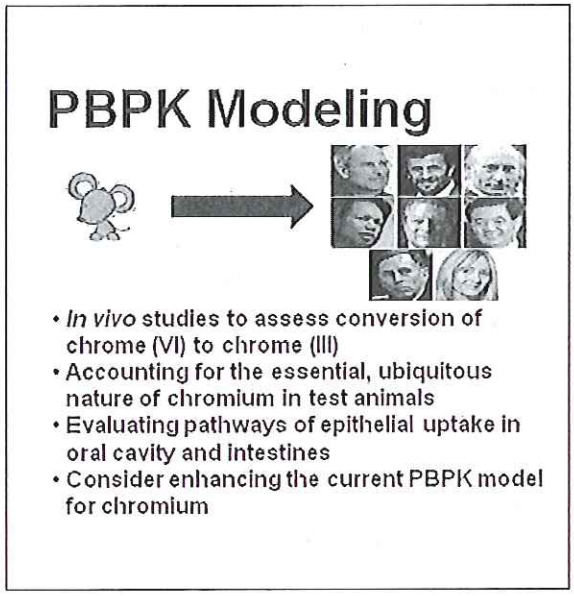
Description:

1) Method Development for RNA Isolation in Target Cell Types.
   a) Isolate epithelial cells from the rat oral cavity using both a standard scraping technique and protease digestion.
      i) Measure the cell yield for each isolation protocol
      ii) Evaluate tissue following isolation protocol to assess the extent the underlying submucosa was also isolated.
      iii) Isolate RNA from the epithelial cells and evaluate RNA quality using an Agilent Bioanalyzer.
      iv) Label RNA using Affymetrix labeling reagents and hybridize to test array to assess performance of the RNA in gene expression microarray analysis.
   b) Isolate epithelial cells from the mouse duodenum using previously established methods (Bjerknes and Cheng, 1981).
      i) Isolate RNA from the epithelial cells and evaluate RNA quality using an Agilent Bioanalyzer.
      ii) Label RNA using Affymetrix labeling reagents and hybridize to test array to assess performance of the RNA in gene expression microarray analysis.

2) Measurement of Gene Expression Changes in Rat and Mouse Target Tissues.
   a) Isolate epithelial cells from the rat oral cavity and mouse duodenum following 7 days and 90 days of chromium exposure (6 doses plus vehicle control).
   b) Purify RNA from five animals per dose and eight animals from control group. The reason for additional control animals is that it is important to precisely define the variability in the control population for the benchmark dose analysis.
   c) Evaluate RNA quality using the Agilent Bioanalyzer.
   d) Label RNA using Affymetrix labeling reagents and hybridize to Rat Genome 230_2 and Mouse Genome 430_2 arrays.

3) Analyze Dose-Response Changes in Gene Expression Using Benchmark Dose Methods
   a) Normalize gene expression data using Robust Multi-array Averaging (RMA).
   b) Fit dose-response changes in gene expression to linear, 2nd order polynomial, 3rd order polynomial, power, and Hill models.
   c) Select least complex model that best describes the data using log-likelihood ratio tests and AIC values.
   d) Group genes based on functional ontologies (GO biological processes) and signaling pathways (KEGG).
   e) Calculate summary values (e.g., median, standard deviation, minimum) for each GO category and signaling pathway.

Section 3 – Pharmacokinetic Modeling



Goals:

- Collect *in vivo* data on the reduction, uptake, and disposition of Cr(VI) in mice using magnetic resonance imaging (MRI).
- Collect *in vivo* data on the uptake and disposition of total Cr in mice at and below the doses used in the NTP bioassays
- Refine the PBPK models for the mouse and rat developed in the pilot study (See Attachment: Chromium Pilot Study) using the experimental data collected in the *in vivo* studies together with information from the literature on the homeostatic control of trivalent chromium.

Rationale: The extracellular and intracellular reduction of Cr(VI) at the tumor sites is a key event in the hypothesized Cr(VI) carcinogenic MOA. Standard analytical procedures cannot differentiate between the oxidation states of Cr in biological tissues. A direct approach to monitor the reduction of Cr(VI) and its distribution using magnetic resonance imaging (MRI) was demonstrated in pilot studies in the mouse. The preliminary data from the pilot studies indicate that "real time" site specific reduction of Cr(VI) can be tracked *in vivo* by studying the reaction rate of the formation of the paramagnetic form Cr(III) (see Figure 3-2 below). By comparing concentrations of Cr(III) from MRI with total Cr concentrations from traditional analytical methods, it should be possible to provide data on the kinetics of both valences in the intestinal lumen and tissue for use in refining the PBPK models developed in the pilot study.

The current PBPK models for chromium do not consider the potential role of essentiality in the transport and storage of chromium in the intestinal tissues. Since trivalent chromium is an essential element, there is undoubtedly some form of homeostatic control of uptake, probably involving active transport. There may also be controls in the enterocytes for switching between uptake (transport) and excretion (storage) that are dependent on the concentration of chromium in the diet, as is the case for iron.

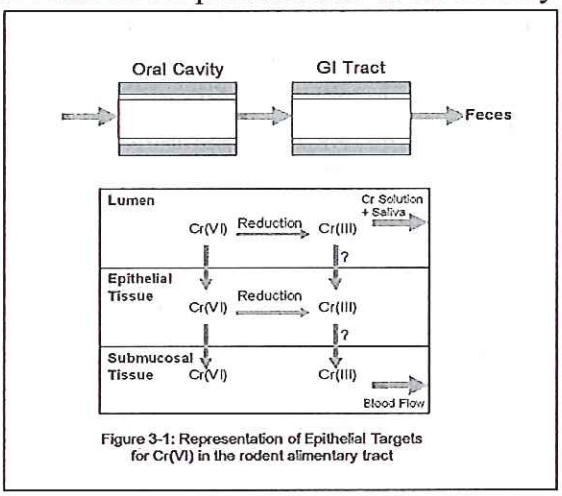


Figure 3-1: Representation of Epithelial Targets for Cr(VI) in the rodent alimentary tract

Figure 3-1. Representation of Epithelial Targets for Cr(VI) in the Rodent Alimentary Tract (also see attachment 1)

These processes have been incorporated into the modeling described in the report of our initial efforts to develop the PBPK model for chromium in the intestinal tract (Figure 3-1). Improved understanding of these processes would provide a stronger foundation for the description of the intestinal tissues in our PBPK model, and should help identify potential dose-dependent transitions in the kinetics and mode of action for the effects of chromium in the intestine.

Evaluation of data on the essentiality of CR(III) will also provide important perspective for assessing the potential role of Cr(III) in the carcinogenicity of Cr(VI). The two most broadly held hypotheses for the mode of action of Cr(VI) carcinogenicity can be differentiated by the posited role of Cr(III). In one hypothesis, the carcinogenic action is attributed to one or more of the reactive valences (VI, V, and IV) or the oxidative stress associated with their reduction. In the competing hypothesis, CR(VI) is considered to be a pro-carcinogen that enhances the delivery of the carcinogenic valence, Cr(III), into the cell. Information on the cellular homeostasis of Cr(III) can be used to evaluate the potential for high levels of Cr(VI) exposure to disrupt normal homeostasis for Cr(III) leading to genotoxicity.

Description:

1. Magnetic Resonance Imaging (MRI) study to investigate the reduction rate of Cr(VI) to Cr(III). This study will be conducted by Dr. Weili Lin and his associates at the Biomedical Research Imaging Center at the University of North Carolina-Chapel Hill under a combination of expert consultant and UNC subcontracts with The Hamner Institutes. Female B6C3F1 mice will be divided into five study groups (four SDD drinking water concentration groups and a control group -- the same concentration groups as the 90-day study except for the environmentally-relevant low dose group) with 10 mice in each group, leading to a total of 50 mice in the study. The Hamner Institutes will prepare, analyze, and deliver the SDD drinking water solutions for the study. Each animal will be imaged using the 9.4T MR scanner at three time points: 7 days, 14 days, and 28 days of exposure. At each time point, anatomical images as well as quantitative measures of T1 and T2* relaxation times will be obtained. Each imaging session is estimated to take about 1.5 hours. Thus, study groups will have to be divided into subgroups (replicates) and dosing with SDD will be staggered over a 10-week period. Animals will return to the animal facility immediately after each imaging session. Following Day 28 imaging, animals will be euthanized and organs, including stomach, small intestine (duodenum), liver, kidney and blood will be collected. Total chromium analysis will be performed on the duodenum, kidney, and blood.

Results of the pilot study demonstrated that it was possible to monitor the reduction of Cr(VI) to Cr(III) (Figure 3-2); however, the MR results provided only qualitative data. To obtain quantitative results, the MR images will be imported into medical imaging software (Mimics, Materialise, Inc., Ann Arbor, MI) by 3-D computational modelers at The Hamner Institutes and UNC-Chapel Hill.

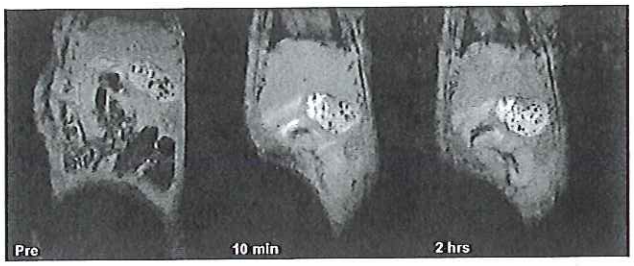
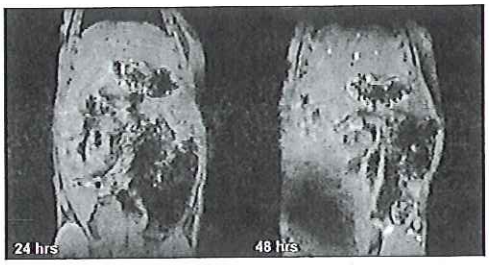


Figure 3-2. MR Images of a Mouse at Different Times After Gavaging with 0.1 ml of 1 mM Cr(VI). The progress through the GI tract and loss from stomach can be visualized over time.



This software uses thresholding and segmentation algorithms to isolate regions of high signal intensity from the MR images. Interpolation methods will then be used to develop 3D reconstructions of these regions of interest. For this study, MR image sets for controls and at various time points following Cr(VI) exposure will be imported into Mimics so that the observed signal of Cr(III) in the duodenum, stomach, kidneys, etc. can be isolated using various thresholding parameters. Volumes of these regions of interest will be then be quantitated and used to compute rates of reduction of Cr(VI) to Cr(III) in these tissues.

Preparation of tissue samples for the analysis of total chromium will be conducted using microwave assisted nitric acid digestion, based on established methods for total chromium in tissue samples (NTP, 2008; Glaser et al., 1985; Kotz et al., 1972; The Hamner SOP ANA-31). Analysis of the prepared samples will be conducted by The Hamner's Analytical Chemistry staff according to EPA method 218.2 "Chromium (AA, Furnace Technique)", and using a Perkin Elmer AAnalyst 800 Graphite Furnace Atomic Absorption Spectrometer.

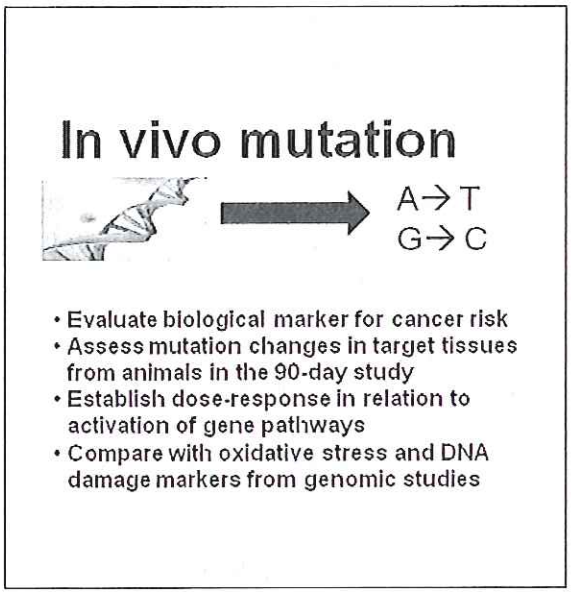
2. Review of the literature on the homeostatic control of chromium

Insights gained from the literature on the essentiality of Cr(III) will be used to inform the further development of the PBPK model for chromium in the mouse as well as the likely mode of action for the carcinogenicity of Cr(VI). A report will also be prepared summarizing the results of the review and identifying critical data gaps. Hamner staff has developed a PBPK model for control of another essential element over the past 5 years (Nong et al., 2008).

3. Refinement of PBPK models

The data and information collected in the previously described studies will be used to refine the PBPK models developed in the pilot study (See Attachment: Chromium Pilot Study).

Section 4 – *In Vivo* Mutation Analysis

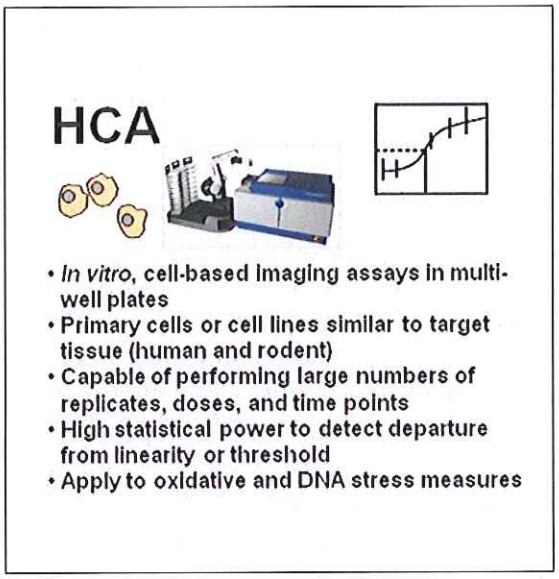


Goal: Characterize the dose-response for *in vivo* mutation from animals in the 90-day drinking water study in relation to other potential factors in the mode of action for Cr(VI) carcinogenicity.

Rationale: The fact that a compound is genotoxic and carcinogenic does not imply that the mode of action for its carcinogenic effect is direct DNA-reactivity mutagenic, and therefore low-dose linear. Hamner scientists recently collaborated with the National Center for Toxicology Research (NCTR) to evaluate the *in vivo* mutagenicity of formaldehyde. Two oncogenes associated with formaldehyde-induced nasal tumors (p53 and K-ras) were sequenced following 90-day inhalation exposures (Meng et al., 2008; See Attachments 3 and 5: Meng et al., 2008, poster presentation and draft manuscript). No increases in mutations were observed for either oncogene at concentrations associated with tumors. The NCTR investigators concluded that: "The lack of induction of p53 mutation at a later time point than is needed to detect changes in cell proliferation and gene expression adds to the body of evidence that formaldehyde is not carcinogenic through a mutagenic mode of action." The proposed task would preserve tissues for a similar analysis with Cr(VI), if it is determined to be possible. The specific oncogenes to be sequenced will be determined from information on the gene mutations associated with Cr(VI)-induced tumors. If it is determined that oncogene sequencing is not appropriate, another analysis, such as the Comet assay, will be considered.

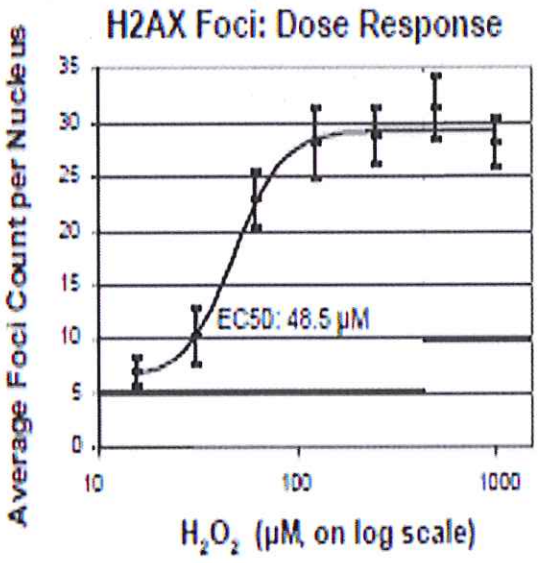
Description: An additional 5 animals per dose will be included in the 90-day study to provide tissues for subsequent analysis of *in vivo* mutagenicity. Following 90 days of exposure, the tissues (intestinal epithelium from mice and the oral cavity epithelium from rats) will be harvested, fresh frozen and stored in a manner that will preserve the tissues for future analysis of *in vivo* mutation.

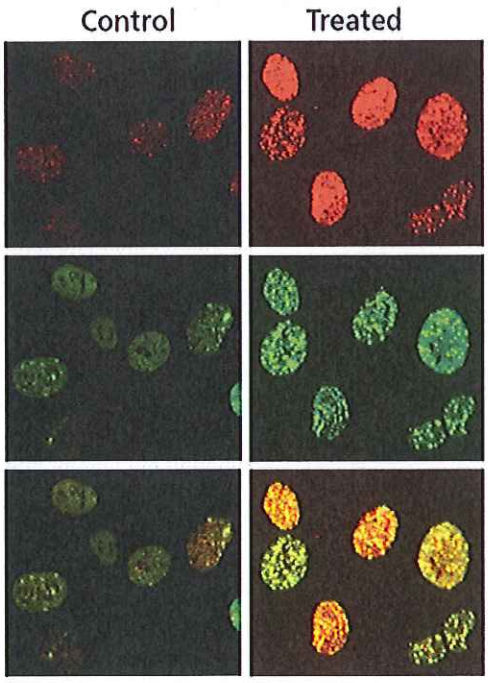
Section 5 – *In Vitro* High-Content Imaging Assays



Goal: Analyze dose-response changes for a suite of imaging-based cytotoxicity, oxidative stress and DNA-damage assays using mouse and human epithelial cell lines derived from alimentary tract tissues.

Rationale: A mode-of-action evaluation for Cr(VI) presented by the U.S. Environmental Protection Agency (Akerman *et al.*, 2009) includes both mutagenic and oxidative stress components. Due to DNA repair capabilities and other adaptive cellular processes, previous studies have suggested that mutagenic chemicals can display a linear dose-response for DNA adducts, but a non-linear response for mutations (Swenberg et al., 2008). In this study, a series of high-content imaging assays (HCA) will be used to assess cytotoxicity, oxidative stress and DNA-damage produced by Cr(VI) in epithelial cells *in vitro*. Due to the high-throughput nature of these assays, a large number of concentrations and replicates provide requisite data to more fully-characterize the dose-response behavior for each endpoint. The photo below (right) is from a BD Biosciences Application Note (see Attachment) and shows H2AX and 53BP1 foci formation in nuclei from HT 1080 (a human fibrosarcoma derived cell line) after treatment with hydrogen peroxide. Red is H2AX; green is 53BP1; and yellow is the overlap of the two indicating DNA-damage and accumulation of these proteins at the strand breaks. These two proteins are important in the DNA-damage response. The dose response for foci per nuclei (below left) would be evaluated and used to produce a dose response curve for





more detailed analysis to assess threshold behaviors. These refined and extended dose response curves can be evaluated with new statistical approaches to examine the likelihood that the results are associated with threshold behaviors (Werner Lutz, personal communication; Lutz and Lutz, in review). The HCA results from the rodent epithelial cells will be integrated with target tissue doses estimated using the PBPK modeling and used to support transcriptional dose-response changes associated with the cytotoxicity, oxidative stress, DNA damage and DNA repair gene ontology categories.

The human epithelial cells will allow comparison with the rodent *in vitro* and assist in a parallelogram approach to assess expected dose response behavior in humans drinking water containing Cr(VI). The current design focuses on cell lines to accelerate the *in vitro* analysis and complete a parallelogram *in vitro* to *in vivo* extrapolation for the human risk assessment. Another option considered was to develop these assays in primary cells from the target tissues in rats and mice. Although there are published methods for isolating primary buccal and duodenum epithelial cells (Autrup and Autrup, 1986; Bjerknes and Cheng, 1981), this option would likely require more assay development to assure ability to harvest these cells and adapt the current, human-antibody based HCA tools to the two rodent species. Our preferred option is using alimentary epithelial cell lines from mice and humans, which would avoid developing and optimizing the primary cell culture methods.

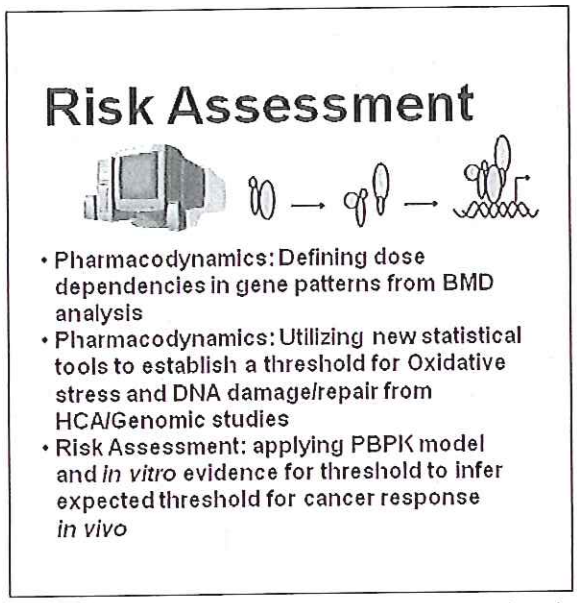
Description:

1) Develop Cell Culture Methods for 96-well High-Content Screening
   a) Obtain mouse intestinal epithelial cell line (m-ICc12) and characterize growth rate and plating density in 96-well plates. NOTE: The mouse m-ICc12 cell line is not currently available from public sources (e.g., ATCC) and will have to be obtained from the original investigator. If we are unable to obtain this cell line from the original investigator, an alternative cell line will be used.
   b) Obtain human colon epithelial cell line (Caco-2) and characterize growth rate and plating density in 96-well plate.
2) Evaluate genotoxicity and oxidative stress in each cell line using imaging-based assays.
   a) Expose each cell type to Cr(VI) in twenty-point dose response and 3 different time points in 96-well plates (6, 24, and 72 hrs).
   b) Process cells using commercially-available high-content imaging assays. The genotoxicity and oxidative stress assays are listed below. It should be noted that the genotoxicity assays all rely on antibodies developed for the human form of the target protein. These assays may or may not work against the rodent protein, so assay qualification will be required.
      i) H2AX nuclear foci formation
      ii) 53BP1 nuclear foci formation
      iii) Nuclear localization of phospho-P53 relative to total P53
      iv) Nuclear localization of phospho-ATM
      v) Nuclear localization of phosho-Chk2
      vi) Conversion of dihydroethidium to ethidium and nuclear localization (oxidative stress)

c) Statistical analysis will be used to assess the non-linearity and threshold behavior of each endpoint.

Section 6 – Integration of Endpoints and Risk Assessment

Goal: To understand the contribution of different modes of action for chromium throughout the broad dose range to which the animals are exposed and provide both statistical and biological understanding of thresholds for chromium carcinogenicity and dose-dependencies in contributing modes of action.



Rationale: While high concentration tumors in laboratory animals and humans indicate some level of genotoxicity; these tumors often arise in the presence of other cellular responses which likely include cytotoxicity, inflammation, and high levels of oxidative stress. These secondary responses are expected to lead to dose dependent transitions where Cr(III) and Cr(VI) DNA reactivity finally leads to DNA damage, mutation, and transformation. The contributions of these various pathways over the range of doses and drinking water concentrations can only be elucidated and quantified for a risk assessment by a combination of genome-wide microarray analyses in intact animals, high data content imaging of activation of key cytotoxic and DNA-damage repair pathways by HCA analysis, and consideration of dose dependencies in dosimetry.

The primary components of our research framework (genomics, high content imaging and PBPK modeling) all play important roles in assessing dose dependencies of individual processes and thresholds for the induction of tumors by Cr(VI). Gene array results, as recently shown for formaldehyde (Thomas et al., 2007; Andersen et al., Tox. Sci, 2008, will allow us to examine whether DNA-damage and clear evidence of cytotoxicity only arise at high drinking water concentrations and that lower concentrations lead to activation of qualitatively distinct pathways from those activated at high, carcinogenic doses. The BMD analysis will provide quantitative measures of the *in vivo* dose response and, by application of multiple models for the curve fitting procedure, provide strong, but indirect evidence for thresholds in activation of specific families of genes. Critically, the *in vitro* HCA studies, relying on multiple dose points and multiple assays, will provide quantitative evidence for a threshold based on statistical evaluation of background repair and repair induced by chromium in these cells (Lutz and Lutz, in review). In addition the HCA analysis and dose response coupled with PK modeling will allow for development of a parallelogram approach for assessing conditions under which

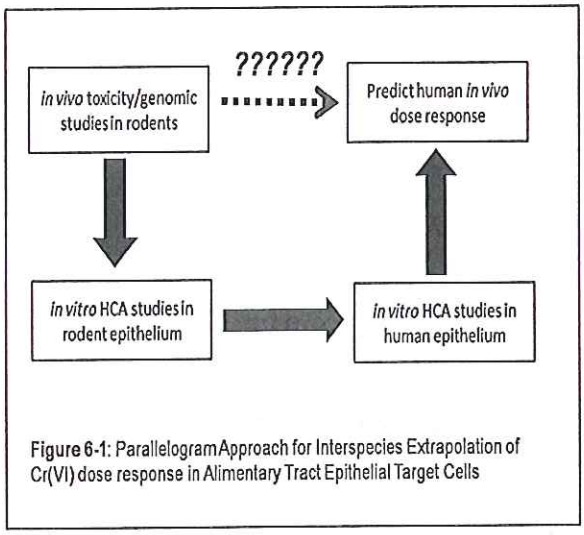


Figure 6-1: Parallelogram Approach for Interspecies Extrapolation of Cr(VI) dose response in Alimentary Tract Epithelial Target Cells

alimentary tract epithelial cells in humans are expected to receive doses of Cr(VI) that overwhelm the capacity of the cell to adapt to low level oxidant stress (Figure 6-1).

Figure 6-1. Parallelogram Approach for Interspecies Extrapolation of Cr(VI) Dose Response in Alimentary Tract Epithelial Target Cells

PBPK modeling will show the differences in chromium processing for background exposures and in dose regions exceeding those associated with chromium's role as an essential element. Only through the risk assessment oriented integration of these studies can the safe region of exposure be established for chromium exposures leading to a margin of exposure calculation and a non-linear cancer risk assessment.

Description: Based on the evidence from these studies, we will develop mode of action framework for the oral carcinogenicity of Cr(VI) in the rodent. The Framework for Mode-of-Action Evaluation in the USEPA cancer guidelines (USEPA, 2005) will be used as the template for this analysis. Particular attention will be given to identifying evidence for dose-dependent transitions in kinetics or mode of action that would argue against the use of simplistic linear low-dose extrapolation from the rodent bioassay results. To the extent possible, the data will be used to differentiate the conditions under which tumors would or would not be expected.

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ATTACHMENT 1

Appendix A

Review of Evidence Regarding the Mode of Action for the Carcinogenicity of Chromium

APPENDIX A

REVIEW OF EVIDENCE REGARDING THE MODE OF ACTION FOR THE CARCINOGENICITY OF CHROMIUM

A two year cancer bioassay was completed for Cr(VI) (i.e., sodium dichromate dihydrate) in drinking water by the US National Toxicology Program (NTP, 2008; Stout et al., 2008). The NTP study design resulted in the development of intestinal tumors in mice and oral mucosal tumors in rats at daily concentrations in excess of 85 mg/L. Qualitative evidence for the human carcinogenicity of chromium is strong following inhalation exposures: a number of epidemiological studies in several different chrome industries have demonstrated an increased incidence of lung cancer mortality in workers chronically exposed to chromium compounds. However, there are no epidemiological studies indicating carcinogenicity following oral exposures.

Chromium is a naturally occurring essential trace element and exists in oxidation states ranging from -2 to 6+; however, the trivalent (+3) and hexavalent (+6) states are the only forms which may be found in the environment (ATSDR 1989; Snow and Xu 1991; Fan and Harding-Barlow 1987; De Flora and Wetterhahn 1989; Tandon 1985), with Cr3+ being more stable than Cr6+ (Shi and Dalal 1988; Petrilli et al. 1986; IARC 1980; Bianchi et al. 1980). Further, Cr6+ and Cr3+, as well as other chromium valences, do not typically exist as simple ions, but rather as molecules compounded with oxygen and other elements (Jones 1990). However, for the purpose of clarity in this paper, we will refer to the various forms of chromium by their valence state, i.e., Cr6+, Cr3+, etc.

Cr6+ is produced through industrial processes in the manufacturing of steel and alloys, chrome plating, and wood and water treatments, while Cr3+ is naturally occurring in the environment (ATSDR 1989; IARC 1980). Cr6+ readily crosses cell membranes and is rapidly reduced to Cr3+ *in vivo* by numerous processes (Mertz 1969).

Cr3+ i an essential nutrient (ILSI 1990) required for the maintenance of normal glucose metabolism (Hopkins et al. 1968), and is essentially nontoxic (Connett and Wetterhahn 1985; NAS 1974). Cr3+ does not cross cell membranes under normal physiological conditions. However, Cr3+ can pass across the membrane if it is bound to ligands. Ligand-binding would allow Cr3+ to cross the cell membrane via passive diffusion, which is much slower than the rate of actively transported Cr6+ (Patierno 1996).

Inhalation exposure to Cr6+ has been associated with an increased incidence of lung cancer in occupational and animal studies (Standeven and Wetterhahn 1989; Rossi et al. 1988; Shi and Dalal 1988). However, the valence state of chromium responsible for the observed tumorigenicity has been the subject of much study, but with very little conclusive results. This dilemma results from the observation that it is Cr+6 (and not Cr+3) that crosses the cell membrane, but Cr6+ has not been shown to interact directly with genetic material, as will be discussed later. However, test results indicate that numerous forms of metabolic intermediates and the ultimate metabolic form, Cr3+, may be capable of interaction with intracellular nuclear material believed to initiate the carcinogenic process. Therefore, an understanding of the metabolic fate and behavior of chromium, both extra- and intracellularly, is critical to understanding possible mechanisms of action.

Effects of Chemical Form and Solubility on Carcinogenicity

Key issues regarding the dose-response for chromium exposure are the impact of the oxidation state (trivalent vs. hexavalent), the specific chromium compound (lead chromate, calcium chromate, chromic oxide, etc.) and solubility. In considering the properties and biological effects of chromium, the two principal oxidation states, Cr3+ (trivalent) and Cr6+ (hexavalent) must be distinguished. Trivalent chromium is by far the most stable form, both in the environment (Cotton and Wilkinson 1966) and in biological systems (Mertz 1969), and for practical purposes oxidation of trivalent to hexavalent does not occur in biological systems (WHO 1988). Hexavalent chromium, on the other hand, is a potent oxidizing agent (Cotton and

Wilkinson 1966), and is readily reduced to the trivalent form *in vivo* (Feldman 1968; Mertz 1969).

The trivalent and hexavalent forms are very different in their observed biological activities (WHO 1988; Grant and Mushak 1989). Hexavalent chromium compounds are corrosive oxidizing agents which cause local irritation and systemic nephrotoxicity (Gad 1989). In contrast, trivalent chromium is an essential nutrient (AMA 1979), required for the maintenance of normal glucose metabolism (Hopkins et al. 1968), and is essentially nontoxic (NAS 1974). Recently, NTP evaluated the toxicity and carcinogenicity of chromium picolinate (CP) in a two year feeding study (Stout et al., 2009). CP, which is a human nutritional supplement and designed to increase absorption of Cr3+, was administered in the feed of both mice and rats. No toxicity or carcinogenicity was observed in this study.

Chromium compounds are generally mutagenic, whereas the evidence for the mutagenicity of trivalent compounds is equivocal (DeFlora et al. 1990). Hexavalent chromium compounds have been associated with the production of lung cancer from inhalation in both animals (Langard 1988) and humans (Langard 1990). The equivocal epidemiological evidence from primarily trivalent chromium exposures in the ferrochromium industry, as contrasted with the clear evidence of carcinogenicity from primarily hexavalent exposures in the chromate production industry, suggests that hexavalent chromium, rather than trivalent, is responsible for the observed induction of lung cancer (Langard et al. 1990).

However, the World Health Organization (WHO), in an attempt to determine if there was sufficient data to determine the relationship between the risk of cancer and exposures to the trivalent or hexavalent forms of chromium compounds, reported conflicting conclusions from epidemiological studies regarding exposures to trivalent chromium and cancer risk (WHO 1988). In their review, WHO noted that in most of the available epidemiological studies, exposures were poorly characterized (i.e., it was difficult to determine if workers had been exposed to trivalent chromium, hexavalent chromium or both). The authors of some epidemiological studies have concluded that exposures to trivalent chromium compounds were not associated with an

increased risk of lung cancer (Korallus et al. 1974a,b,c; Axelsson et al. 1980; Langard et al. 1980; Norseth 1980). However, other authors have not reached the same conclusions (Essing et al. 1971; Mancuso 1975; Zober 1979). Based on a review of epidemiological data, including case reports, collected from studies of the chromate-producing, chromium-plating, chromate-pigment and ferrochromium industries, IARC has concluded that there is sufficient evidence of cancer of the respiratory tract in workers occupationally exposed to chromium in the chromate plating industry; however, the data are insufficient to draw conclusions regarding lung cancer risk in other occupations with potential exposures to chromium (WHO 1988). IARC has also concluded that the data are inadequate to determine the relative contributions of metallic chromium, trivalent chromium, hexavalent chromium, soluble chromium and insoluble chromium to cancer risk (WHO 1988).

WHO (1988) also reviewed the inhalation carcinogenicity studies conducted in animals. Nettesheim et al. (1971) exposed 136 mice to13 mg calcium chromate/m^3, 5 hours/day, 5 days/week for lifetime. There were 14/136 lung adenomas reported in the treated group, compared to 5/136 in the controls. There were no carcinomas reported. In a study where rats, mice and guinea pigs (number of animals/group not specified) were exposed to mixed chromate dust, 3-4 mg $Cr0_3/m^3$, 4-5 hours/day, 4 days/week for lifetime or 50 weeks, respectively, via inhalation, only three alveologenic adenomas were identified in 50 exposed guinea pigs (Steffee and Baetjer 1965). In rats exposed to 2 mg calcium chromate/m^3, for 589 exposures over 891 days, one lung squamous cell carcinoma, one squamous cell carcinoma of the larynx, and one peritruncal tumor were reported (Laskin et al. 1970; Laskin 1972). In hamsters exposed using the same protocol as for the rats, one papilloma and one squamous cell carcinoma of the larynx were reported. The number of animals/group was not specified for either study.

Mechanistically, the difference in the effects of the two valence states can be understood as resulting from two factors: cellular transport and oxidation potential. Although trivalent chromium has been shown to react with DNA in solution, it is to a large extent inactive with intact cells and tissues (DeFlora et al. 1990). The relative *in vivo* inactivity of trivalent chromium appears to result from the fact that it does not readily cross the cell membrane, in

contrast with hexavalent chromium, which readily crosses the cell membrane (Levis et al. 1978). It has been suggested that the rapid cellular uptake of hexavalent chromium is mediated by the sulfate transport system and results from the similarity of the chromate ion to sulfate ion (Wetterhahn 1981). At any rate, once hexavalent chromate reaches the interior of the cell, it is reduced to the trivalent form, which is then trapped inside the cell (Grogan 1958; Mertz 1969). Alternative theories for the carcinogenic effects of hexavalent chromium are based on (1) the intracellular production of trivalent chromium, which reacts directly with DNA or inhibits DNA repair enzymes (Tamino et al. 1981), or (2) the formation of reactive intermediates (e.g., pentavalent chromium) produced in the intracellular reduction of hexavalent chromium (Wetterhahn 1981; Wetterhahn and Hamilton 1989), as will be discussed later.

Epidemiological studies were also reviewed to determine if data regarding the carcinogenicity of specific chromium compounds were presented (Alexander et al. 1996; Aw 1997; Dalager et al. 1980; Davies 1984; Davies et al. 1991; Franchini et al. 1983; Hayes 1988; Langard 1990, 1993; Langard and Norseth 1975; Lees 1991; Mancuso 1997; Moulin 1997; Rafnsson et al. 1997). Excess lung cancer risk was reported in several of the studies reviewed (Aw 1997; Dalager et al. 1980; Davies 1984; Davies et al. 1991; Franchini et al. 1983; Langard and Norseth 1975; Mancuso 1997; Moulin 1997; Rafnsson et al. 1997). Alexander et al. (1996) reported there was no clear association between chromium exposures and lung cancer risk in workers in the aerospace industry exposed to various chromium pigments that include zinc, strontium and lead chromate or to chromium trioxide. In the epidemiological studies reviewed, exposures were poorly characterized and exposures to specific chromium compounds were determined based on the various materials used and processes performed at the plants. It should be noted that none of these studies evaluated a cohort where exposures were exclusively to a specific chromium compound, i.e., the workers in these study cohorts were exposed to more than one chromium compound. In addition, other confounding factors, such as smoking habits and exposures to other chemicals, were not evaluated in all of the studies. Therefore, from the epidemiological data reviewed, the potential carcinogenicity of a specific chromium compound cannot be stated with certainty, although Langard (1993) noted that based on a review of the epidemiological data, zinc and calcium chromate appeared to be more potent than lead chromate. However, in a

comprehensive review of the epidemiological studies of workers in the various chromium industries, Lees (1991) concluded that due to inadequate worker exposure data, the risks associated with exposures to a particular chromium compound could not be determined.

Additionally, a change in the refining process of chromite ore may have resulted in a decreased risk of lung cancer in workers employed after this change (Aw 1997; Davies et al. 1991). The previous "high lime" method involved the addition of calcium carbonate or calcium magnesium carbonate to chromite ore resulting in the formation of calcium chromate, which was reported to be carcinogenic following implantation into the lungs of rats (Laskin et al. 1970; Levy and Vennitt 1975; Levy and Martin 1983). In the current "low lime" methods, these calcium-containing materials are not used. Therefore, workers were not exposed to calcium chromate (Aw 1997; Davies et al. 1991).

Differences in the solubilities of inhaled hexavalent chromate compounds have also been found to affect their carcinogenic potency (Langard 1990). Evidence from epidemiological studies and animal studies support the generalization that the less soluble chromate salts (e.g., Zn, Ca, and Sr chromate) are the most potent respiratory carcinogens, while the highly soluble salts (e.g., Na, K, and ammonium chromate) are less active (Gibb and Chen 1989; Hathaway 1989). Presumably, the less soluble salts serve as a persistent source of locally available chromium. For example, following the implantation of cholesterol pellets containing different chromium compounds into the bronchi of rats, bronchogenic carcinomas were produced in rats that received implants containing calcium or zinc chromate; however, bronchogenic carcinomas were not observed in rats that received soluble chromates or trivalent chromium chemicals (Laskin et al. 1970; Levy and Vennitt 1975). Levy and Martin (1983) reported that sparingly soluble chromates, including strontium and calcium chromate and to a lesser extent some forms of zinc chromate, were carcinogenic in rats following implantation as described above. In contrast, a much weaker response was observed in rats that received barium or lead chromate, when compared with the response observed in rats that received calcium or strontium chromate. However, based on reviews of the epidemiologic data, Mancuso (1997) and Langard (1993) noted that there was some evidence that exposures to soluble chromium may result in lung cancer.

Extracellular Reduction of Chromium VI

Following exposure to chromium, several mechanisms exist for extracellular reduction of Cr6+ to Cr3+. Much of Cr6+ ingested or inhaled is reduced to poorly absorbed Cr3+ before the Cr6+ reaches membranes through which it might be absorbed. When ingested, Cr6+ is rapidly reduced by saliva and gastric juices. When inhaled, Cr6+ is reduced by interaction with fluids and cells of the lung (DeFlora and Wetterhahn 1989; Snow 1992; Jones 1990; Petrilli et al. 1986). The reduction of Cr6+ to Cr3+ is an important defense mechanism, since Cr3+ readily complexes with other extracellular components and does not permeate the cell membrane (Bianchi and Levis 1985; Petrilli et al. 1986; Suzuki 1988; Korallus et al. 1984). Studies have shown that the reduction of Cr6+ to Cr3+ is through saturable kinetic processes, when tested at physiological pH (Connett and Wetterhahn 1985; Snow 1992; Petrilli et al. 1986; Standeven and Wetterhahn 1989). Therefore, if these processes were to be saturated, a greater proportion of Cr6+ would be available to cross the cell membrane and enter the cell (Snow 1992; Jones 1990; Petrilli et al. 1986). However, it is likely that relatively high doses of Cr6+ (dose levels well above those typically encountered in the environment) would have to be ingested or inhaled to saturate these defense mechanisms. These extracellular reduction mechanisms that are operative following ingestion or inhalation of Cr6+ are discussed below.

Inhalation

The fate of chromium in the lung is important since the lung is the only proven target of chromium-induced cancer in humans (DeFlora and Wetterhahn 1989). When Cr6+ enters the body via inhalation, the first extracellular mechanism to reduce Cr6+ to Cr3+ is contact with the epithelial lining fluid of the lung which contains ascorbic acid and glutathione (GSH). DeFlora et al. (1997) reported that the acute capacity for Cr6+ reduction by the epithelial lining fluid (ELF) of the lung ranged from 0.9 to 1.8 mg Cr6+.

Ascorbic acid is recognized as an extremely important chromium-reducing agent and is found in the surfactant layers of the lung (Snow 1992; Suzuki 1988; Korallus et al. 1984; Jones 1990). Suzuki (1988) reported that ascorbic acid was an important reducing agent in rat lung lavage fluids. In these fluids, the mean molar ratio of oxidized ascorbic acid and reduced chromium (VI) was 3:2.3. Based on this ratio, the amount of Cr6+ reducible by ascorbic acid was 8.4 μg/g tissue. On this basis, it has been suggested that a 10-hour inhalation exposure to 1 mg Cr6+/m^3 could occur before an accumulation of Cr6+ would take place in the lining of the lungs and absorbed into the cell (Suzuki 1988). Similarly, the alveolar region of the lung contains high levels of GSH (Jones 1990), an extracellular reducing agent of Cr6+ (Snow 1992; Jones 1990; Shi and Dalal 1988). When chromium particles less than 0.5 μm are inhaled, they can reach the alveolar region of the lung. However, GSH has the ability to reduce much of the Cr6+ to the trivalent form before absorption into the cell can occur (Jones 1990).

A secondary reducing mechanism in the alveolar region of the lungs of humans and animals is via pulmonary alveolar macrophages (PAMs). PAMs rapidly phagocytize hexavalent particles and through enzymatic process, reduce them to Cr3+ within cytoplasmic phagosomes (Jones 1990; Petrilli et al. 1986). This reduction process is considered irreversible due to the long life span of these cells, the efficient removal by the mucociliary elimination process, and the inability of cells to oxidize Cr3+ back to Cr6+. DeFlora et al. (1997) reported that the acute capacity for reduction of Cr6+ by PAMs was 136 mg/day/individual, based on the results of studies conducted with PAMs collected from volunteer smokers and nonsmokers. However, Cr6+ that bypasses extracellular reduction is absorbed into the vasculature where GSH in erythrocytes and protein complexes in plasma have the opportunity to reduce Cr6+ to Cr3+ (Jones 1990). Also within the lung are the peripheral parenchymal cells that have the capacity to reduce as much as 260 mg Cr6+ entering the lung per individual (DeFlora et al. 1997). Because Cr3+ is largely impermeable to the cell membrane, it binds to other components and is then excreted in the urine (Korallus et al. 1984). However, Cr6+ which escapes extracellular reduction is available to penetrate the cell membrane and enter the cell. Although the studies just described only estimate capacities for extracellular reduction of Cr6+, the extracellular reduction and cellular uptake of Cr6+ are parallel, competing processes. Evaluation of the dose-response for the cellular uptake

of Cr6+ would also require information on the relative kinetics of extracellular reduction and cellular uptake.

Ingestion

When chromium ingestion occurs, saliva, gastric juice, and intestinal bacteria act to reduce Cr6+ to Cr3+ (Jones 1990; Snow 1992; DeFlora et al. 1985, 1997; Petrilli et al. 1986; Tandon 1985; IARC 1980). Reduction by gastric juices also reduces Cr6+ following reflux from the lung. Kerger et al. (1996) reported that more than 80 mg Cr6+/day may be reduced to Cr3+ by gastric juice of the stomach. DeFlora et al. (1997) reported that 8.3- 12.5 mg Cr6+/day/individual could be reduced by gastric juices during interdigestive periods and 25.1 mg Cr6+/individual may be reduced following ingestion of a meal. DeFlora et al. (1997) also reported that between 0.7 and 2.1 mg Cr6+/day/individual may be reduced by saliva. This capacity for Cr6+ reduction by saliva has been reported to be sufficient for detoxifying Cr6+ found in drinking water at the recommended standards (0.05 - 0.1 mg/L) of several countries.

Intestinal bacteria are also known to contain high levels of GSH. DeFlora et al. (1997) reported that a range of 1 to 24 mg Cr6+/individual may be reduced by intestinal bacteria and eliminated daily with feces. The Cr6+ which escapes reduction by saliva, gastric juices, or intestinal bacteria and is absorbed into the portal blood stream may reach the liver. However, the liver has been reported to have the capability of reducing 3300 mg Cr6+/individual (DeFlora et al. 1997).

Systemic

Cr6+ that crosses the vasculature into the blood is reduced by red blood cells to Cr3+. Inhalation of higher doses results in excess hexavalent chrome in the plasma. The capacity of the human plasma to reduce Cr6+ has been confirmed by monitoring workers at manufacturing plants of various chromium compounds (Korallus 1986). Cr6+ reaching the systemic circulation reacts with GSH found in the erythrocytes and plasma (Jones 1990; DeFlora et al. 1997). Cr6+ is then reduced to Cr3+ by GSH, leaving the Cr3+ to bind to hemoglobin and serum proteins (Tandon

1985; Jones 1990). The overall capacity of whole human blood to reduce Cr6+ has been reported to be 234 mg/individual for males and 187 mg/individual for females. The overall reduction of Cr6+ by red blood cells was reported to be 128 mg/individual for males and 93 mg/individual for females (DeFlora et al. 1997).
Duringthe reduction of Cr6+ within the blood stream, Cr5+ intermediates may also form and complex with the GSH to form GSH-Cr5+ species (Goodgame and Joy 1986; Jones 1990). However, like Cr3+ species, Cr5+ is also impermeable to biological membranes (Arslan et al. 1987).

Intracellular Reduction of Chromium VI

Solubility is an important issue in considering the bioavailability of chromium to target tissues (DeFlora and Wetterhahn 1989). It has been reported that chromium compounds with lower solubilities are less easily transported into the cell (Jones 1990). Once Cr6+ reaches the cell membrane, the transport of Cr6+ into the cell is believed to mimic the sulfate and phosphate anion transport systems (Standeven and Wetterhahn 1989; Arslan et al. 1987; Snow 1992). This assumption is based on the fact that Cr6+ is mainly found in the tetrahedral form as chromate (CrO_4^{2-}). Therefore, since sulfate and phosphate anions are also in tetrahedral form, it is assumed that Cr6+ is transported in the same manner (Standeven and Wetterhahn 1989). Trivalent chromium is found exclusively as octahedral complexes. This form does not have a distinct cellular uptake mechanism and therefore, makes Cr3+ unable to penetrate the cell membrane under normal physiological conditions (Cohen and Costa 1992).

Once Cr6+ crosses the cell membrane, a rapid reduction takes place (Snow 1992). This rapid intracellular reduction of Cr6+ to Cr3+ helps maintain a concentration gradient and allows for a steady-state influx of Cr6+. A number of intracellular reducing factors act on Cr6+ to reduce it to Cr5+ and Cr4+, which are relatively unstable species, and then to Cr3+, a stable form (Snow 1992). Thus, Cr6+ is short-lived inside the cell, due to the intracellular reducing factors within the cytosol, endoplasmic reticulum, and mitochondria (Snow 1992; Standeven and Wetterhahn 1989; Connett and Wetterhahn 1983; Mikalsen et al. 1989, 1991). The major intracellular

reductants include NAD(P)H, cytochrome P-450, ascorbate, and thiols (i.e., GSH, cysteine, and penicillamine), with ascorbate and GSH being the most significant reductants (Connett and Wetterhahn 1985; Snow 1992, DeFlora et al. 1988, 1985; Shi and Dalal 1990; Cupo and Wetterhahn 1985). The following is an overview of the reduction of Cr6+ to Cr3+ once Cr6+ reaches the cytoplasm, mitochondria and endoplasmic reticulum.

Cytoplasm

Upon entering the cytoplasm, ascorbate, DT-diaphorase, and GSH are the major contributors to the reduction of Cr6+ to Cr3+. Connett and Wetterhahn (1983) were the first to note that the intracellular metabolism of chromate is under kinetic rather than thermodynamic control. This means that the most plentiful and kinetically active electron donor will be of primary importance in the reduction of chromate, rather than the one producing the most stable product. Enzymatic reduction of Cr6+ by microsomal enzymes or the mitochondrial electron transport system is slow and therefore of less overall importance than reduction by various small compounds, such as GSH and ascorbic acid, which can be present in millimolar concentration.

GSH plays a significant role in the reduction of Cr6+ in the cytoplasm. GSH is the most abundant thiol found within the cell and takes part in a two-phase reduction of Cr6+ to Cr3+ as discussed below (Connett and Wetterhahn 1985; Shi and Dalal 1988; Suzuki and Fukuda 1990). In the presence of GSH, a Cr6+-thioester intermediate is formed as an initial reducing step. A two-phase intracellular reduction then follows. The first phase, in which the Cr6+-thioester reduces to form Cr5+ intermediates, is very rapid (Connett and Wetterhahn 1985; Snow 1992; Shi and Dalal 1988; Rossi et al. 1988; Suzuki and Fukuda 1990). During this step of reduction, a glutathionyl radical (GS·) also forms (Shi and Dalal 1988; Cupo and Wetterhahn 1985). At this point, if sufficient GSH is present, a second reduction phase occurs, reducing Cr5+ to Cr4+ and then to Cr3+ (Snow 1992; Connett and Wetterhahn 1985). However, the second step in the reduction process is much slower. The Cr3+ may then bind to other components, such as protein complexes or possibly with DNA. If there is not enough GSH present to further reduce the Cr5+ to Cr3+, Cr5+-thioester intermediates may form or Cr5+ may possibly interact with DNA

(Connett and Wetterhahn 1985; Snow 1992; Shi and Dalal 1988). An *in vitro* study conducted by Suzuki (1990), supports the two-phase reduction of Cr6+ by GSH. Suzuki (1990) reported that during the first phase of the reduction, the half-life of Cr6+ was 9.2 minutes with only a 7% decrease in Cr6+ concentration, when compared to the initial Cr6+ concentration. It was also reported that the second phase of the reduction was the main process and followed pseudo-first-order kinetics.

Ascorbate is found in high concentrations within the cell, and is one of the most important intracellular reducing agents (Snow 1992; Suzuki 1988). Extracellular ascrobic acid serves to detoxify Cr6+ by decreasing cellular uptake. On the other hand, the reduction of chromate by intracellular ascorbic acid and the resultant formation of intracellular active oxygen species increases Cr6+ toxicity. Suzuki (1990) reported that ascorbic acid, when tested *in vitro* at high concentrations, followed pseudo-first-order redox processes in a single phase, with respect to Cr6+. When tested at lower concentrations, ascorbic acid followed pseudo-first-order redox processes for only a short duration. In the presence of ascorbate, Cr6+ is more commonly reduced to Cr3+ than Cr5+. However, upon reduction of Cr6+ to Cr3+ within the presence of ascorbate, reactive oxygen species (e.g., HO, singlet oxygen, and superoxide anion [O_2A-]) may also form (Cohen et al. 1993; Snow 1992). Ascorbic acid and GSH have also been shown to work in combination with a synergistic effect on the reduction of Cr6+. Suzuki (1990) compared the effect of GSH and ascorbic acid on the reduction of Cr6+ and found that the reducing ability of ascorbic acid is much higher than that of GSH. However, when tested *in vitro* with mixed solutions of GSH and ascorbic acid at physiological pH, Cr6+ reduction was markedly accelerated.

DT-diaphorase, an intracellular enzyme, also participates in the reduction of Cr6+ to Cr3+ within the cytoplasm by catalyzing the NADPH and NADH-dependent reduction of Cr6+. However, due to the limited availability of NADPH and NADH in the cytoplasm, the DT-diaphorase mediated reduction of Cr6+ is not as significant as GSH reduction.

Mitochondria

Within the mitochondria, amino acids, such as succinate and glutamate, as well as DT-diaphorase, participate in the reduction of Cr6+ to Cr3+. Succinate and glutamate activate the production of NADPH by contributing electrons from attached sulfhydryl groups (Petrilli and De Flora 1988). DT-diaphorase again acts as a mediator of this reduction by assisting the NADPH in donating electrons to the Cr6+. This process is similar to the reduction of Cr6+ activated by GSH within the cytosol. A two-phase process takes place, with an initial rapid phase followed by a slower reduction phase (Ryberg and Alexander 1984). However, the reduction of Cr6+ activated by succinate works via a different mechanism than the reduction activated by glutamate (Arillo et al. 1987). Succinate is more active in the reduction of Cr6+ when in the presence of ADP or an uncoupler. Glutamate only contributes to the reduction of Cr6+ in the presence of respiratory-chain inhibitors (Arillo et al. 1987).

Endoplasmic Reticulum

The endoplasmic reticulum also plays a role as an intracellular site for Cr6+ reduction through an NADPH-dependent cytochrome P-450 system (DeFlora et al. 1985). Studies indicate that in the presence of NADPH, the microsomal cytochrome P-450 contributes to the reduction of Cr6+ to Cr3+. However, the contribution of cytochrome P-450 is very small and is inhibited by the presence of oxygen (Standeven and Wetterhahn 1989; 1991). Studies also indicate that cytochrome P-450 in the presence of NADPH may reduce Cr6+ to form Cr5+ complexes. These Cr5+ complexes may then interact with H_2O_2 to form OH- radicals (Shi and Dalal 1990).

These intracellular sites contribute to the overall reduction of Cr6+ to Cr3+. The specific reductants may be found in one or more of the intracellular compartments as seen with DT-diaphorase assisting in Cr6+ reduction in both the cytoplasm and within the mitochondria. However, the cytosolic fractions containing GSH have been shown to have the most significant rate of Cr6+ reduction (Connett and Wetterhahn 1985; De Flora et al. 1985). A

Genotoxicity/Mutagenicity

The mutagenicity of chromium in mammalian and bacterial species has been widely documented. Results differ for Cr+3 and Cr+6 tested with intact cells compared to tests with cells in which the cell membrane has been disrupted (i.e., "simplified systems"), due to the ability of Cr+6 to cross cell membranes. Overall, Cr+6 compounds are positive in cellular (intact) systems, whereas Cr+3 compounds have been shown to be inactive unless a direct interaction with DNA is permitted in the course of the test, as in simplified systems.

Studies of Cr6+ have shown that when tested in intact bacterial and mammalian cell cultures, results were positive for mutagenic activity. In bacterial systems, Cr6+ produced base-pair substitutions (mutations favoring A-T sequences rather than G-C), frameshift mutations, and mutations related to error-prone DNA-repair pathways (Cohen et al. 1993; Bianchi and Levis 1985; Cupo and Wetterhahn 1985; Becking 1981; Snow 1992; Mikalsen et al. 1991). Bianchi et al. (1983) reported that Cr+6 was mutagenic, based on the induction of base substitutions and frame-shift mutations, in *Salmonella typhimurium* strains when tested without metabolic activation; however, the addition of rat microsomes suppressed Cr+6 mutagenicity.

In mammalian (intact) test systems, Cr+6 produced a variety of genetic effects including mitotic cycle alterations, nucleotide pool imbalance, and sister chromatid exchange (Bianchi et al. 1983; Bianchi and Lewis 1985; DeFlora et al. 1990). Cr+6 has also been reported to induce morphological cell transformation in mouse and hamster cells (Tsuda and Kato 1977; Bianchi et al. 1983). Cr+6 has been reported to significantly increase the frequency of sister chromatid exchanges in four rodent cell lines (Bianchi et al. 1983). The frequency of chromosomal aberrations, consisting primarily of single chromatid breaks and chromatid exchanges, was significantly increased in hamster embryo cells incubated with Cr+6 compounds ($K_2Cr_2O_7$ and CrO_3); however, when Cr+6 was tested in isolated nuclei or purified DNA, it was found to be inactive (Bianchi and Levis 1985). When tested in cultured hamster cells, Cr+6 induced gene mutations indicated by 6TG resistance (Bianchi et al. 1983). An increase in nuclear protein/DNA crosslinks was reported in Novikoff ascites hepatoma cells incubated with K_2CrO_4 (Wedrychowski et al. 1985). However, the authors concluded that after the hexavalent

chromium entered the cell, it was reduced in the cytoplasm to trivalent chromium that subsequently induced the DNA crosslinks.

Bianchi et al. (1983) reported that when Cr+6 was tested in intact mammalian cells, DNA repair synthesis was not induced. When tested *in vitro* using highly purified DNA, Cr+6 was reported to inhibit DNA replication; however direct DNA damage was not observed (Bianchi et al. 1983). The authors noted that Cr+3 is capable of complexing with nucleotide precursors, polynucleotides and proteins at concentrations much lower than Cr+6 is active, and concluded that the inhibition of DNA replication by chromium compounds was attributed to Cr+3. Cr+6 was also positive for genotoxicity when tested *in vivo*, producing DNA-repair inhibition and various indicators of DNA damage (DeFlora et al. 1990).

Recent *in vitro* studies have evaluated the potential of induction and modulation of genotoxicity by hexavalent chromium and the role of apoptosis in chromium-induced carcinogenesis (Manning et al. 1994; Xu et al. 1996; Blankenship et al. 1997). Internucleosomal DNA fragmentation, typical of cells undergoing apoptosis, inhibition of cell growth and delay in the progression through the S-phase of the cell cycle were observed in intact mammalian cells exposed to hexavalent chromium as Na_2CrO_4 (Manning et al. 1994; Xu et al. 1996; Blankenship et al. 1997). Evidence of direct effects on DNA included single strand breaks, DNA adducts and interstrand crosslinks. The DNA adducts were associated with the suppression of RNA and mRNA synthesis and suppression of the expression of the GRP78 gene (Manning et al. 1994). In cells exposed to Cr+6 *in vitro* guanine-guanine interstrand DNA crosslinks, inhibition of DNA repair and suppression of thymidine incorporation into DNA, indicating an arrest in DNA replication, were reported (Xu et al. 1996). Pretreatment with vitamin C or vitamin E reduced the clastogenic effects produced by Cr+6 in cell cultures (Blankenship et al. 1997). In addition, pretreatment with vitamin C protected cells from chromium-induced toxicity and apoptosis; however, this effect was not observed in cells pretreated with vitamin E. The authors concluded that apoptosis, likely initiated by a guanine-specific termination of DNA replication that is sensitive to the effects of vitamin C but not vitamin E, is the mechanism responsible for chromium cytotoxicity. Cells that escape apoptosis may contain genes damaged by chromium, and replication of these cells could subsequently result in carcinogenesis.

An intermediate product of Cr+6 reduction, Cr+5, is capable of producing biological effects via direct interaction with DNA or enhanced reduction to Cr+3, when complexed with GSH (Goodgame and Joy 1986; Jones 1990; Stearns et al. 1995). The interaction of Cr+6 with GSH has been shown to produce both the long-lived Cr+5 intermediate and the reactive glutathionyl radical (GS·) (Shi and Dalal 1988). Reaction of Cr+6 with GSH has also been shown to result in the formation of the Cr+4-GSH intermediate and hydroxyl radicals (Liu et al. 1997). When Cr+6 was reduced with varying concentrations of ascorbate, Cr+5, Cr+4 and carbon free radicals were formed, with Cr+3 the final product (Stearns and Wetterhahn 1994; Stearns et al. 1995). Cr+5 was associated with Cr/DNA adducts and carbon radicals were associated with DNA single strand breaks. Cr+5 may also be reduced to Cr+4, which causes DNA damage and generate hydroxyl radicals (Luo et al. 1996; Liu et al. 1997).

When tested for mutagenic or genotoxic effects in intact bacterial and mammalian cell systems, Cr3+ was found to be inactive due to its impermeability to the cell membrane. For example, in strains of *S. typhimurium*, Cr+3 was nonmutagenic with and without microsomal activation (Bianchi et al. 1983). There were no increases in the frequency of chromosomal aberrations in hamster embryo cells incubated with trivalent chromium salts [$CrCl_3$ and $Cr_2(SO_4)_3$] (Tsuda and Kato 1977). DNA repair synthesis was not induced by Cr+3, and the frequency of sister chromatid exchanges was not increased when tested in intact mammalian cells (Bianchi et al. 1983). However, when Cr3+ was tested in isolated nuclei or purified DNA, it was found to produce DNA-protein cross-links, sister chromatid exchanges, chromosomal aberrations, and interruptions in DNA replication (Bianchi et al. 1983; Bianchi and Levis 1985; Cohen et al. 1993). Among the various valence states of chromium, only Cr3+ has been shown to complex directly with DNA (Jones 1990), although it is hypothesized that other chromium species and complexes may be able to interact with DNA (DeFlora and Wetterhahn 1989). Cr3+ will form chromium-DNA adducts and can produce DNA strand breaks, interference with DNA restriction enzymes, and other genotoxic effects (DeFlora and Wetterhahn 1989; Snow 1992).

Studies have also evaluated the potential for chromium to generate free radicals and for these free radicals to interact with DNA as a possible mode of carcinogenesis. As discussed above, it has been demonstrated that Cr+6 may react with reduced GSH forming the reactive intermediates, Cr+5 and Cr+4, and a glutathionyl radical (Shi and Dalal 1988; Shi et al. 1992). It

has been demonstrated that Cr+6 may be reduced to Cr+5 *in vivo* (Liu et al. 1994, 1996), with an NADPH/flavoenzyme complex the major reductant (Liu et al. 1996). The Cr+5 intermediate may interact directly with DNA (Shi et al. 1992). However, it has also been shown that reactive free oxygen species, such as the hydroxyl radical, may be formed from hydrogen peroxide or lipid hydroperoxides when Cr+6 is reduced to Cr+5 (Shi et al. 1992; Shi and Dalal 1994; Shi et al. 1994a,b; Ye et al. 1995; Luo et al. 1996; Liu et al. 1997; Shi et al. 1997; Chen et al. 1997), likely via a Fenton-type reaction (Shi and Dalal 1994; Shi et al. 1994a,b). The reaction of hydrogen peroxide with Cr+5 bound to DNA may result in the generation of hydroxyl radicals within close proximity to the DNA increasing the possibility of damage to a specific DNA site (Shi et al. 1992).

Hydroxyl radicals that could damage DNA at specific sites may also be produced by the reduction of Cr+5 to Cr+4 and Cr+3 in Fenton-like reactions (Shi et al. 1993; Shi et al. 1994a; Luo et al. 1996; Liu et al. 1996). Cr+3 may also form hydroxyl radicals via the Haber-Weiss cycle (Shi et al. 1997). As discussed above, Cr+3 is capable of binding with DNA. Therefore, it is possible that the formation of these hydroxyl radicals in close proximity to DNA could result in DNA damage. Free radicals may also be formed when chromium is reduced by intracellular thiols, such as cysteine (Shi et al. 1994b) or α-lipoic acid (Chen et al. 1997). The incubation of Cr+6 with cysteine or penicillamine resulted in the formation of thyil radicals and hydroxyl free radicals, via the Fenton reaction. The authors concluded that these data provided evidence that sulfhydryl groups present on thiols are an important functional group in the reduction of Cr+6.

Chromium may induce carcinogenesis by a mechanism other than direct interaction with DNA or by free radical generation. The reduction of Cr+6 to Cr+4 and the free radical generation associated with this reaction have been shown to activate nuclear factor κB (NF-κB) (Ye et al. 1995; Chen et al. 1997), a transcription factor that induces the overexpression of the c-*myc* gene, which in turn has been associated with the formation of Burkitt's lymphoma (Ji et al. 1994). Therefore, the activation of NF-κB by the reduction of chromium may be involved in chromium-induced carcinogenesis (Ye et al. 1995).

Mode of Action for Carcinogenicity

Even if it is accepted that the carcinogenicity of chromium is via a genotoxic mechanism, two issues concerning this mechanism remain unresolved. These are: 1) the form(s) of chromium responsible for interaction with DNA leading to genotoxic effects and ultimately to carcinogenic effects; and 2) the location where, in the intracellular environment, the active moiety is formed.

Despite the extensive study of chromium and its genetic activity, very little can be said concerning the ultimate genotoxic form. Several authors concede, however, that there may be several genetically reactive moieties (DeFlora and Wetterhahn 1989; Snow 1992). The various active forms are due to differences in a target cell's metabolic capacity within the cytoplasm and compartmentalized cell structures. Putative DNA-damaging species include: Cr5+, Cr4+ and Cr3+; active oxygen species ($HO\cdot$, $\Delta_g 0_2$, 0_2^-); and reactive radicals including $RS\cdot$ and $R\cdot$ (DeFlora and Wetterhahn 1989). All of the above, with the exception of Cr3+, are intermediate or other highly reactive species and are thought to be genetically active only when in close proximity to the nuclear envelope/material. However, a notable exception is demonstrated by binding of Cr5+ to ligands, increasing its stability and lifetime in the cell. The final stable form is Cr3+ which may form chromium-DNA adducts, and mediate cross-linking of DNA strands and DNA to protein.

Issues on intracellular sites of metabolism can be further explored by two proposed theories, as defined by DeFlora and Wetterhahn (1989), of chromium-induced carcinogenesis. The first theory is called the uptake-reduction or uptake-activation mechanism. It involves movement of Cr6+ across the target cell membrane after which it is reduced in cytoplasmic structures to the ultimate genotoxic species. The active genotoxic form(s) would then migrate into the nucleus and cause DNA damage and initiation of cancer (DeFlora and Wetterhahn 1989). This theory infers that Cr6+ is not the genetically active species, but other reduced intermediate species, including the ultimate reduced form, Cr3+, could be the genotoxic agents.

The other theory is the uptake-detoxification mechanism. This theory holds that genotoxic effects would result only when Cr6+ taken into the cell exceeds the reducing mechanisms of the cytoplasm thereby escaping detoxification via reduction. As Cr6+ approaches the nucleus, it, or a reduced form, could interact with DNA.

The authors note that "...a crucial point is the site of the cytoplasm where reduction is achieved. An activating effect is more likely to ensue when reduction occurs in proximity of the nuclear envelope...or even inside the nucleus," whereas production of reduced forms is favored when reduction takes place in organelles or in cytoplasmic sites at distance from the nucleus (DeFlora and Wetterhahn 1989). Both theories may be valid; however, both leave unresolved the issue of the ultimate species which reacts with DNA material to cause genotoxicity and potential carcinogenicity.

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