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Review Article

An Evaluation of the Mode of Action Framework for Mutagenic Carcinogens Case Study II: Chromium (VI)

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In response to the 2005 revised U.S Environmental Protection Agency's (EPA) Cancer Guidelines, a strategy is being developed to include all mutagenicity and other genotoxicity data with additional information to determine whether the initiating step in carcinogenesis is through a mutagenic mode of action (MOA). This information is necessary to decide if age-dependent adjustment factors (ADAFs) should be applied to the risk assessment. Chromium (VI) [Cr (VI)], a carcinogen in animals and humans via inhalation, was reassessed by the National Toxicology Program (NTP) in 2-year drinking water studies in rodents. From these data, NTP concluded that the results with Cr (VI) showed clear evidence of carcinogenicity in male and female mice and rats. Cr (VI) is also mutagenic, in numerous in vitro assays, in animals (mice and rats) and in humans. Accordingly, Cr (VI) was processed through the MOA framework; postulated key steps in tumor formation were interaction of DNA with Cr (VI) and reduction to Cr (III), mutagenesis, cell proliferation, and tumor formation. Within the timeframe and tumorigenic dose range for early events, genetic changes in mice (single/double-stranded DNA breaks) commence within 24 hr. Mechanistic evidence was also found for oxidative damage and DNA adduct formation contributing to the tumor response. The weight of evidence supports the plausibility that Cr (VI) may act through a mutagenic MOA. Therefore, the Cancer Guidelines recommend a linear extrapolation for the oral risk assessment. Cr (VI) also induces germ cell mutagenicity and causes DNA deletions in developing embryos; thus, it is recommended that the ADAFs be applied. Environ. Mol. Mutagen. 00:000–000, 2009. © 2009 Wiley-Liss, Inc.

Key words: mutagenicity; hexavalent chromium; Cr (VI); mode of action

INTRODUCTION

Elemental chromium (Cr) is a naturally occurring element found in rocks, animals, plants, soil, and volcanic dust and gases [ATSDR, 2000]. In the environment, Cr exists in two stable oxidation states: +3, trivalent or Cr (III), and +6, hexavalent or Cr (VI). Reductive metabolism of Cr (VI) in mammalian cells through the redox system leads to the formation of the intermediate and unstable forms [Cr (V) and Cr (IV)] that ultimately yield Cr (III) [Shrivastava et al., 2005]. Cr (III), which is less toxic than Cr (VI), is considered to be kinetically inert when introduced to a cell but is an essential nutrient in the human body. Cr (VI) is largely introduced into the environment by man-made processes such as the manufacture of steel and other alloys. Chrome plating, dye and pigment manufacturing, leather tanning, and wood preserving are other major uses of Cr (VI). The Agency for Toxic Substances and Disease Registry

The views expressed in this article are those of the authors and do not reflect the policies of the US Environmental Protection Agency.

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[ATSDR, 2000] estimated that in the United States, ~2,700–2,900 tons of Cr are annually emitted to the atmosphere and ~35% is in the form of Cr (VI). Recent analyses have revealed detectable levels of Cr (VI) in 38% of the of the drinking water sources in California [Sedman et al., 2006]. The general population is exposed to Cr through eating food or food supplements, drinking water, and inhaling air contaminated with Cr. Dermal exposure can also occur from skin contact with certain consumer products that contain Cr such as wood preservatives, cement, and cleaning materials [ATSDR, 2000, 2008]. Because of this potential widespread exposure and the lack of an adequate assessment of chronic oral exposure, the California Congressional Delegation, the California Environmental Protection Agency, and the California Department of Health Services nominated Cr (VI) to the National Toxicology Program (NTP) for study.

Accordingly, NTP conducted 2-year rodent drinking water studies with Cr (VI). Based on the results, NTP stated that Cr (VI) showed clear evidence of carcinogenic activity in male and female mice and rats [NTP, 2008]. The Environmental Protection Agency's (EPA's) Cancer Assessment Review Committee (CARC) of the Office of Pesticide Programs (OPP), using the same data classified Cr (VI) as ''Likely to be Carcinogenic in Humans'' via the oral route, based on oral cavity tumors in male and female rats and tumors of the small intestine in male and female mice [Kidwell, 2008]. Because the CARC also concluded that Cr (VI) is positive in a wide range of mutagenicity studies, causing gene mutations in bacteria, mammalian cells and transgenic mice, chromosome aberrations, and a variety of other types of DNA damage both in vitro and in vivo, the possibility that Cr (VI) is a rodent carcinogen via a mutagenic mode of action (MOA) was explored. The purpose of this data analysis was to determine if the gastrointestinal tumors induced by Cr (VI) in both mice and rats occurred via a mutagenic MOA. The analysis is in accordance with the EPA's 2005 Guidelines for Carcinogen Risk Assessment [USEPA, 2005a] and the Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens, particularly, agents with a mutagenic MOA [USEPA, 2005b].

EPA's Risk Assessment Forum's Technical Panel [USEPA, 2007] is developing a strategy/framework in which mutagenicity and genotoxicity data combined with additional information relevant to mutagenicity are assessed to determine whether a carcinogen operates through a mutagenic MOA. This information is necessary for proper implementation of the 2005 revised U.S. EPA Cancer Guidelines and deciding whether age-dependent adjustment factors (ADAFs) should be applied to the cancer risk assessment. Because there is some controversy regarding the definition of certain terms used throughout this document, the following definitions are provided to describe mutagenicity and genotoxicity, as the authors use them:

Genotoxicity—Refers to the ability of agents to interact with and damage DNA.
Mutagenicity—Refers to the ability of agents to cause gene mutations and/or chromosome aberrations.

Within the context of these definitions, agents that are mutagenic are also genotoxic but agents that are listed only as being genotoxic only cause damage to DNA. Examples of assays designed to detect genotoxicity include: sister chromatid exchange (SCE), unscheduled DNA synthesis (UDS), DNA strand breaks, or DNA adducts.

MATERIALS AND METHODS

Data Collection

A framework for the analysis of carcinogens with a mutagenic MOA has been developed which uses a decision tree as part of the general approach for analysis of a mutagenic MOA (Fig. 1). As outlined in the decision tree, the first step in this process is to gather and organize genotoxicity data and determine if the criteria established to provide sufficient evidence of mutagenicity could be satisfied. The second step is to determine whether a mutagenic MOA for carcinogenesis can be demonstrated in animals and, if so, to determine whether a mutagenic MOA is plausible and/or supported in humans. For Cr (VI), data were extracted from the relevant literature on mutagenicity, toxicity, and carcinogenicity to decide if a link could be made between Cr (VI)-induced tumors and mutagenicity. It should be note that most of the studies evaluated in this document were primarily designed for hazard identification and, consequently, studies that specifically address various aspects of the MOA analysis were not available for review. Nevertheless, we have taken a weight-of-the-evidence approach to analyze the data and determine whether missing data influence the final conclusion. Similarly, the findings presented in this document do not represent an exhaustive list of all of the mutagenicity or carcinogenicity data available on Cr (VI). The reader is referred to the excellent reviews prepared by Sedman et al. [2006], Costa and Klein [2006] and the toxicological profile on Cr published by ATSDR in 2000 as well as the updated draft toxicological profile on Cr [ATSDR, 2008]. Data from all cited literature were also briefly reviewed for general quality; no major study deviations from regulatory guidelines were seen in the cited literature used in this undertaking. Our basic approach for preparing this document has been presented in earlier publications [McCarroll et al., 2002, 2008].

Genetic Toxicology Data

In vitro and in vivo genetic toxicology data from the open literature were surveyed as previously described [Dearfield et al., 2005], and the genetic activity profile (GAP) used was developed jointly with the International Agency for Research on Cancer (IARC) to graphically display genetic toxicology data as a function of concentration or dose. Details for the schematic representation of the GAP for Cr (VI) and Cr (III), depicted in Figure 2, can be found in Waters et al. [1988], as updated by Lohman and Lohman [2000].

Carcinogenicity/Mode of Action Data

Carcinogenicity data were extracted from the NTP 2-year rat and mouse drinking water studies [NTP, 2008]) and the open literature.

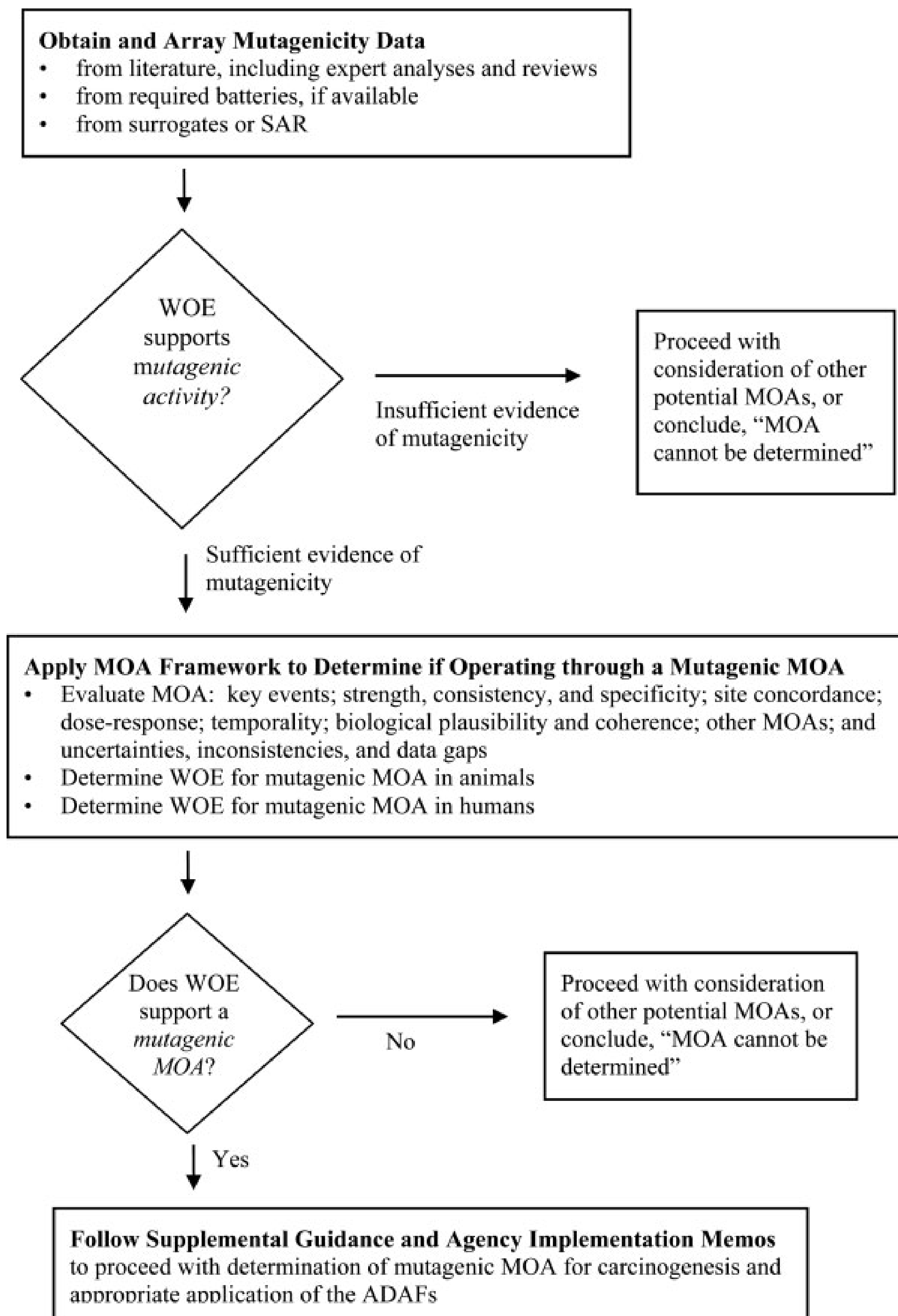


Fig. 1. Framework for the analysis of carcinogens with a mutagenic mode of action. Adapted from Dearfield and Moore [2005].

Epidemiological studies of carcinogenicity and mutagenicity were derived from the reviews of Costa and Klein [2006] and Sedman et al. [2006]. Relevant data pertaining to metabolism, various phases of the MOA analysis (i.e., description of the postulated MOA, key events, dose response, temporal associations, biological plausibility, other MOAs, and relevance to humans) were derived from studies reviewed by Costa and Klein [2006] and Sedman et al. [2006] and from the open literature.

CRITICAL ANALYSIS OF THE AVAILABLE DATA

Genetic Toxicology

In Vitro

Mutagenicity studies. Based on the GAP developed by Waters et al. [1988], updated by Lohman and

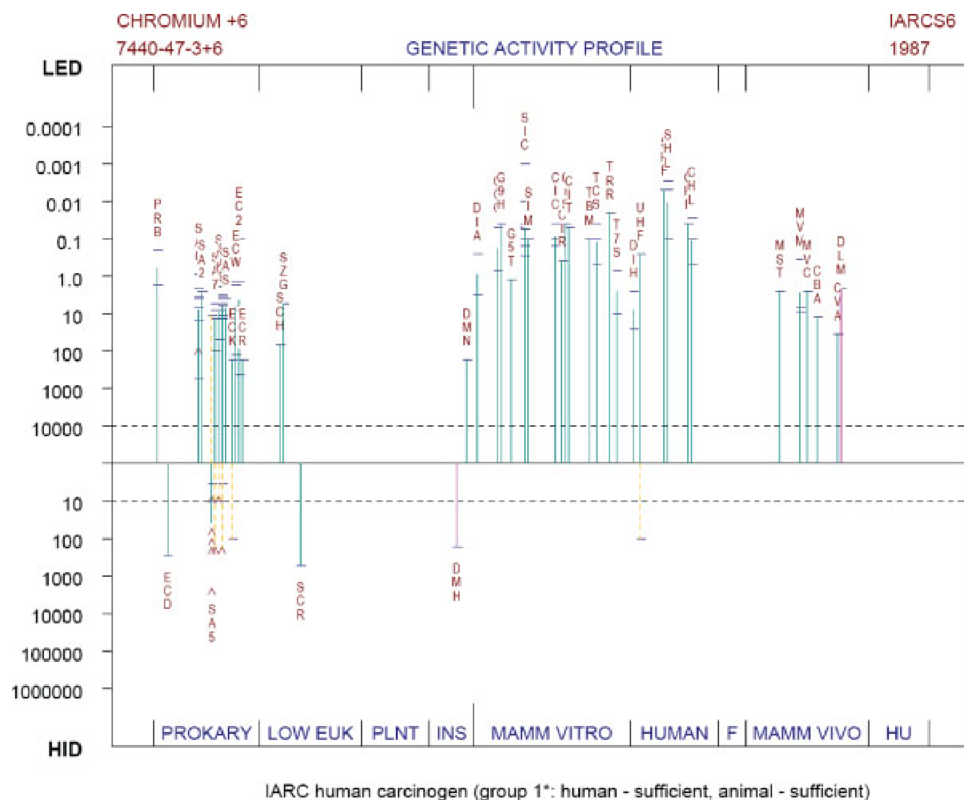


Fig. 2. Genetic activity profile (GAP) for chromium (VI) (extracted from Waters et al., 1988 and Lohman and Lohman [2000]). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Lohman [2000] and presented in Figure 2, there is a wealth of genetic toxicology data showing that Cr (VI) is an in vitro mutagen, causing point mutations in *Salmonella typhimurium* and *Escherichia coli*. Microbial data compiled from the work of NTP [2008], Haworth et al. [1983], and Watanabe et al. [1998] are presented in Table I and illustrate the point that Cr (VI) induces a clear linear concentration response in mutant colonies at nonactivated concentrations down to relatively low-noncytotoxic levels in bacterial strains (*S. typhimurium* and *E. coli*), which are sensitive to agents inducing base pair substitutions, point mutations, as well as oxidative damage and DNA crosslinks. It is also mutagenic in *Saccharomyces cerevisae* [Singh, 1983] and in mammalian cell lines [Chinese hamster ovary (CHO), Chinese hamster lung V79, and mouse lymphoma cells [Paschin et al., 1983; McGregor et al., 1987]. Clastogenic activity is induced in cultured CHO cells [Seoane and Dulout, 1999], mouse mammary FM3A carcinoma cells [Umeda and Nishmura, 1979] and human lymphocytes [Nakamuro et al., 1978; Sarto et al., 1980; Stella et al., 1982]. Morphological cell transformation has also been found in BALB/C3T3, Syrian hamster embryo, and the Rauscher leukemia virus-infected Fischer rat embryo cell lines and rat liver epithelial cells [Dunkel et al., 1981; Briggs and Briggs, 1988]. In general, S9 activation is not required to detect the mutagenic effects seen in the in vitro studies. Thus, the weight-of-the-evidence from these in vitro data suggests that Cr (VI) is a direct-acting mutagen. By contrast, Cr (III) is largely devoid of mutagenic activity [ATSDR, 2008]). However, there are data indicating that Cr (III) does induce DNA-protein crosslinks in cell-free nuclei and in solutions of DNA and protein while Cr (VI) was not active [Fornace et al., 1981]. The authors suggest that these findings indicate that the trivalent state may be the final active entity of the hexavalent form. Similarly, Snow and Xu [1991] found that low concentrations (0.4–2 μM) of Cr (III) as $CrCl_3$ increased the rate of nucleotide incorporation (2-to 6.3-fold, respectively) in the DNA of single-stranded M14mp2 bacteriophage by altering DNA polymerase-template interactions, thus increasing DNA polymerase processivity and decreasing fidelity during replication. On the basis of these findings, the authors proposed that Cr (III) ''may act synergistically to enhance the mutagenic potential of DNA lesions produced during the intracellular reduction of Cr (VI)....''

DNA damage studies. As further indicated in the GAP, Cr (VI) is genotoxic in the absence of S9 activation, causing DNA damage/repair in bacteria, alkaline

TABLE I. Mutant Colony Counts (MCC) and Fold Increases (FI) in *Salmonella typhimurium* Strains and *Escherichia coli* Strains Exposed to Nonactivated Cr (VI) in the Standard Plate Incorporation Assay

	Revertants/plate[a]											
	Salmonella typhimurium strains								*Escherichia coli* strains			
Dose	TA100[b]		TA100[c]		TA102[d]		TA2638[d]		WP_2/pKM101[d]		WP_2 *uvrA*/pKM101[d]	
(μg/plate)	MCC	FI	MCC	FI	MCC	FI	MCC	FI	MCC	FI	MCC	FI
0	65	–	103		464		43		54		105	
2.5					821	1.8	52		63		154	1.5
5					916	2.0	83	1.9	101	1.9	361	3.4
10	117	1.8	119		1283	2.8	129	3.0	331	6.1	839	8.0
20					1714	3.7	465	10.8	945	17.5	1702	16.2
25	272	4.2										
40					735[e]	1.6	172[e]	4.0	1555	28.8	1909	18.2
50	386	5.9	285	2.8								
75	154	2.4	464	4.3								
80									1311	24.3	1385[e]	13.2
100			791	7.7								
160									629[e]	11.6	330[e]	3.1

[a]Standard Plate Incorporation Assay; triplicate plates/dose/strain; positive controls included with all assays. All assays were repeated.
[b]Data from the NTP study [NTP, 2008]; Cr[VI] as $Na_2Cr_2O_7$.
[c]Data from the Haworth et al. study [1983]; Cr[VI] as $CaCr_2O_4$.
[d]Data from the Watanabe et al. study [1998]; Cr[VI] as CrO_3.
[e]Cytotoxic.

elution in rat hepatocytes, DNA–protein crosslinks, and DNA strand breaks in mammalian and/or human cells. Venier et al. [1982] showed a good response for SCE induction in CHO cells with significant activity ($P < 0.001$) detected after treatment for two division cycles (30 hr) with $K_2Cr_2\ O_4$ at a concentrationas low as 0.1 μg/ml. UDS has also been demonstrated in similar mammalian cell lines and human cells, such as lymphocytes and fibroblasts. Likewise, many investigators [Zhitkovich et al., 1998, 2002, 2005; Voitkun et al., 1998; Quievryn et al., 2003] have found evidence of DNA adduct formation in mammalian and human cells. The works of Zhitkovich et al. [2002], and Zhitkovich [2005], indicate that Cr-DNA adducts are the most abundant form of Cr (VI)-induced genetic lesions in mammalian cells and are largely responsible for all of the mutagenic damage generated during Cr (VI) reduction with cysteine and ascorbate.

The overall assessment from these in vitro findings is that Cr (VI) is generally positive in vitro for multiple genetic toxicology endpoints in an increasingly higher order of phylogenetically distinct species.

Whole animal studies

Gene mutation assays. Until recently, the only in vivo assay that could detect gene mutations was the somatic cell mouse spot test. Using this test system, Knudsen [1980] showed positive results in C57BL/6J/BOM9 female mice administered intraperitoneal (i.p.) doses (thus, initially bypassing the liver) of 10 or 20 mg/kg/day $K2CrO_4$ or 100 mg/kg/day welding fume particles at days 8, 9, and 10 of pregnancy. Positive results were also obtained for the welding fume particles and both doses of K_2CrO_4. Since that time, Itoh and Shimada [1998] have evaluated Cr (VI) in the transgenic Muta^{TM} Mouse assay with the *lacZ* gene as the mutational target. In this study, 40 mg/kg K_2CrO_4 was administered via a single i.p. injection to groups of five male mice. Mutation frequencies in the bone marrow and liver were significantly increased by days 1 or 7, respectively. These in vivo data, showing gene mutations in mouse bone marrow and livers, suggest a possible systemic response. In contrast, the occurrence of gene mutations in the in vitro data clearly demonstrates that Cr (VI) is a direct-acting mutagen, suggesting a portal of entry effect. A plausible explanation for these unexpected findings is that under these conditions, Cr (VI) administered i.p. bypasses the metabolic capabilities of the gastrointestinal tract and is perhaps only marginally reduced to Cr (III), thus leaving a portion of the Cr (VI) dose available to other tissues such as the liver and the bone marrow. Ample proof of how well Cr (VI) can induce adverse effects at sites distant from the portal of entry comes from the study of Davidson et al. [2004] showing significantly increased skin tumors in female mice receiving 2.5 or 5.0 ppm (~360 or 714 μg/kg/day) K_2CrO_4 in drinking water for 1 month followed by radiation treatment (see carcinogenicity data). Nevertheless, data generated via the i.p. route are not relevant to the assessment of oral exposure; these findings do, however, support the argument that Cr (VI) is positive in vivo and do provide qualitative information that can affect the hazard identification.

TABLE II. Summary of In Vivo Genotoxicity of Chromium (VI)

Endpoint	Dose/route	Time of evaluation	Tissue (strain)	Response	References
Rat					
Chromosome aberrations	1 mg/kg/day K_2CrO_4/oral gavage	1 year	Bone marrow	+	Bigaliev et al. [1977][a]
	15 mg/kg K_2CrO_4/single dose oral gavage	2, 4, 6, 8, and 12 hr	Bone marrow (unspecified)	+	
DNA protein crosslinks	100 or 200 ppm (6.1 or 8.7 mg/kg/day) K_2CrO_4/drinking water	3 weeks	Liver Lymphocytes (Fisher 344)	+ −	Coogan et al. [1991][a]
DNA single strand breaks	10 mg/kg/day $Na_2Cr_2O_7$/oral gavage	15, 30, 45, 60, 75, or 90 days	Liver (Sprague–Dawley)	+	Bagchi et al. [1995a][a]
DNA single strand breaks	25 mg/kg $Na_2C_2rO_7$/ single dose/oral gavage	48 hr	Liver (Sprague–Dawley	+	Bagchi et al. [1995b][a]
DNA single strand breaks	2.5 mg/kg/day $Na_2Cr_2O_7$/oral gavage	120 days	Liver and brain (Sprague–Dawley)	+	Bagchi et al. [1997][a]
Mice					
Single and double DNA strand breaks (Comet Test)	0.59–9.5 mg/kg $Na_2Cr_2O_7$ single dose/oral gavage	1–14 days	Leucocytes (Swiss)	+	Dana Devi et al. [2001][a]
DNA fragmentation	1.9–95 mg/kg $Na_2Cr_2O_7$ Single dose/oral gavage	24 hr	Liver Brain (C547BL/6Ntac and C57BL/TSG p 53)	+ +	Bagchi et al. [2002][a]
Gene mutation	10 and 20 mg/kg/day K_2CrO_4or 100 mg welding fume/IP	8, 9, and 10 days of pregnancy	Skin (C57BL/6J/BOM9)	+	Knudsen [1980]
Gene mutation	40 mg/kg $K_2Cr_2O_7$ Single dose/IP	1 and 7 days	Bone marrow Liver (transgenic muta mouse)	+ (Day 1) + (Day 7)	Itoh and Shimada [1998]
Mice					
Micronucleus induction	30, 40, 50 mg/kg/day K_2CrO_4/IP X2	24 hr after second injection	Bone marrow (Slc:ddY)	+	Itoh and Shimada [1996]
Micronucleus induction	12.12–48.5 mg/kg/day K_2CrO_4/IP X2	6 hr after second injection	Bone marrow (NMRI)	+	Wild [1978]
Micronucleus induction	12 mg/kg/day K_2CrO_4/IP X2	24 hr after second injection	Bone marrow (ICR Pregnant ♀)	+	Chorvatovičová and Ginter [1989]
Micronucleus induction	21 mg/kg K_2CrO_4/single dose/IP	30 hr	Bone marrow (BALB/c)	+	Wronska–Nofer et al. [1999]
Micronucleus induction	10–320 mg/kg K_2CrO_4/single dose/IP	24 hr	Bone marrow (CD-1 and MS/Ae)	+	Shindo et al. [1989][a]
Micronucleus induction	10–320 mg/kg K_2CrO_4/single dose/oral gavage	24, 48, and 72 hr	Bone marrow (CD-1)	_	Shindo et al. [1989][a]
Micronucleus induction	1–20 ppm (0.05–1.0 mg/kg/day) K_2CrO_4 drinking water and oral gavage	3 months	Bone marrow (Swiss Webster)	−	Mirsalis et al. [1996][a]
Micronucleus induction	62.5–1000 mg/kg/day Na_2CrO_4 drinking water	3 months	Bone marrow (B6C3F1)	−	NTP [2007]
	62.5–1000 mg/kg/day Na_2CrO_4 drinking water	3 months	Bone marrow (BALB/c and am3-C57BL/6)	−	
Chromosome aberrations	20 mg/kg CrO_3/single oral gavage	24 hr	Bone marrow (Swiss)	+	Sarkar et al. [1993][a]

[a]Adapted from Sedman et al. [2006].

Chromosomal aberration assays. Numerous in vivo studies in rats and mice also exist in the literature for Cr (VI). Results from these assays have been summarized by Sedman et al. [2006] and appear in Table II. As shown, Cr (VI) administered via oral gavage is positive for inducing chromosome aberrations in the bone marrow, livers,

or brains of rats, as well as DNA–protein crosslinks and DNA single strand breaks in these tissues. Only one study using drinking water as the route of exposure was available for rats. In this study, Coogan et al. [1991] found that DNA–protein crosslinks were produced in the livers of F 344 rats receiving 100 or 200 ppm (6.1 or 8.7 mg/kg/day) K_2CrO_4 for 3 weeks. These levels approximate the doses causing tumors in the rats (~6–7 mg/kg/day) in the NTP 2-year drinking water study in rats.

In mice, a slightly different picture emerges and generally indicates that Cr (VI) induces mutagenic events (i.e., gene mutations, chromosome aberrations, or micronuclei) when it is administered via the i.p. route. For example, the results from the study of Shindo et al. [1989] indicate that when Cr (VI) was administered either by oral gavage or by i.p. injection at 10–320 mg/kg, a dose-dependent increase in the incidence of micronucleated polychromatic erythrocytes (MPCEs) was obtained by the i.p. route but not when the oral route of exposure was employed. These conflicting results led the investigators to consider that Cr (VI) may not be ''distributed to the bone marrow because of metabolic inactivation or difficulty of absorption.'' Similarly, Mirsalis et al. [1996] observed no increase in the incidence of MPCEs in mouse bone marrow after a drinking water exposure to 0.05–1.0 mg/kg/day for 3 months that exceeded ''the maximum level expected in human consumption.'' They speculated that the negative results occurred because the animals in this study had sufficient ''reductive capacities in their gastrointestinal tracts to prevent uptake of Cr (VI) into the blood and/or distribution in to the target tissues assessed.'' This rationale probably accounts for the three negative drinking water peripheral blood micronucleus assays conducted by NTP [2007] in mice.

De Flora et al. [2006] undertook a comprehensive study of micronucleus detection using drinking water, intragastric, or i.p. administration of $K_2Cr_2O_7$ or $NA_2Cr_2O_7$ to the male and female BDF1 mouse strain or pregnant Swiss mice. In the drinking water phase of testing, 10 and 20 mg/l of either Cr compound were administered for 20 days ($K_2Cr_2O_7$) or up to 210 consecutive days ($NA_2Cr_2O_7$) to male and female BDF1; no increases in the frequency of micronuclei were observed in either the bone marrow or peripheral blood. Similarly, negative results were obtained in the bone marrow of dams and the livers of fetuses receiving 5 or 10 mg/l $NA_2Cr_2O_7$ or $K_2Cr_2O_7$ in drinking water until day 18 of pregnancy. The single intragastric administration of 50 mg/kg of Cr (VI) as $K_2Cr_2O_7$ on day 19 also failed to produce any evidence of micronucleus induction. By contrast, positive results were achieved in the bone marrow of the BDF1 adult mice and the Swiss dams as well as in the liver and peripheral blood of the fetuses following the single i.p. administration of 50 mg/kg $NA_2Cr_2O_7$ or $K_2Cr_2O_7$ on day 17 of pregnancy. These findings led De Flora et al. to conclude that Cr (VI) is not genotoxic to the hematopoietic cells of either adult mice or transplacentally exposed fetuses when administered in drinking water at doses that exceed drinking water standards because Cr (VI) ''is efficiently detoxified in the gastrointestinal tract.'' Nevertheless, these authors did concede that the micronuclei induction seen in the peripheral blood of fetuses is a noteworthy finding and state that ''evidently, detoxification in the blood, which normally prevents adverse effects of Cr (VI) at a distance from the portal of entry into the organisms, was overwhelmed at the dose of 50 mg of either $NA_2Cr_2O_7$ or $K_2Cr_2O_7$ per kg body weight.''

A similar argument can be proposed for the chromosomal aberrations reported by Sarkar et al. [1993] in mouse bone marrow 24 hr after oral gavage exposure to 20 mg/kg Cr (VI) oxide (CrO_3). The majority of aberrations were chromatid breaks; however, rearrangements (i.e. exchanges) were also increased. It is of note that the dose in this study exceeds the high doses in the NTP drinking water study (6–9 mg/kg/day) that produced adenomas and carcinomas in mice. It does, however, support the earlier comments of Sedman et al. [2006] that when Cr (VI) is not completely converted to Cr (III) in the stomach, a portion of the oral dose may be available for other tissues that would then be subjected to the mutagenic action of Cr (VI).

Other studies showing genetic damage. There are data from other assays indicating that Cr (VI) can overcome the blood testicular barrier and induce mutagenicity in germ cells. For example, Cr (VI) induced dominant lethal mutations in male hybrid mice (CBA × C57Bl/6J) following acute i.p. exposure to 20 mg/kg and following ''chronic exposure'' to 2.0 mg/kg (1 × daily for 21 days; i.p.) [Paschin et al., 1982]. Similarly, Kirpnick-Sobol et al. [2006] reported that Cr (VI) can be absorbed and pass through the placental barrier of pregnant C57BL/6J p^{un}/p^{un} mice receiving 62.5 or 125 mg/l Cr (VI) as $K_2Cr_2O_7$ in drinking water and cause weak but significant ($P < 0.01$) increases (27 or 38%, respectively) in the number of eyespots on the retinal epithelium (indicative of DNA deletions) of the developing embryos. The authors also reported the unexpected significant increases (36 or 53%, $P < 0.001$) in DNA deletions in pups exposed to much higher doses (1875 or 3750 mg/l) of Cr (III). Despite the above evidence of genotoxicity in several drinking water studies, De Flora et al. [2008] disputed the finding that Cr (VI) is mutagenic via the oral route. This argument is based on the lack of 8-oxo-dG adducts or DNA protein crosslinks in the forestomach, glandular stomach or the duodenum of female SKH-1 hairless mice receiving 5 or 20 mg/L $Na_2Cr_2O_7 \cdot 2\ H_2O_2$ (equivalent to 1.2 or 4.7 mg/kg/day) in drinking water for 9 months. However, the levels tested in this study may in part explain the negative conclusion, because they are

below the doses causing tumors in the NTP 2-year drinking water studies. Throughout the discussion of in vivo genetic toxicology studies (oral vs. i.p. injection), several authors have alluded to the possibility that once the reductive capacity of the cell is exhausted, the mutagenic and tumor responses of Cr (VI) may be augmented. It is of note that De Flora et al [2008] are in agreement with this remark and also with the earlier comments of Sedman et al [2006] that Cr (VI) is mutagenic when tested in vitro or in vivo if it can reach remote tissues and be reduced to Cr (III). Costa and Klein [2006] suggest that this is likely to occur after chronic or high dose exposures.

Human studies. The ATSDR [2000] reported conflicting or equivocal results for genotoxicity assays conducted in humans occupationally exposed to Cr (VI). However, in the recent revisit of the toxicological profile for Cr, ATSDR states that the more recent occupational exposure studies have identified genotoxic effects in exposed workers [ATSDR, 2008]. For example, Danadevi et al. [2004] applied simple and multiple linear regression analysis to the data from 102 welders (matched for age, smoking habits, alcohol consumption, duration of exposure, and medical treatment with 102 negative controls) and revealed that the levels of Cr (VI) in the workplace correlated positively with DNA damage (Comet Assay) in peripheral blood leukocytes. A similar correlation was found for nickel. Additional testing revealed a significant link between blood Cr levels and micronucleus induction in the buccal epithelial cells harvested from 58 welders exposed to both Cr and nickel, but no correlation could be made between micronucleus induction and nickel levels. These data confirm the earlier results reported by Benova et al. [2003] of significant increases in micronucleus induction in the peripheral blood lymphocytes and buccal cells of Cr workers. However, no significant effects on the frequency of chromosome aberrations or SCE were observed in the lymphocytes of the exposed workers. Because these studies are from occupational exposures, the influence of confounding factors could be disputed; nevertheless, they do provide suggestive evidence of mutagenic effects in humans.

CARCINOGENICITY

Animals

Davidson et al. [2004] were the first to show that Cr (VI) is carcinogenic via drinking water. In this study, CRL: SK1-hrBR hairless female mice (15–19/group) were initially exposed to 0.5, 2.5, or 5.0 ppm (~71, 360, or 714 μg/kg/day) K_2CrO_4 in drinking water for 1 month. Mice were irradiated with 1.1 kJ/m^2 ultraviolet radiation (UVR) 3×/week for the first 3 months, 2×/week for the next 3 months, and terminated at day 120. Results show significant ($P < 0.05$) increases in skin tumors (majority were squamous cell carcinomas, 1 sarcoma, and 1 basal cell carcinoma) at 2.5 and 5.0 ppm in the irradiated mice; tumors started to appear at day 120. As mentioned earlier, this study is the first to illustrate ''how well hexavalent Cr, delivered by ingestion, can penetrate to distant sites such as the skin.''

Data from the NTP mouse and rat drinking water studies [NTP, 2008], which were the subject of the HED CARC meeting in November 2007 [Kidwell, 2008], confirmed the carcinogenicity of Cr (VI). In these studies, B6C3F1 mice were exposed to 0, 14.3, 28.6, 85.7, or 257.4 mg/l Na_2CrO_4 (M) or 0, 14.3, 57.3, 172, or 516 mg/l (F) [average daily doses of Cr (VI) equivalent to 0, 0.45, 0.9, 2.4, or 5.7 mg/kg (M) or 0, 0.3, 1.2, 3.2, or 8.8 mg/kg (F)] for 2 years. As shown in Table III, the incidence of neoplasms of the small intestines (duodenum, jejunum, or ileum combined) at 257.4 mg/l (M, 34%) and 172 and 516 mg/L (F, 34 and 44%, respectively) were significantly ($P < 0.01$) increased. Significant increases in the incidence of carcinomas and the combined tumor incidences for adenomas and carcinomas were also observed at 257.4 mg/l (M) and 172 and 516 mg/l (F) or exceeded the historical control ranges at lower doses of $\geq$85.7 mg/l (M) or $\geq$57.3 mg/l (F). The time to tumor for both sexes was ~451 days.

In the NTP rat drinking water study [NTP, 2008], F344 rats were exposed to 0, 14.3, 57.3, 172, or 516 mg/l Na_2CrO_4 [average daily doses of Cr (VI) equivalent to 0, 0.21, 0.77, 2.10, or 5.95 mg/kg (M) or 0, 0.26, 0.95, 2.45, or 7 mg/kg (F)] for 2 years. The incidence of squamous cell papillomas or squamous cell carcinomas in the oral mucosa or tongue of the 516-mg/l male and female rats were significantly (trend and pairwise at $P < 0.01$) increased. The incidence at 172 mg/l (F) and 516 mg/l (M F) exceeded the historical control range for drinking water studies and for all other routes of administration. The time to tumor for both sexes was ~543 days (Table IV).

De Flora et al. [2008] questioned these findings and argued that the increased tumors in the oral cavity of the rat may be due to local irritation and oxidation possibly combined with ''some mechanical stimulus'' from the water bottle cannula. If the water bottle cannulae were associated with tumor formation, it would be reasonable to assume that similar findings would be seen in the untreated controls. We note that there were no signs of irritation or oral tumors in the negative control males; no signs of irritation and only one squamous cell papilloma were recorded for the oral cavity of the control females. Similarly, no evidence of compound irritation was reported in either sex up to the highest dose tested. In fact, CARC concluded that the high dose (516 mg/l) was adequate but not excessive in both sexes, based on non-neoplastic liver lesions and histiocytic infiltration in the

TABLE III. Incidence of Neoplasms and Nonneoplastic Lesions in the Small Intestine of Mice Receiving Sodium Dichromate Dihydrate in the 2-Year Drinking Water Bioassay[a]

	Dosage: mg/l (mg/kg)				
Male	0	14.3 (0.45)	28.6 (0.9)	85.7 (2.4)	257.4 (5.7)
No. Necropsied	50	50	50	50	50
Duodenum					
Epithelium, hyperplasia, focal	0	0	0	1 (3.0)[b]	2 (3.5)
Epithelium, hyperplasia, diffuse	0	11** (2.0)	18** (1.6)	42** (2.1)	32** (1.7)
Infiltration cellular, histocytes	0	2 (1.0)	4 (1.0)	37** (1.2)	35** (1.7)
Adenoma, multiple	0	0	0	0	6*
Adenoma (includes multiple)[c]					
Overall rate[d]	1/50 (2%)	0/50 (0%)	1/50 (2%)	5/50 (10%)	15/50 (30%)
Adjusted rate[e]	2.2%	0.0%	2.3%	10.8%	32.9%
First incidence (days)	665	–	729 T	729 T	451
Poly-3 test[f]	$P < 0.001$	$P = 0.505$	$P = 0.751$	$P = 0.106$	$P < 0.001$
Carcinoma[g]					
Overall rate	0/50 (0%)	0/50 (0%)	0/50 (0%)	2/50 (4%)	3/50 (6%)
Adjusted rate	0.0%	(0.0%)	0.0%	4.3%	6.8%
First incidence (days)	–	–	–	729 T	729 T
Poly 3 test	$P = 0.011$	–	–	$P = 0.243$	$P = 0.113$
Jejunum adenoma[h]					
Overall rate	0/50 (0%)	0/50 (0%)	0/50 (0%)	0/50 (0%)	3/50 (6%)
Adjusted rate	0%	0%	0%	0%	6.8%
First incidence (days)	–	–	–	–	714
Poly-3 test	$P = 0.002$	–	–	–	$P = 0.114$
Carcinoma, multiple	0	1	0	0	0
Carcinoma (includes multiple)[i]	0	2	0	1	2
Duodenum, jejunum, or illeum adenoma[j]					
Overall rate	1/50 (2%)	1/50 (2%)	1/50 (2%)	5/50 (10%)	17/50 (34%)
Adjusted rate	2.2%	2.3%	2.3%	10.8%	37.2%
First incidence (days)	665	729 T	729 T	729 T	451
Poly-3 test	$P < 0.001$	$P = 0.755$	$P = 0.751$	$P = 0.106$	$P < 0.001$
Carcinoma[k]					
Overall rate	0/50 (0%)	2/50 (4%)	1/50 (2%)	3/50 (4%)	5/50 (10%)
Adjusted rate	0.0%	4.5%	2.3%	6.5%	11.4%
First incidence (days)	–	729 T	729 T	729 T	729 T
Poly 3 test	$P = 0.014$	$P = 0.233$	$P = 0.492$	$P = 0.123$	$P = 0.028$
Combined adenoma and carcinoma[l]					
Overall rate	1/50 (2%)	3/50 (6%)	2/50 (4%)	7/50 (14%)	20/50 (40%)
Adjusted rate	2.2%	6.8%	4.6%	15.1%	43.8%
First incidence (days)	665	729 T	729 T	729 T	541
Poly-3 test	$P < 0.001$	$P = 0.296$	$P = 0.485$	$P = 0.032$	$P < 0.001$
Female	0	14.3 (0.3)	57.3 (1.2)	172 (3.2)	516 (8.8)
No. Necropsied	50	50	50	50	50
Duodenum					
Epithelium, hyperplasia, focal	0	0	1 (2.0)	1 (3.0)	0
Epithelium, hyperplasia, diffuse	0	16** (1.6)	35** (1.7)	31** (1.6)	42** (2.2)
Infiltration cellular, histocytes	0	0	4 (1.3)	33** (1.2)	40** (2.0)
Adenoma, multiple	0	0	0	1	6*
Adenoma (includes multiple)[m]					
Overall rate	0/50 (0%)	0/50 (0%)	2/50 (4%)	13/50 (26%)	12/50 (24%)
Adjusted rate	0.0%	0.0%	4.2%	27.8%	25.2%
First incidence (days)	–	–	729 T	729 T	693
Poly-3 test	$P < 0.001$	–	$P = 0.251$	$P < 0.001$	$P < 0.001$
Carcinoma[n]					
Overall rate	0/50 (0%)	0/50 (0%)	0/50 (0%)	1/50 (2%)	6/50 (12%)
Adjusted rate	0.0%	0.0%	0.0%	2.1%	12.6%
First incidence (days)	–	–	–	729 T	625
Poly 3 test	$P < 0.001$	–	–	$P = 0.507$	$P = 0.019$

TABLE III. ***continued***

	Dosage: mg/l (mg/kg)				
Jejunum					
Epithelium, hyperplasia, diffuse	0	2 (2.0)	1 (1.0)	0	8^{**} (1.9)
Infiltration cellular, histocytes	0	0	0	2 (1.0)	8^{**} (1.6)
Adenoma, multiple	0	0	0	0	1
Adenoma (includes multiple)[o]					
Overall rate	0/50 (0%)	1/50 (2%)	0/50 (0%)	2/50 (4%)	5/50 (10%)
Adjusted rate	0.0%	2.2%	0.0%	4.3%	10.6%
First incidence (days)	–	729 T	–	729 T	729 T
Poly-3 test	$P = 0.002$	$P = 0.504$	–	$P = 0.246$	$P = 0.035$
Carcinoma[p]	1	0	2	2	1
Duodenum, jejunum, or illeum adenoma[q]					
Overall rate	0/50 (0%)	1/50 (2%)	2/50 (4%)	15/50 (30%)	16/50 (32%)
Adjusted rate	0.0%	2.2%	4.2%	32%	33.7%
First incidence (days)	–	729 T	729 T	729 T	693
Poly-3 test	$P < 0.001$	$P = 0.504$	$P = 0.251$	$P < 0.001$	$P < 0.001$
Carcinoma[r]					
Overall rate	1/50 (2%)	0/50 (0%)	2/50 (4%)	3/50 (6%)	7/50 (14%)
Adjusted rate	2.2%	0.0%	4.2%	6.4%	14.7%
First incidence (days)	729 T	–	729 T	729 T	625
Poly 3 test	$P < 0.001$	$P = 0.496$	$P = 0.521$	$P = 0.319$	$P = 0.037$
Combined adenoma and carcinoma[s]					
Overall rate	1/50 (2%)	1/50 (2%)	4/50 (8%)	17/50 (34%)	22/50 (44%)
Adjusted rate	2.2%	2.2%	8.3%	36.3%	45.9%
First incidence (days)	729 T	729 T	729 T	729 T	625
Poly-3 test	$P < 0.001$	$P = 0.756$	$P = 0.198$	$P < 0.001$	$P < 0.001$

T, terminal sacrifice.
*Significantly different ($P \leq 0.05$) from control by the Poly-3 test.
**Significantly different ($P \leq 0.01$) from control by the Poly-3 test.
[c]Extracted from the NTP 2-year drinking water study with sodium dichromate dihydrate, pp. 70–72, Table 13 [NTP, 2008].
[b]Average severity grade of lesions in affected animals: 1, minimal; 2, mild; 3, moderate; 4, marked.
[c]NTP historical incidence for drinking water studies with controls (mean ± standard deviation): 6 of 299 (2.0% ± 2.2%; range, 0–6%), all routes; 9 of 1549 (0.6% ± 1.3%); range, 0–6%.
[d]Number of animals with neoplasms per number of animals necropsied.
[e]Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality.
[f]Beneath the control incidence is the *P* value associated with the trend test. Beneath the exposed group incidences are the *P* values associated with the pairwise comparison between the control and that exposed group. The Poly-3 test accounts for differential mortality in animals that did not reach terminal sacrifice.
[g]NTP historical incidence for drinking water studies: 1 of 299 (0.3% ± 0.8%); range, 0–2%; all routes: 3 of 1549 (0.2% ± 0.8%); range, 0–4%.
[h]NTP historical incidence for drinking water studies: 0 of 299; all routes: 1 of 1549 (0.1% ± 0.4%); range, 0−2%.
[i]NTP historical incidence for drinking water studies: 5 of 299 (1.7% ± 1.5%); range, 0−4%; all routes: 25 of 1,549 (1.6% ± 2.2%); range 0−8%.
[j]NTP historical incidence for drinking water studies: 6 of 299 (2.0% ± 2.2%); range, 0−6%; all routes: 10 of 1,549 (0.7% ± 1.3%); range, 0−6%.
[k]NTP historical incidence for drinking water studies: 6 of 299 (2.0% ± 1.8%); range, 0−4%; all routes: 30 of 1,549 (2.0% ± 2.2%); range, 0−8%.
[l]NTP historical incidence for drinking water studies: 11 of 299 (3.7% ± 3.7%); range, 0−10%; all routes: 39 of 1,549 (2.6% ± 2.7%); range, 0−10%.
[m]NTP historical incidence for drinking water studies: 1 of 350 (0.3% ± .0.8%), range 0−2%; all routes: 3 of 1,648 (0.2% ± 0.6%); range 0−2%.
[n]NTP historical incidence for drinking water studies: 0 of 350; all routes: 1 of 1,648 (0.1% ± 0.4%); range, 0−2%.
[o]NTP historical incidence for drinking water studies: 0 of 350; all routes: 0 of 1,648.
[p]NTP historical incidence for drinking water studies: 2 of 350 (0.6% ± 1.0%); range, 0−2%; all routes: 5 of 1,648 (0.3% ± 0.7%); range, 0−2%.
[q]NTP historical incidence for drinking water studies: 1 of 350 (0.3% ± 0.8%); range, 0−2%; all routes: 3 of 1,648 (0.2% ± 0.6%); range, 0−2%.
[r]NTP historical incidence for drinking water studies: 3 of 350 (0.9% ± 1.1%); range, 0−2%; all routes: 8 of 1,648 (0.5% ± 0.8%); range, 0−2%.
[s]NTP historical incidence for drinking water studies: 4 of 350 (1.1% ± 1.6%); range, 0−4%; all routes: 11 of 1,648 (0.7% ± 1.1%); range, 0−4%.

duodenum, mesenteric, or pancreatic lymph nodes [Kidwell, 2008].

De Flora et al. [2008] further stated that the induction of oral tumors in rats has ''nothing to do with possible human exposures, and no association between occupational exposure to Cr (VI) and oral cancers has ever been reported in the literature.'' These investigators also challenged the tumor findings of the NTP mouse drinking

TABLE IV. Incidence of Neoplasms of the Oral Cavity in Rats Receiving Sodium Dichromate Dihydrate in the 2-Year Drinking Water Bioassay[a]

	Dosage: mg/l (mg/kg)				
Male	0	14.3 (0.21)	57.3 (0.77)	172 (2.10)	516 (5.95)
No. necropsied	50	50	49	50	49
Oral mucosa squamous cell papilloma	0	0	0	0	1
Squamous cell carcinoma[b]					
Overall rate[c]	0/50 (0%)	0/50 (0%)	0/49 (0%)	0/50 (0%)	0/50 (0%)
Adjusted rate[d]	0.0%	0.0%	0.0%	0.0%	13.6%
First incidence (days)	–	–	–	–	543
Poly-3 test[e]	$P < 0.01$	–	–	–	$P = 0.015$
Tongue squamous cell papilloma	0	0	0	0	1
Squamous cell carcinoma	0	1	0	0	0
Combined oral mucosa or tongue papilloma or carcinoma[f]					
Overall rate	0/50 (0%)	1/50 (2%)	0/49 (0%)	0/50 (0%)	7/49 (12%)
Adjusted rate	0.0%	(2.4%)	0.0%	0.0%	15.7%
First incidence (days)	–	729 T	–	–	543
Poly 3 test	$P < 0.01$	$P = 0.49$	–	–	$P = 0.007$
Female	0	14.3 (0.26)	57.3 (0.95)	172 (2.45)	516 (7)
No. necropsied	50	50	50	50	50
Oral mucosa squamous cell carcinoma[g]					
Overall rate	0/50 (0%)	0/50 (0%)	0/50 (0%)	2/50 (4%)	11/50 (22%)
Adjusted rate	0.0%	0.0%	0.0%	4.6%	23.9%
First incidence (days)	–	–	–	646	506
Poly 3 test	$P < 0.001$	–	–	$P = 0.233$	$P = 0.001$
Tongue squamous cell papilloma	1	1	0	0	0
Squamous cell carcinoma	0	0	0	1	0
Combined oral cavity or tongue papilloma or carcinoma[h]					
Overall rate	1/50 (2%)	1/50 (2%)	0/50 (0%)	2/50 (4%)	11/50 (22%)
Adjusted rate	2.2%	(2.3%)	0.0%	4.6%	23.9%
First incidence (days)	618	729 T	–	646	506
Poly 3 test	$P < 0.001$	$P = 0.756$	$P = 0.503$	$P = 0.49$	$P = 0.002$

[a]Extracted from the NTP 2-year drinking water study with sodium dichromate dihydrate, p. 53, Table VI [NTP, 2008].
[b]NTP historical incidence for drinking water studies with (mean ± standard deviation): 0 of 350, all routes; 5 of 1,499 (0.3% ± 0.7%; range, 0–2%).
[c]Number of animals with neoplasms per number of animals necropsied.
[d]Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality.
[e]Beneath the control incidence is the *P* value associated with the trend test. Beneath the exposed group incidences are the *P* values associated with the pairwise comparison between the control and that exposed group. The Poly-3 test accounts for differential mortality in animals that did not reach terminal sacrifice.
[f]NTP historical incidence for drinking water studies: 1 of 300 (0.3% ± 0.8%); range, 0–2%; all routes: 10 of 1,449 (0.6% ± 0.8%); range, 0−2%.
[g]NTP historical incidence for drinking water studies: 0 of 300; all routes: 5 of 1400 (0.4% ± 0.8%); range, 0−2%.
[h]NTP historical incidence for drinking water studies: 3 of 250 (1.2% ± 1.1%); range, 0−2%; all routes: 14 of 1,350 (1.1% ± 1.6%); range, 0−6% T-terminal sacrifice.

water study, stating that some Cr (VI) ''may have escaped reduction and detoxification upstream in the alimentary tract'' at very high doses that are unrealistic for human exposure. We agree that the doses used in the NTP study were high, but not excessive. The CARC concluded that the highest dose (257.4/516 mg/l, males/females) used in this bioassay was considered adequate but not excessive, based on the presence of diffused epithelial hyperplasia and cellular histiocytic infiltration in the duodenum and jejunum of male and female mice [USEPA, CARC, 2008]. It is of note that the high dose selected for this assay was in conformance with the Office of Prevention, Pesticides and Toxic Substances (OPPTS) Harmonized Test Guideline for Carcinogenicity, recommending that the ''highest dose should elicit signs of toxicity without substantially altering the normal life span due to effects other than tumors'' [USEPA OPPTS, 1998].

Humans

The carcinogenic activity of Cr has been known since the late 19th century when the first cases of nasal tumors were reported in Scottish chrome pigment workers [Costa and Klein, 2006]. Several occupational studies have linked human exposure to Cr (VI) by inhalation to increased rates of cancer [Sedman et al., 2006]. Addition-

ally, a number of retrospective analyses have associated significant increases in respiratory cancer to Cr (VI) worker exposure in the chromate and chromate pigment production industry. The Gibb et al. [2000] investigation of 2,357 chrome production workers is considered to be the most comprehensive and firmly established Cr (VI) as a human lung carcinogen [Costa and Klein, 2008].

Although many studies exist in the open literature showing that Cr (VI) is carcinogenic in exposed workers via the inhalation route [Langård, 1990], there is one study suggesting that oral ingestion may also be implicated as an exposure route associated with Cr (VI)-induced cancer. In the study of Zhang and Li [1986], 155 subjects were exposed to ≈20 mg/l Cr (VI) through drinking water in the Liaoning Province of northeastern China. The source of contamination was a Cr ore smelting facility located in a rural area outside of Jinzhou. Cr (VI) was detected in 28% of the area wells in 1965. Contamination levels in 55% of these wells were >20 mg/l Cr (VI). Higher per capita rates of cancers, including lung and stomach cancers, were found. In a retrospective analysis of the Zhang and Li study, Beaumont et al. [2008] confirmed that there was a substantial association between stomach cancer and mortality and Cr (VI) contaminated drinking water in these subjects when compared to a nearby population whose source of water was from an uncontaminated area.

Based on the findings of tumors in the small intestines of mice (both sexes) and tumors of the oral cavity tumors in rats (both sexes) from these 2-year drinking water studies, NTP concluded that the results with Cr (VI) showed clear evidence of carcinogenic activity in male and female rats and mice. CARC classified Cr (VI) as ''Likely to be Carcinogenic in Humans'' via the oral route [Kidwell, 2008]. These data are supported by the results of epidemiological bio-monitoring in humans showing an association between stomach cancers and mortality and Cr (VI)-contaminated drinking water. When these data are viewed in conjunction with the established mutagenesis of Cr (VI), the analysis of whether Cr (VI) induces tumors via a potential mutagenic MOA should move forward.

APPLICATION OF THE CANCER GUIDELINES MOA FRAMEWORK

The revised U.S EPA Cancer Guidelines describe a framework that can be used to determining whether a postulated MOA is operative. Steps in the Cancer Guidelines framework include: an outline of the sequence of events leading to cancer, identification of the key events, and determination of whether there is a causal relationship between events and cancer (e.g., dose-response relationship and temporal associations). Additionally, the plausibility of the hypothesis and the examination of other potential MOAs are explored. Finally, the relevance to humans is assessed. As mentioned earlier, it is important to note that many of the studies discussed in this analysis were conducted long before the Agency adopted the framework analysis. Consequently, the ''ideal data'' to support many of the aspects of the MOA framework are not available. Nevertheless, the process can move forward and sound scientific judgment can be used when data gaps are identified.

TABLE V. Proposed Key Events in the Mutagenic Mode of Action for Cr (VI)

Key events:
Reduction of Cr (VI) to Cr (III) via interaction of cellular components (DNA) with Cr (VI)
Mutagenesis
Cell proliferation (hyperplasia)
Gastrointestinal tumors

Postulated MOA and Evidence to Support the Key Events

Based on the available data, it is postulated that Cr (VI) induces tumors in the gastrointestinal tract of male and female mice and rats via a mutagenic MOA. The analysis focuses on the tumor response in mice, because this species appears more sensitive to Cr (VI). However, rat data are discussed when appropriate to support or refute the mouse findings. The postulated key precursor events involve: the reduction of Cr (VI) to Cr (III) via interaction with cellular components (DNA), mutagenesis, cell proliferation, and tumor formation (Table V). Data supporting each proposed/postulated key event are discussed in the following section of this article.

Reduction of Cr (VI) to Cr (III) via Interaction with Cellular Components (DNA)

Many studies have focused on elucidation of mechanism(s) that might be responsible for Cr's mutagenicity. It has been shown that Cr (VI) compounds were genotoxic only in the presence of appropriate reducing agents in vitro or in vivo [Jennette, 1979; Fornace et al., 1981]. The bulk of evidence indicates that Cr (VI) readily crosses the cell membrane through the sulfate anion channel transport system [Jennette, 1979; Sugiyama, 1992], because the tetrahedral anionic structure of Cr (VI) is structurally similar to phosphate or sulfate ions and allows it to mimic these ions [Sugden and Stearns, 2000]. By contrast, Cr (III), which is octahedral [Shrivastava et al., 2005], does not readily cross the cell membrane. Once Cr (VI) is inside the cell, the studies of Gruber and Jennette [1978] indicate that the enzymes bound to the endoplasmic reticulum reduce it to Cr (III). Toxicokinetic studies also indicate that a similar pattern of Cr (VI) reduction, absorption, and distribution occurs in humans and animals [Baetjer et al., 1959; De Flora et al., 1997; Edel and Sabbioni, 1985; Febel et al., 2001; Procter et al., 2002].

Cr (VI), an oxidant, is possibly reduced directly to Cr (III) or reduced to the reactive but unstable intermediates [Cr (V) and Cr (IV)] before conversion to Cr (III). Nevertheless, Cr (VI) and Cr (III) are the major stable oxidation states of Cr and have biological relevance to humans [EC, 2002]. Salnikow and Zhitkovich [2008] state, ''inside the cell, Cr (VI) reduction is the activation event that is responsible for the generation of genotoxic damage and other forms of toxicity.'' Thus, Cr (VI) is not the agent responsible for the induction of DNA damage but rather the process of reduction to Cr (III) is the punitive event. The central tenet of Cr (VI)-induced formation of DNA damage is that reductive metabolism must take place intracellularly [Sugden and Stearns, 2000]. Because DNA damage is thought to result through either generation of reactive oxygen species (ROS) or from direct binding of Cr (III) to cellular constituents (i.e., DNA adduct formation), these two mechanisms, which may play a key role in Cr (VI)-induced carcinogenesis are discussed before the key event of mutagenesis.

Oxidative Damage

During the process of reduction, free radicals generated by these reactions can cause DNA strand breaks [Shi and Dalal, 1992, 1994; Shi et al., 1999], base modifications [Shi and Dalal, 1992, 1994; Luo et al., 1996], lipid peroxidation, and nuclear transcription factor-κB activation [Ye et al., 1995; Chen et al., 1997]. As stated by Gambelunghe et al. [2003], Cr (VI) induces cell death via apoptosis (an indicator of oxidative damage) that is triggered by damage to genetic material that exceeds the repair capacity of the cell. To illustrate the point, these investigators harvested peripheral lymphocytes from a group of 19 individuals working in the chrome-plating industry (matched for age, body mass, and smoking habits to 18 hospital workers and 20 university staff, negative control groups). These peripheral blood lymphocytes were assessed for primary DNA damage and DNA-strand breaks using the Comet assay and for apoptosis using flow cytometry; Cr (VI) in the urine was also measured. Results show that comet tail moments were significantly increased in lymphocytes ($P < 0.01$) of Cr-exposed workers, indicating increased DNA damage. During the workshift, concentration of Cr (VI) in the urine of the exposed workers was ~44 times higher than the negative control group (5.3–7.3 μg/g creatinine, $P < 0.001$) and approximately nine times and 1.9 times higher in the red blood cells and lymphocytes, respectively, than the negative control group. Increased percentages of apoptosis, however, were minimal. This finding led the authors to conclude that the detection of apoptosis may have been hindered by the very low Cr body burden in the chrome-plating workers in this study. Although apoptosis was not demonstrated by Gambelunghe et al. [2003], in this in vivo human study, primary DNA damage was seen in peripheral lymphocytes and several authors have reported that Cr (VI) induces apoptosis in mammalian and human cell lines. For example, apoptosis was found in the murine keratinocytes, Pam 212-ras cells at the IC50 of 76 μM [Flores and Perez, 1999], in CHO cells [Manning et al., 1994], and in the human lung epithelial cell line, A546 at concentrations ranging from 75 to 300 μM [Ye et al., 1999]. Similarly, enhanced apoptosis was seen in the p53−/−, p21−/− HCT116 colon carcinoma cells exposed in vitro to 30 μM Cr (VI) for 12 hr [Hill et al., 2008]. The authors commented that loss of the tumor suppressor gene p53 compromises the cell's ability to repair damage to DNA and loss of the p21 gene from these cells correlates with an increased sensitivity to DNA damaging agents, promoting apoptosis. Although no in vivo animal studies evaluating apoptosis were found in the open literature and the human study of Gambelunghe et al. [2003] was negative, the results from the in vitro assays suggest that apoptosis may be a plausible mechanism contributing to the Cr (VI)-induced DNA damage.

Likewise, the findings of Danadevi et al. [2001], who investigated the genotoxic effects of K_2CrO_4, on Swiss albino male mice, support the findings by Gambelunghe et al. [2003] of increased DNA damage in Cr-exposed workers. The data of Danadevi et al. [2001] are noteworthy, because they illustrate how early and at what low doses direct DNA damage can occur. Approximate values from the three independent experiments are presented in Figure 3. As shown, dose-related and significant ($P < 0.05$) increases in DNA damage (Comet assay) were seen in the harvested leukocytes of mice exposed over an oral gavage dose range of 0.59–9.5 mg/kg. The peak response was observed 48 hr after treatment with 9.5 mg/kg.

Bagchi et al. [2002] also showed dose- and time-dependent increases in lipid peroxidation, cytochrome c reduction (i.e., superoxide anion production), and DNA fragmentation in the liver and brain of female C57BL/6NTac mice receiving single oral doses of Na_2CrO_4 equivalent to 1, 10, or 50% of the LD_{50}. The investigators listed the oral LD_{50} for this mouse strain as 190 mg/kg for Cr (VI); thus, the applied doses were ~1.9, 19, or 95 mg/kg, respectively. Peak and significant ($P < 0.05$) responses were observed in mice administered the 0.5 LD_{50} at 48 hr for cytochrome c reduction (6.0-fold, liver; 4.1-fold, brain), lipid peroxidation (3.3-fold, liver; 3.6-fold, brain), and DNA fragmentation (2.2-fold, liver; 2.1-fold, brain). Significant increases were also seen at 0.5 LD_{50} (19 mg/kg) for lipid peroxidation, DNA fragmentation, and cytochrome c reduction in both organs. As discussed below, Cr (VI) also induces the formation of the 8-oxo-deoxyguanosine adduct (8-OH-dG), which is one of the most studied biomarkers for oxidative damage [Sander et al., 2005]. Nevertheless, the reliance on this single DNA lesion as critical to the mutagenic MOA must be

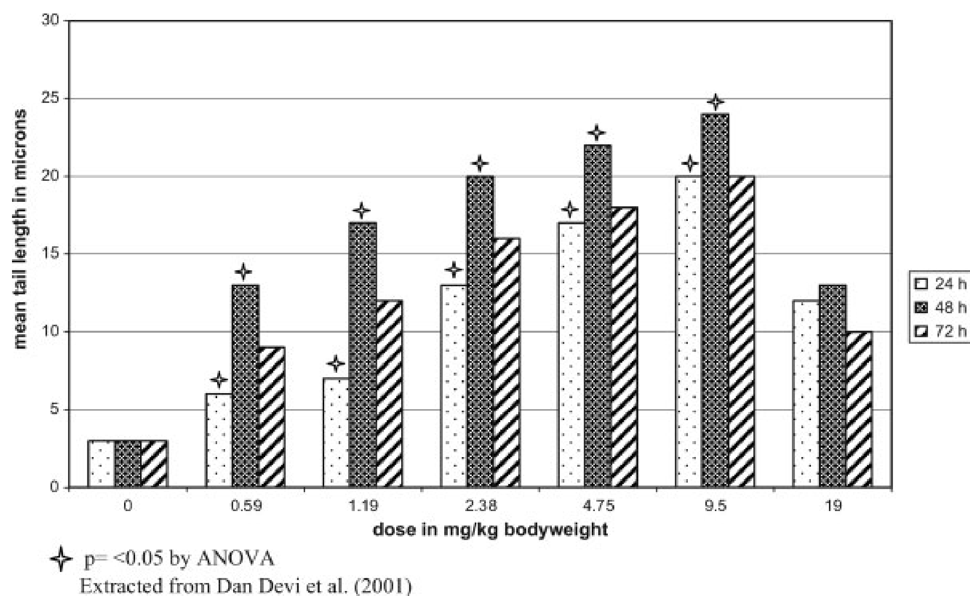


Fig. 3. DNA damage in leukocytes harvested from mice treated with potassium dichromate: Comet assay extracted from Devi et al. [2001].

considered in the perspective of DNA repair [Swenberg et al., 2008]. For example, Bagchi et al. in an earlier study [2001] determined that a single oral dose of ~95 mg/kg administered to p53-deficient mice induced marked increases in hepatic cytochrome c reduction (11 times higher), lipid peroxidation (11 times higher), and DNA fragmentation (four times higher) compared to the three to sixfold increases in these parameters in C57BL/6Ntac (p53-proficient). These data indicate that the inactivation of the p53 tumor suppressor gene, which plays a major role in DNA repair, allows enhancement of oxidative stress.

DNA Damage

Cr (VI) itself is unreactive toward DNA; however, reductive metabolism of Cr (VI) to Cr (III) produces the DNA-damaging species [Zhitkovich et al., 2002]. As stated earlier, this reductive metabolism occurs only within the cell where the transport of Cr (VI) is made possible by its tetrahedral anionic structure [Sugden and Stearns, 2000; Shivastava et al., 2005]. Intracellular reduction of Cr (VI) is facilitated by ascorbate and non-protein thiols, (glutathione or cysteine) to form Cr (III) [Zhitkovich et al., 1998]. It is this product of Cr (VI) reduction that has an affinity for macromolecules such as DNA and causes DNA strand breaks, alkali-liable sites, DNA–protein, and DNA–DNA crosslinks, and the formation of 8-OH-dG adducts as well as other DNA adducts [Zhitkovich et al., 2002]. As stated earlier, several investigators have presented data [Quievryn et al., 2002; Zhitkovich et al., 2002; Zhitkovich, 2005] indicating that Cr-DNA adducts are the most abundant form of Cr (VI)-induced genetic lesions in mammalian cells and are responsible for all of the mutagenic damage generated during Cr (VI) reduction with cysteine and ascorbate. Cr appears to preferentially bind to the N7 position of guanine on DNA [Bridgewater et al., 1994, 1998], as cited in Costa and Klein [2006]. Additionally, Costa and Klein [2006] cite the work of several authors in support of their claim that the N7 position adducts formed by Cr are probably unrepaired and, hence, responsible for the DNA alterations and cell transformation leading to cancer. However, Voitkun et al. [1998] in their analysis of ternary DNA adducts[1] [Cr (III)-mediated crosslinks of cysteine, histidine, or glutathione (GSH)] also found that these adducts were mutagenic.

In this study, $CrCl_3$ was reacted with cysteine, histidine, or glutathione for 5 hrs. Binary and ternary adducts were formed by treating the pSP189 plasmid with varying concentrations of Cr (III), Cr (III)-cysteine, Cr (III)-histidine, or Cr (III)-glutathione for 30 min and DNA adducts were measured. Results show a dose-dependent increase in Cr (III)-cysteine, Cr (III)-histidine, and Cr (III)-GSH complexes. The greatest increases occurred for Cr (III)-cysteine and Cr (III)-histidine. For the mutation phase of the study, plasmids were reacted with Cr (III), Cr (III)-cysteine, Cr (III)-histidine, and Cr (III)-GSH complexes and recovered. Adducted and control plasmids were transfected into SV-40 transformed human fibroblasts (normal human fibroblasts immortalized with SV virus) for 48 hr. Plasmids were recovered, treated with Dpnl restrictase to eliminate unreplicated molecules, electotransfected into the indicator microorganism, *E. coli* MBL50 (to select

[1]Ternary DNA adduct: Binding of the test chemical or a metabolite to DNA and an amino acid, whereas a binary DNA adduct is the binding of the test material or metabolite directly to DNA.

and sequence the *supF* gene), and the mutant colony frequency was determined. Results indicate that the bulky Cr (III)-GSH adducts induced the highest mutation frequency in *E. coli* MBL50, causing a fivefold increase at ~15 adducts/1,000 bp while binary adducts were only weakly mutagenic.

The authors also showed that adduct formation and the mutagenic response could be blocked by the addition of 10 mM $MgCl_2$ to mask the DNA phosphate ion on the treated plasmids. From these findings, they concluded that the induction of mutagenicity was dependent on the binding of the Cr (III) complexes to DNA. Analysis of the sequence alterations in the *sup*F gene indicated that the overall distributions of mutations were significantly changed for Cr (III)-GSH treatment and the majority of the point mutations were G: C base pair substitutions (95%). The same general distribution was found for plasmids adducted with the Cr (III)-cysteine or histidine complexes but to a lesser extent. The majority of the point mutations in treated and control plasmids were G: C base pair substitutions and ~78% of the Cr (III)-GSH ''induced mutations were G: C → T: A transversions''. This is of note, because the authors point out that ''guanines are by far the most frequent sites of DNA adduct formation for the majority of carcinogens, whereas, uracil paired with adenine was the preferred site for the spontaneous mutations in the single-stranded shuttle vector.''

The formation of mutagenic adducts was confirmed by Zhitkovich et al. [2002] using a similar approach of treating pSP189 plasmid DNA with a Cr (VI)/Cys reduction mixture, followed by purifying and transfecting the adducted and control plasmids into human fibroblasts. The ability of the Cr (VI)/Cys adducts to cause mutation was tested in *E. coli* MBL50. These investigators found that the yield of binary and ternary DNA adducts was relatively constant (54 and 45%, respectively) regardless of the Cr (VI) concentration. They also showed that the cysteine-dependent metabolism of Cr (VI) caused the formation of mutagenic and replication-blocking DNA lesions. Additionally, the authors noted that these cysteine adducts do not have the typical profile of a DNA-damaging oxidant (i.e., did not cause oxidative damage to the sugar-phosphate backbone of DNA as indicated by the lack of significant DNA breakage or production of abasic sites). Similarly, the ability of the Cr adducts to induce genotoxicity can be abolished in the presence of EDTA or phosphate, which indicated to these authors that ''nonoxidative, Cr (III)-dependent reactions were responsible for the induction of both mutagenicity and replication blockage by Cr (VI).'' These data indicate that reduction of Cr (VI) is required to set the stage for ternary Cr (III)—DNA adduct formation. These adducts, which are mutagenic in human fibroblasts, are formed in the absence of oxidative damage to DNA [Zhitkovich, 2000].

As Sander et al. [2005] stated in the workshop on the biological significance of DNA adducts, ''DNA adducts are one of many types of DNA damage that accumulate in the genome due to ongoing exposure to endogenous and exogenous compounds and chemicals. When unrepaired, different DNA adducts differentially generate mutations in replicating cells.''

Although no studies investigating DNA adduct formation in mouse gastrointestinal tissue were found in the open literature, there is in vivo evidence from the Swiss mouse study of Danadevi et al. [2001] showing DNA damage in leukocytes 24 hr after the male animals received oral gavage doses as low at 0.59 mg/kg Cr (VI). This dose is lower than the lowest tumorigenic level (2.4 mg/kg/day) in the 2-year mouse drinking water study. In rats, Coogan et al. [1991] found that DNA protein cross-links were produced in the livers of the F 344 strain receiving 100 or 200 ppm (6.1 or 8.7 mg/kg/day) K_2CrO_4 in drinking water for 3 weeks. These levels approximate the doses causing tumors in the rats (~6–7 mg/kg/day) in the NTP 2-year drinking water study. Thus, both of these studies show a causal relationship for tumor induction.

Mutagenesis

As stated earlier, there is a vast array of data showing that Cr (VI) is mutagenic both in vitro and in vivo. For example, gene mutations were induced in the liver and bone marrow of transgenic mice injected i.p. with 40 mg/kg K_2CrO_4 [Itoh and Shimada, 1998]. Similarly, induction of micronuclei and chromosome aberrations in mouse bone marrow was seen in mice receiving i.p. or oral doses of Cr (VI) but not when the dose was delivered via drinking water [Shindo et al., 1989]. Several possibilities have already been put forth to explain this unexpected result. Either Cr (VI) is not adequately distributed to the bone marrow [Shindo et al, 1989] or the reductive capacities of the gastrointestinal tract of the treated mice were sufficiently active to prevent Cr (VI) uptake in the blood and/or distribution to the target tissue [Mirsalis et al., 1996]. The latter explanation may in part apply to the positive findings of Sarkar et al. [1993] in which chromosome aberrations were observed in mouse bone marrow 24 hr after oral gavage administration of 20 mg/kg CrO_3. It is conceivable that once the reductive capacity of the cell is exhausted, the mutagenic and tumor responses may be augmented. Costa and Klein [2006] suggest that this is likely to occur after chronic or high doses exposure. They point out that there is mounting evidence that exposure to Cr (VI) by either inhalation or ingestion can have systemic effects at sites that are distant from the site of exposure. Furthermore, they state that ''all cells and organs possess the ability to take up hexavalent chromate, and any cell has the capacity to reduce the Cr (VI) intracellu-

larly to trivalent Cr, which reacts with protein to produce toxicity and with DNA to potentially cause cancer.''

Cell Proliferation (Hyperplasia)

No quantitative studies, such as assays measuring DNA synthesis [e.g., 5-bromo-2′-deoxyuridine incorporation or proliferating cell nuclear antigen] as an indicator of cell proliferation were available. However, hyperplasia was considered an appropriate measure of cell proliferation. As shown in Table III from the NTP 2-year mouse drinking water study [NTP, 2008], the incidence of diffuse duodenal hyperplasia was significantly increased in males and females of all treatment groups ($\geq$14.3 mg/l). The incidence of hyperplasia in the duodenum was also significantly increased in all exposure groups of mice ($\geq$62.5 mg/l, equivalent to $\sim$10 mg/kg/day) in the 90-day NTP mouse drinking water study [NTP, 2007]. This subchronic study was used to set the doses for the chronic exposure. Hyperplasia was not reported in the rat 2-year drinking water study (Table V) or in the 90-day drinking water subchronic study in rats. Nonetheless, the evidence of treatment-related papillomas and carcinomas of the oral mucosa and the tongue in male and female rats is not in doubt.

Tumors

Animals. As noted earlier, significant ($P < 0.01$) increases in the incidence of neoplasms of the small intestines (duodenum, jejunum, or ileum combined) at 257.4 mg/l (M, 34%) and 172 and 516 mg/l (F, 34 and 44%, respectively) Na_2CrO_4 were seen in mice in the NTP 2-year drinking water study (Table III). Additionally, the NTP reported that the incidence of squamous cell papillomas or squamous cell carcinomas in the oral mucosa or tongue of the 516 mg/l Na_2CrO_4 male and female rats was significantly (trend and pairwise at $P < 0.01$) increased (Table IV).

Causal Relationship Between Key Events and Cancer: Dose-Response Relationship/Temporal Associations

Data derived from the mouse studies using the oral route (oral gavage for the mutagenic events), the NTP subchronic or chronic (showing hyperplasia and neoplasia) drinking water studies and the in vivo mutagenicity assays were used to develop the causal relationships for the dose response and temporal associations. These data are listed in Figure 4 for the key events and are discussed as follows.

Mutagenesis

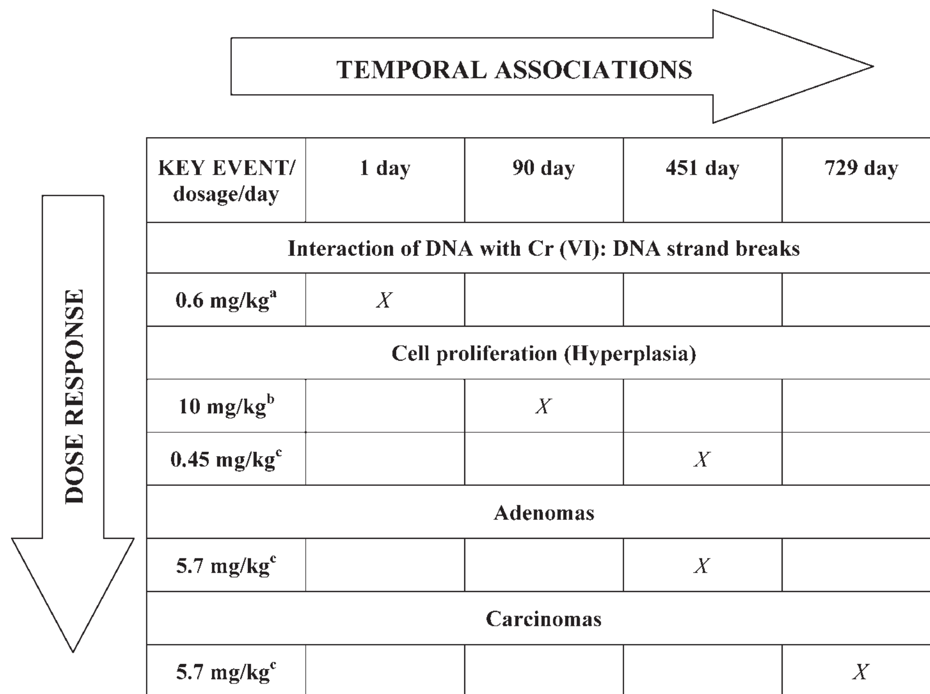
No in vivo studies employing the drinking water exposure route were found on gene mutation or chromosome aberrations at Cr (VI) doses within the tumorigenic range or on the target tissue. Because the evidence of in vitro and in vivo mutagenicity is compelling and interaction of cellular components, such as DNA, with other chemicals is a basis for mutagenicity [Preston and Williams, 2005], an assay of DNA damage was selected for the temporal and dose response concordance. As shown earlier, the lowest effective dose for induction of adverse genetic events (e.g. DNA damage) in animals comes from the Comet assay performed by Danadevi et al. [2001] showing reproducible and significant ($P < 0.05$) dose-related increases in single-/double-stranded DNA breaks in leukocytes harvested from Swiss male mice within 24 hr of oral dosing with 0.59, 1.19, 2.38, 4.75, or 9.5 mg/kg (see Fig. 3). A clear and significant dose response is observed at levels ranging from 0.59 to 9.5 mg/kg. Although the high dose exceeds the tumorigenic levels, the low dose (0.59 mg/kg) is 10$\times$ lower that the high-tumorigenic dose (5.7 mg/kg) in the 2-year drinking water mouse study [NTP, 2008]. This dose response analysis strengthens the causal relationship. The causality of adverse genetic events in mice is also reinforced by the findings of Coogan et al. [1991] demonstrating that DNA crosslinks do occur in the livers of F344 rats receiving 100 or 200 ppm K_2CrO_4 ($\sim$6.1 or 8.7 mg/kg/day) in drinking water for 3 weeks. These levels approximate the doses causing tumors ($\sim$6–7 mg/kg/da) in the NTP 2-year rat study.

Cell Proliferation (Hyperplasia) and Tumors

As previously stated, no quantitative DNA synthesis data were available. Consequently, the incidence of hyperplasia was viewed as an adequate measure of cell proliferation. Treatment-related diffused duodenal hyperplasia was recorded for both male and female mice at levels down to the lowest dose tested (0.45 mg/kg/day) in the 2-year drinking water study [NTP, 2008]. Diffused duodenal hyperplasia was seen at 451 days, whereas adenomas and carcinomas were observed at 5.7 mg/kg/day by 451 or 729 days, respectively.

Plausibility and Coherence of the Database

As shown in the GAP (Fig. 2), Cr (VI) is positive in many in vitro genetic toxicology assays, inducing a wide array of adverse mutagenic events in a wide variety of species. Cr (VI) is mutagenic in vivo in the liver, brain, and bone marrow of rats and/or mice and shows DNA-damaging activity and micronucleus induction in humans. However, no genetic toxicology data were found for the mouse target tissues of interest. As mentioned earlier, negative results were obtained by De Flora et al. [2008] in the study of 8-oxo-dG and DPXL induction in mouse duodenum. It is likely that the doses used in this assay (5 or 20 mg/l via drinking water for 9 months) were effectively reduced. However, it is equally plausible that the higher chronic exposure (172 or 516 mg/l for 729 days), used in the NTP drinking water study with female mice would exhaust the reductive capacity of the cell and enhance both the



KEY EVENT/ dosage/day	1 day	90 day	451 day	729 day
Interaction of DNA with Cr (VI): DNA strand breaks				
0.6 mg/kg[a]	X			
Cell proliferation (Hyperplasia)				
10 mg/kg[b]		X		
0.45 mg/kg[c]			X	
Adenomas				
5.7 mg/kg[c]			X	
Carcinomas				
5.7 mg/kg[c]				X

[a]Data obtained from the oral gavage study of Devi et al. (2001).
[b]Data obtained from the subchronic drinking water study of NTP (2007).
[c]Data obtained from the carcinogenicity drinking water study of NTP (2008).

Fig. 4. Causal Relationships: Temporal Associations and Dose Response for Key Events of Cr (VI)-Induced Tumors

mutagenic and tumorigenic response as evidenced by DNA damage at oral doses lower than the levels causing tumor formation. We contend that higher doses and a longer exposure may be necessary to demonstrate site concordance when drinking water is the exposure route. Because of the absence of site concordance data, we chose to adopt a weight of the evidence approach to evaluate the body of information available for Cr (VI). On the basis of these considerations, we conclude, in the face of the overwhelming positive in vitro and in vivo (at sites other than the gastrointestinal tract) mutagenicity, that the lack of acceptable site concordance results for rat oral tumors or mouse small intestine tumors via drinking water is not a major limitation to the overall assessment, as Costa and Klein [2006] state in their review of the toxicity and carcinogenicity of Cr compounds:

''Mounting experimental evidence points to the fact that hexavalent Cr exposure, by either inhalation or ingestion, can have systemic effects that are distant from the site of exposure. Since hexavalent Cr is isostructural with sulfate and phosphate at physiological pH, it can be carried throughout the body and even into the brain. There is likely a competition between the extracellular reducing capacity and the uptake of chromate by cells.''

Costa and Klein [2006] further affirm that all cells and all organs in rodents and humans are potentially at risk for DNA damage and cancer from Cr (VI). Accordingly, for risk assessment purposes, it was decided that the data are adequate for key events leading to tumors in animals and humans, and most of these key events have been demonstrated qualitatively in animals and humans. Finally, there is agreement between the tumor profile for other mutagenic carcinogens and Cr (VI). Typically, mutagenic carcinogens induce tumors in multiple sites of trans-species and trans-sex [Kirkland et al., 2006], and the time to tumor is generally reduced as compared to non-mutagenic carcinogens. With the exception of the extended period before the first tumors appeared, these characteristics fit the tumor profile for Cr (VI).

Alternative Modes of Action

Because Cr (VI) has a tumor profile of trans-species, trans-sex, and multi-sites, it is likely to be a mutagenic carcinogen. Nevertheless, the possibility that cytotoxicity may be the MOA for Cr (VI) was considered. Although NTP [2008] believes the diffused hyperplasia seen in the duodenum of male and female mice in the 2-year study to be consistent with regenerative cell growth secondary to tissue injury, no histological evidence of cytotoxicity was noted in either the 90-day subchronic or 2-year rat or mouse drinking water bioassays. These findings do not

TABLE VI. Human Relevance: Comparison of Key Events in Rodents and Humans for Cr (VI)

Key event	Evidence in rodent	Evidence in humans
Interaction of cellular components with Cr (VI)	Toxicokinetic studies indicate a similar pattern of Cr (VI) reduction, absorption, distribution in humans and animals	Toxicokinetic studies indicate a similar pattern of Cr (VI) reduction, absorption, distribution in humans, and animals [De Flora et al., 1997; Febel et al., 2001; Proctor et al., 2002]
Mutagenesis	In vivo DNA damage in mouse leukocytes and rat livers [Coogan et al., 1991; Devi et al., 2001]; in vivo gene mutations, chromosome aberrations, micronuclei in rats and mice [Shindo et al., 1989; Sarkar et al., 1993; Itoh and Shimada, 1998; GAP, 2000]	Mutagenic DNA adducts in human fibroblasts; DNA damage and micronuclei in PBL and buccal epithelial cells of Cr (VI) workers [Benova et al., 2003; Voitkun et al., 1998; Danadevi et al., 2004]
Cell Proliferation	Epithelial hyperplasia in mouse duodenum, 2-year drinking water study [NTP, 2008]	Biologically plausible
Tumors	Small intestine tumors in mouse and tumors of oral mucosa or tongue, 2-year drinking water study [NTP, 2008]	Stomach cancer associated with Cr (VI) contaminated drinking water in China [Zhang and Li, 1986; Beaumont et al., 2008]

preclude the possibility of localized cytotoxicity but Cr (VI)-induced mutagenicity is observed in the absence of cytotoxicity. Likewise, DNA-protein cross-links were observed in human Burkitt lymphoma cells transformed with Epstein–Barr virus at concentrations of 100 μM K_2CrO_4 with no detectable cytotoxicity [Zhitkovich et al., 1998]. Furthermore, the overwhelming evidence of mutagenicity in vitro and in vivo without cytotoxicity and tumorigenesis in the absence of frank toxicity in the long-term rodent studies suggest that cytotoxicity may be ruled out as the MOA for Cr (VI)-induced tumors.

Relevance to Humans

The overall data show persuasive evidence that Cr (VI) is a mutagenic carcinogen in rodents; therefore, the relevance to humans was assessed. Qualitative aspects of the Human Relevance Framework, developed by Meek et al. [2003] were used to establish whether this proposed mutagenic animal MOA for Cr (VI) is biologically relevant to humans (Table VI). Taking into account toxicokinetic studies, several investigators [Baetjer, 1959; Edel and Sabbioni, 1985; De Flora et al., 1997; Febel et al., 2001; Procter et al., 2002] have demonstrated that reduction, absorption, and distribution of Cr (VI) are similar in humans and rodents; therefore, the interaction of cellular components with DNA is plausible in humans. Similarly, mutagenic DNA adducts in human fibroblasts and micronucleus induction in the peripheral blood lymphocytes and buccal epithelial cells of Cr (VI) workers have been mentioned in the earlier discussion and establish the concern that this key event will likely occur in humans. Cell proliferation is a component of tumor progression and is expected in humans and rodents. Finally, Cr (VI) was designated a human carcinogen by IARC in 1990 and 1997, NTP stated that there is clear evidence of rodent carcinogenicity [NTP, 2008] and OPP concluded that Cr (VI) is ''Likely to be Carcinogenic in Humans'' via the oral route [Kidwell, 2008]. These decisions are supported by the findings of the epidemiological analysis of stomach cancer in Chinese residents ingesting drinking water contaminated with Cr (VI) [Zhang and Li, 1986; Beaumont et al., 2008]. Both IARC [see Cr (IV) GAP, Fig. 2] and the CARC also concluded that Cr (VI) is mutagenic. Thus, the weight of the evidence from this phase of the analysis suggests that the key events of Cr (VI) carcinogenicity in rodents are biologically plausible and, therefore, relevant to humans.

TABLE VII. Strengths and Weaknesses of the Database for Cr (VI)

Strengths
- Mutagenic DNA adducts and clear evidence of point mutations observed.
- Multiple positive results at most in vitro genetic toxicology endpoints confirmed.
- DNA damage, gene mutations, and chromosome aberrations induced in vivo.
- Dose response and time concordance established.
- Key events demonstrated in rodents and plausible in humans.
- An MOA alternative to mutagenicity not found.

Weaknesses
- Target tissue studies, for temporal and dose response concordance for the key event, mutagenicity, were not available.
- No studies that evaluate cell proliferation or the point of saturation for the reductive capacity of the target cells were found.
- Studies on the dose comparison for exposure routes (e.g., i.p. vs. drinking water) have not been performed for Cr (VI).

Uncertainty in the Database

The strengths and weaknesses in the Cr (VI) database are presented in Table VII. Cr (VI) forms mutagenic DNA adducts, inducing point mutations in human fibroblasts and bacteria. Consistent positive findings from multiple in vitro and in vivo genetic toxicology studies have

been presented. Dose response and time concordance have been established, and the plausibility of all key events occurring in rodent and humans has been demonstrated. Finally, the overwhelming evidence of mutagenicity in vitro and in vivo (in the absence of cytotoxicity) along with tumorigenesis (in the absence of frank toxicity) rule out cytotoxicity as a likely alternative MOA.

By contrast, there are no adequate target tissue studies for temporal and dose response concordance for mutagenic events. Site concordance is a major consideration in the MOA framework; however, in this instance, positive in vivo mutagenic results have been demonstrated in tissues generally assayed (bone marrow and liver) in genetic toxicology studies. Furthermore, clear and significant dose-related increases in DNA damage have been demonstrated in mouse leukocytes within 24 hr of oral treatment with 0.59, 1.19, 2.38, 4.75, and 9.5 mg/kg of $K_2Cr_2O_4$. These findings show reasonable time and dose concordance and strengthen the argument for causality. The lack of positive findings for mutagenic effects in the target tissue of several Cr (VI) drinking water studies could be due to procedural issues related to dosing and/or length of exposure but it is, nevertheless, an uncertainty in the database. It has been argued that agents which are DNA reactive are generally carcinogenic and most human carcinogens are DNA reactive [Pitot and Dragan, 2001]. For risk-assessment purposes, therefore, the data on DNA damage are considered adequate in building the causality case for Cr (VI) operating via a mutagenic MOA. Nevertheless, in vivo gene mutation assays with adequate dose selection, sampling and harvest times, and with consideration of site concordance and exposure conditions would help inform the overall analysis.

No quantitative estimates on cell proliferation were found in the open literature. Although data for this event are also preferred, evidence of duodenal hyperplasia was indeed detected in male and female mice at the lowest dose tested (0.45 mg/kg/day) in the NTP 2-year drinking water study. We believe that the occurrence of significantly increased duodenal hyperplasia in male and female mouse in the 2-year drinking water bioassay at all treatment levels is adequate to support the evidence of cell proliferation as a key event. Nevertheless, studies that more clearly define the dose response would more adequately inform the risk-assessment process. In accordance with the EPA Cancer Guidelines, however, the linear approach is recommended for the risk assessment when the MOA indicates a linear response when a conclusion is made that an agent causes direct alterations in DNA.

Finally, no data were found that address dose comparison for the exposure routes of i.p. injection vs. drinking water. Although these studies are also needed, it is discussed in the Supplemental Guidance for Assessing Susceptibility from Early-Life Exposures to Carcinogens that compounds that are ''reasonably well absorbed orally'' may have dose ranges comparable to injection routes [USEPA, 2005b]. Because Cr (VI) salts are relatively soluble in aqueous environments, and hence well absorbed, the above statement can be applied to the studies using the i.p. injection exposure route.

Thus, using the weight of the evidence and sound scientific judgment, it was concluded that confidence in the overall quality and quantity of the studies in the database is high and that the weaknesses that have been uncovered should not adversely influence the outcome of the MOA analysis.

APPLICATION OF THE SUPPLEMENTAL GUIDANCE

It is recommended that ADAFs be applied because Kirpnick-Sobol et al. [2006] showed ''large scale irreversible'' DNA deletions in mouse offspring following transplacental exposure to Cr (VI). Similarly, there is evidence of dominant lethal mutations in mice as well as micronucleus induction in the peripheral blood and liver of mouse fetuses. Although no data were found for children in the 2 to <16 years of age group, it is likely, given the ability of Cr (VI) to penetrate cellular membranes through a nonspecific anion channel and the intracellular mechanisms leading to mutations that children in this age group would be at risk.

SUMMARY AND CONCLUSIONS

The above information was presented to the CARC [Kidwell, 2009], and it was concluded that the evidence of mutagenicity as the MOA for carcinogenesis induced by Cr (VI) can be summarized as follows:

1. Cr (VI) readily crosses the cell membrane through the sulfate transport system, interacts with cellular components, such as DNA, is reduced to Cr (III), and produces oxidative damage and persistent ternary DNA adducts that are mutagenic.
2. Cr (VI) is a direct-acting mutagen in vitro, causing concentration-related base pair substitution point mutations in *S. typhimurium* and *E. coli*.
3. Multiple adverse genetic effects are seen in an increasingly higher order of phylogenetically distinct species.
4. Consistent dose-related increases in gene mutations in mice and chromosome aberrations in rats and mice have been reported. Similarly, there is reproducible evidence of DNA damage and micronucleus induction in humans occupationally exposed to Cr (VI).
5. Mutagenesis in somatic cells is supported by similar evidence in germ cells such as: positive dominant lethal mutations in male mice, DNA deletions in developing mouse embryos, and micronucleus induction in mouse fetuses.

6. Cr (VI) has been associated with malignancies of the lung in rats and humans following inhalation exposure and is classified as a known human carcinogen via the inhalation route. Carcinomas and sarcomas have also been found in mouse skin.
7. In 2-year drinking water studies, Cr (VI) induces tumors of the gastrointestinal tract in both sexes of mice and rats.
8. Using EPA's 2005 Cancer Guidelines, the MOA framework was applied to these data to determine if mutagenicity is the MOA for the rodent tumors induced by Cr (VI).
9. The postulated key precursor events linked to tumor induction by Cr (VI) via drinking water involve: the interaction of cellular components (DNA) with Cr (VI), mutagenesis, cell proliferation, and tumor formation.
10. With the exception of hyperplasia (indicative of regenerative cell proliferation) in rats, these events have been demonstrated qualitatively and/or quantitatively in rodents.
11. With the currently available information and the acknowledgement that certain pieces of the database are missing (quantitative estimates of cell proliferation and mutagenic events in the target tissue), a mutagenic MOA for the tumors in mice and rats remains plausible and coherent, because there is convincing evidence that Cr (VI) exposure, via inhalation, dermal, or oral exposure, induces tumors. Similarly, convincing evidence of in vitro and in vivo mutagenicity has been presented.
12. There is concordance between doses causing tumors and dose response and temporal association for most of the key events.
13. No convincing data were found for an alternative MOA.
14. The data are relevant to humans because of the established link between Cr (VI) inhalation and drinking water exposure and cancer in humans.
15. There is also concordance between the tumor profile for mutagenic carcinogens and Cr (VI), namely, tumors in multiple sites, multiple species, and both sexes.

Despite the uncertainty that have been identified in the database, it is concluded that there is plausible evidence that Cr (VI), administered via drinking water, may act via a mutagenic MOA for carcinogenicity.

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REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR). 2000. Toxicological Profile for Chromium, prepared by Syracuse Research Corporation Under Contract No. 205-199-00024 for U.S. Department of Health and Human Services, Public Health Service, Atlanta, Georgia. pp 1–149.

Agency for Toxic Substances and Disease Registry (ATSDR). 2008. Toxicological profile for Chromium (Draft for Public Comment). Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

Baetjer AM, Damron C, Sudacz V. 1959. The distribution and retention of chromium in men and animals. AMA Arch Ind Health 20:136–150.

Bagchi D, Hassoun EA, Bagchi M, Muldoon DF, Stohs SJ. 1995a. Oxidative stress induced by chronic administration of sodium dichromate. Comp Biochem Physiol C 110:281–287.

Bagchi D, Hassoun EA, Bagchi M, Stohs SJ. 1995b. Chromium-induced excretion of urinary lipid metabolites, DNA damage, nitric oxide production, and generation of reactive oxygen species in Sprague-Dawley rats. Comp Biochem Physiol C 110:177–187.

Bagchi D, Vuchetich PJ, Bagchi M, Hassoun EA, Tran L, Stohs SJ. 1997. Induction of oxidative stress by chronic administration of sodium dichromate [Chromium VI] and cadmium chloride [Cadmium II] to rats. Free Radical Biol Med 22:471–478.

Bagchi D, Bagchi M, Stohs SJ. 2001. Chromium (VI)-induced oxidative stress, apoptotic cell death and modulation of p53 tumor suppressor gene. Mol Cell Biochem 222:149–158.

Bagchi D, Balmoori J, Bagchi M, Ye X, Williams CB, Stohs SJ. 2002. Comparative effects of TCCD, endrin, naphthalene and chromium (VI) on oxidative stress and tissue damage in the liver and brain tissues of mice. Toxicology 175:73–82.

Beaumont J, Sedman RM, Stephen D, Reynolds SD, Sherman CD, Li L, Howd RA, Sandt MS, Zeise L, Alexeeff GV. 2008. Cancer mortality in a Chinese population exposed to hexavalent chromium in drinking water. Epidemiology 19:12–23.

Benova D, Hadjidekova V, Hristova R, Nikolova T, Boulanova M, Gerogieva I, Grigorova M, Popov T, Paney T, Gerogieva R, Natarajan AT, Darroudi F, Nilsson R. 2003. Cytogenetic effects of hexavalent chromium in Bulgarian chromium workers. Mutat Res 538:181–182.

Bigaliev A, Turebaev M, Elemesova M. 1977. Cytogenetic study of the in vivo mutagenic properties of chromium compounds. In: Dubinin NP, editor. Genet Posledstviya Zagryanenia Okruzhayushehei Sredy (Proceedings of the Symposium). Moscow: Nauka. pp.173–176.

Bridgewater LC, Manning FC, Patierno SR. 1994. Base-specific arrest of in vitro DNA replication by carcinogenic chromium: Relationship to DNA interstrand crosslinking. Carcinogenesis 15:2421–2427.

Bridgewater LC, Manning FC, Patierno SR. 1998. Arrest of replication by mammalian DNA polymerases α and β caused by chromium-DNA lesions. Mol Carcinogen 23:201–206.

Briggs JA, Briggs RC. 1988. Characterization of chromium effects on a rat liver epithelial cell line and their relevance to in vitro transformation. Cancer Res 48:6484–6490.

Chen F, Ye J, Zhang X, Rojanasakul Y, Shi X. 1997. One-electron reduction of chromium (VI) by α-lipoic acid and related hydroxyl radical generation, dG hydroxylation and nuclear transcription factor-κB activation. Arch Biochem Biophys 338:165–172.

Chorvatovičová D, Ginter E. 1989. Effects of Cr VI, vitamin C in transplacental micronucleus test in mice. Biologia 44:1033–1038.

Coogan TP, Motz J, Snyder CA, Squibb KS, Costa M. 1991. Differential DNA-protein crosslinking in lymphocytes and liver following chronic drinking water exposure of rats to potassium chromate. Toxicol Appl Pharmacol 109:60–72.

Costa M, Klein CB. 2006. Toxicity and carcinogenicity of chromium compounds in humans. Crit Rev Toxicol 36:115–163.

Danadevi K, Rozati R, Saleha Banu B, Jamil K, Grover P. 2001. In vivo genotoxic effects of potassium dichromate in mice leukocytes using comet assay. Food Chem Toxicol 39:859–865.

Danadevi K, Rozati R, Saleha Banu B, Grover P. 2004. Genotoxic evaluation of welders occupationally exposed to chromium and nickel using the Comet assay and micronucleus assays. Mutagen 19:35–41.

Davidson T, Kluz T, Burns F, Rossman T, Zhang Q, Uddin A, Nadas A, Costa M. 2004. Exposure to chromium (VI) in drinking water increases susceptibility to UV-induced skin tumors in hairless mice. Toxicol Appl Pharmacol 196:431–437.

Dearfield KL, Moore MM. 2005. Use of genetic toxicology information for risk assessment. Environ Mol Mutagen 46:236–245.

De Flora S, Camoirano A, Bagnasco M, Bennicelli C, Corbett GE, Kerger BD. 1997. Estimates of the chromium (VI) reducing capacity in human body compartments as a mechanism for attenuating its potential toxicity and carcinogenicity. Carcinogenesis 18:531–537.

De Flora S, Ilcheva M, Balansky RM. 2006. Oral chromium (VI) does not affect the frequency of micronuclei in hematopoietic cells of adult mice and of transplacentally exposed fetuses. Mutat Res 610:38–47.

De Flora S, D'Agostini FD, Balansky R, Micale R, Baluce B, Izzotti A. 2008. Lack of genotoxic effects in hematopoietic and gastrointestinal cells of mice receiving chromium (VI) with drinking water. Mutat Res 659:60–67.

Dunkel VC, Pienta RJ, Sivak A, Traul KA. 1981. Comparative neoplastic transformation responses of Balb/3T3 cells, Syrian hamster embryo cells, and Rauscher murine leukemia virus-infected Fischer 344 rat embryo cells to chemical carcinogens. J Natl Cancer Inst 67:1303–1315.

Edel J, Sabbioni E. 1985. Pathways of Cr (III) and Cr (VI) in the rat after intratracheal administration. Hum Toxicol 4:409–416.

European Commission DG ENV. E3. 2002. Heavy metals in waste. Final Report, Project ENV.E. 3/ETU/2000/0058.

Febel H, Szegedi B, Huszar S. 2001. Absorption of inorganic, trivalent and hexavalent chromium following oral and intrajejunal doses in rats. Acta Vet Hung 49:203–209.

Flores A, Perez JM. 1999. Cytotoxicity, apoptosis, and in vitro DNA damage induced by potassium chromate. Toxicol Appl Pharmacol 161:75–81.

Fornace AY, Seres DS, Lanchner SF, Harrie CC. 1981. DNA-protein cross-linking by chromium salts. Chem Biol Interact 36:345–354.

Gambelunghe A, Piccinini R, Ambrogi M, Villarini M, Moretti M, Marchetti C, Abbritti G, Muzi G. 2003. Primary DNA damage in chrome-plating workers. Toxicology 188:187–195.

Gibb HJ, Lees PSJ, Pinsky PF, Rooney B. 2000. Lung cancer among workers in chromium chemical production. Am J Ind Med 38:115–126.

Gruber JE, Jennette KW. 1978. Metabolism of the carcinogen chromate by rat liver microsomes. Biochem Biophys Res Commun 82:700–706.

Haworth S, Lawlor T, Mortelmans K, Speck W. 1983. Salmonella mutagenicity test results for 250 chemicals. Environ Mutag Suppl 1 1:3–142.

Hill R, Leidal AM, Madureira PA, Gillis LD, Cochrane HK, Waisman DM, Chiu A, Lee PW. 2008. Hypersensitivity to chromium-induced DNA damage correlates with constitutive deregulation of upstream p53 kinases in p21−/− HCT116 colon cancer cells. DNA Repair (Amst) 7:239–52.

IARC. 1990. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Chromium and chromium compounds, Vol. 49. Lyon: International Agency for Research on Cancer, Lyon. pp. 49–214.

IARC. 1997. Monographs on Chromium, Nickel, and Welding. Lyon: International Agency for Research on Cancer, pp. 17–34 (Updated November 5, 1997).

Itoh S, Shimada H. 1996. Micronucleus induction by chromium and selenium, and suppression of metallothionein inducer. Mutat Res 367:233–236.

Itoh S, Shimada H. 1998. Bone marrow and liver mutagenesis in lacZ transgenic mice treated with hexavalent chromium. Mutat Res 412:63–67.

Jennette KW. 1979. Chromate metabolism in liver microsomes. Biol Trace Elem Res 1:55–62.

Kanojia RK, Junaid M, Murthy RC. 1996. Chromium induced teratogenicity in female rats. Toxicol Lett 89:207–213.

Kidwell J. 2008. Inorganic hexavalent chromium (Cr(VI)): Report of the Cancer Assessment Review Committee. TXR 0054811 (dated March 12, 2008).

Kidwell J. 2009. Inorganic hexavalent chromium (Cr(VI)): Revised report of the Cancer Assessment Review Committee. TXR 0055224 (dated July 1, 2009).

Kirkland D, Aardema M, Muller l, Hayashi M. 2006. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens II. Further analysis of mammalian cell results, relative predictivity and tumor profiles. Mutat Res/Gen Toxicol Environ Mutat 608:29–42.

Kirpnick-Sobol Z, Reliene R, Schiest RH. 2006. Carcinogenic Cr(VI) and the nutritional supplement Cr(III) induce DNA deletions in yeast and mice. Cancer Res 66:3480–3484.

Knudsen I. 1980. The mammalian spot test and its use for the testing of potential carcinogenicity of welding fume particles and hexavalent chromium. Acta Pharm Toxicol 47:66–70.

Langård S. 1990. One hundred years of chromium and cancer: A review of epidemiological evidence and selected case reports. Am J Ind Med 17:189–215.

Lohman PHM, Lohman WJA 2000. Genetic activity profiles of short-term testing data from the US EPA and from IARC monographs. GAP2000 version 1.3 for Windows, 2000.

Luo H, Lu YD, Shi X, Mao Y, Dalal NS. 1996. Chromium (VI)/H_2O_2 Fenton-like reaction causes DNA damage: Implications to chromate's genotoxicity. Ann Clin Lab Sci 26:185–191.

Manning FCR, Blankenship LJ, Wise JP, Xu J, Bridgewater LC, Patierno SR (1994). Induction of internucleosomal DNA fragmentation by carcinogenic chromate: Relationship to DNA damage, genotoxicity, and inhibition of macromolecular synthesis. Environ Health Perspect 102 (Suppl. 3):159–167.

Meek ME (Bette), Bucher JR, Cohen SM, Dellarco V, Hill RN, Lehman-McKeeman LD, Longfellow DG, Pastoor T, Seed J, Patton DE. 2003. A framework for human relevance analysis of information on carcinogenic modes of action. Crit Rev Toxicol 33:591–654.

McCarroll NE, Protzel A, Ioannou Y, Stack HF, Jackson MA, Waters MD, Dearfield KL. 2002. A survey of EPA/OPP and open literature on selected pesticide chemicals III. Mutagenicity and carcinogenicity of benomyl and carbendazim. Mutat Res 512:1–35.

McCarroll N, Keshava N, Cimino M, Chu M, Dearfield K, Keshava C, Kligerman A, Owen R, Protzel A, Putzrath R, Schoney R. 2008. An evaluation of the mode of action framework for mutagenic carcinogens case study: Cyclophosphamide. Environ Mol Mutag 49:117–131.

McGregor DB, Martin R, Cattanach P, Edwards I, McBride D, Caspary WJ. 1987. Responses of the L5178Y tk+/tk− mouse lymphoma cell forward mutation assay to coded chemicals. I. Results for nine compounds. Environ Mutag 9:143–160.

Mirsalis JC, Hamilton CM, O'Loughlin KG, Paustenbach DJ, Kerger BD, Patierno S. 1996. Chromium (VI) at plausible drinking water concentrations is not genotoxic in the in vivo bone marrow micronucleus or liver unscheduled DNA synthesis assays. Environ Mol Mutag 28:60–63.

National Toxicology Program (NTP). 2007. NTP technical report on the toxicity studies of sodium dichromate dihydrate (CAS No. 7789-12-0) administered in drinking water to male and female F344/N rats and B6C3F1 mice and male BALB/c and *am3*-C57BL/6 mice. Southern Research Institute, Birmingham, AL. NTP TR 72 (NIH Publication No. 07-5964), January 2007. Published by the National Institutes of Health, U.S. Department of Health and Human Services. MRID 47325704.

National Toxicology Program (NTP). 2008. NTP toxicology and carcinogenesis studies of sodium dichromate dihydrate (CAS No. 7789-12-0) in F344/N rats and B6C3F1 mice (drinking water studies). Natl Toxicol Prog Tech Rep Ser 546:1–192. MRID No. 47325703.

Paschin YV, Zacepilova TA, Kozachenko VI. 1982. Induction of dominant lethal mutations in male mice by potassium dichromate. Mutat Res 103:345–347.

Paschin YV, Kozachenko VI, Sal'nikova LE. 1983. Differential mutagenic response at the HGPRT locus in V79 and CHO Chinese hamster cells after treatment with chromate. Mutat Res 122:361–365.

Pitot HC III, Dragan YP. 2001. Chemical carcinogenesis. In: Klaassen CD, editor. Cassarett and Doull's Toxicology: The Basic Science of Poisons, 5th ed. New York: McGraw-Hill. pp. 201–267.

Preston RJ, Williams GM. 2005. DNA-reactive carcinogens: Mode of action and human cancer hazard. Crit Rev Toxicol 35:673–683.

Procter DM, Otani JM, Finley BL, Paustenbach DJ, Bland JA, Speizer N, Sargent EV. 2002. Is hexavalent chromium carcinogenic via ingestion? A weight of the evidence review. J Toxicol Environ Health A 65:701–746.

Quievryn G, Messer J, Zhitkovich A. 2002. Carcinogenic chromium (VI) induces cross-linking of vitamin C to DNA in vitro and in human lung A549 cells. Biochemistry 41:3156–3167.

Salnikow K, Zhitkovich A. 2008. Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: Nickel, arsenic and chromium. Chem Res Toxicol 21:28–44.

Sander M, Cadet J, Casciano DA, Galloway SM, Marnett LJ, Novak RF, Pettit SD, Preston RJ, Skare JA, Williams GM, Van Houten B, Gollapudi BB. 2005. Proceedings of a workshop on DNA adducts: Biological significance and applications to risk assessment Washington DC, April 13–14, 2004. Toxicol Appl Pharmacol 208:1–20.

Sarkar D, Sharma A, Talukder G. 1993. Differential protection of chlorophyllin against chromium and chlordane in mouse bone marrow in vivo. Mutat Res Lett 301:33–38.

Sarto F, Levis AG, Paulon C. 1980. Clastogenic activity of hexavalent and trivalent chromium in cultured human lymphocytes. Caryologia 33:239–250.

Sedman RM, Beaumont J, McDonald TA, Reynolds S, Keowech G, Howd R. 2006. Review of the evidence regarding the carcinogenicity of hexavalent chromium in drinking water. J Environ Sci Health Part C 24:155–182.

Seoane AI, Dulout FN. 1999. Contribution of the validation of the anaphase-test: Aneugenic and clastogenic effects of cadmium sulfate, potassium dichromate and nickel chloride in Chinese hamster ovary cells. Genet Mol Biol 22:551–555.

Shi X, Dalal NS. 1992. The role of superoxide radical in chromium (VI) generated hydroxyl radical: The Cr(VI) Haber-Weiss cycle. Arch Biochem Biophys 292:323–327.

Shi X, Dalal NS. 1994. On the mechanism of generation of hydroxyl radical by chromate in biologically relevant systems: Role of Cr(VI) complexes versus tetraperoxochromate (V). Environ Health Perspect 102:231–236.

Shi X, Chiu A, Chen CT, Halliwell B, Castranova V, Vallyathan V. 1999. Reduction of chromium (VI) and its relationship to carcinogenesis. J Toxicol Environ Health B 2:87–104.

Shindo Y, Toyoda Y, Kawamura K, Kurebe M, Shimada H, Chidharu H, Satake S. 1989. Micronucleus test with potassium chromate (VI) administered intraperitoneally and orally to mice. Mutat Res 223:403–406.

Shrivastava HY, Ravikumar T, Shanmugasundaram M, Babu M, Nair BU. 2005. Cytotoxicity studies of chromium (III) complexes on human dermal fibroblasts. Free Radical Biol Med 38:58–69.

Singh I. 1983. Induction of reverse mutations and mitotic gene conversion by some metal compounds in *Saccharomyces cerevisae*. Mutat Res 117:149–152.

Snow ET, Xu L-S. 1991. Chromium (III) bound to DNA templates promotes increased polymerase processivity and decreased fidelity during replication in vitro. Biochemistry 30:11238–11245.

Stella M, Montaldi A, Rossi R, Rossi G, Levis AG. 1982. Clastogenic effects of chromium on human lymphocytes in vitro and in vivo. Mutat Res 101:151–164.

Sugiyama M. 1992. Role of physiological antioxidants in chromium VI-induced cellular injury. Free Biol Med 12:397–407.

Sugden KD, Stearns DM. 2000. The role of chromium (V) in the mechanism of chromate-induced oxidative DNA damage and cancer. J Environ Path Toxicol Onco 19:215–230.

Swenberg JA, Fryar-Tita E, Jeong Y-C, Boysen G, Starr T, Walker VE, Albertini RJ. 2008. Biomarkers in toxicology and risk assessment: Informing critical dose-response relationships. Chem Res Toxicol 21:253–265.

Umeda M, Nishimura M. 1979. Inducibility of chromosomal aberrations by metal compounds in cultured mammalian cells. Mutat Res 67:221–229.

USEPA. 2005a. Guidelines for carcinogenic risk assessments, EPA/630/P-03/001F, March 2005 (http://www.epa.gov/cancer guidelines).

USEPA. 2005b. Supplemental guidance for assessing cancer susceptibility from early-life exposure to carcinogens, EPA/630/R-03/003F, March 2005 (http://www.epa.gov/cancer guidelines).

USEPA. 2007. Framework for determining a mutagenic mode of action for carcinogenicity using EPA's 2005 cancer guidelines and supplemental guidance for assessing susceptibility from early-life exposure to carcinogens: External Peer Review Drafthttp://www.epa.gov/osa/mmoaframework/pdfs/MMOA-ERD-FINAL-83007.pdf.

USEPA OPPTS. 1998. OPPTS Harmonized Test Guidelines Series 870 Health Effects Vol I of III Guideline OPPTS 870.1000-OPPTS 870.4300.

Venier P, Montaldi A, Majone F, Bianchi V, Levis AG. 1982. Cytotoxic, mutagenic and clastogenic effects of industrial chromium compounds. Carcinogenesis 3:1331–1338.

Voitkun V, Zhitkovich A, Costa M. 1998. Cr(III)-mediated crosslinks of glutathione or amino acids to the DNA phosphate backbone are mutagenic in human cells. Nucleic Acids Res 26:2024–2030.

Watanabe K, Sakamoto K, Sasali T. 1998. Comparisons on chemically-induced mutagenicity among four bacterial strains, *Salmonella*

typhimurium TA102 and TA2638, and *Escherichia coli* WP2/pKM101 and WP2 *uvr*A/pKM101: Collaborative study I. Mutat Res 361:142–155.

Waters MD, Stack HF, Brady AL, Haroun I, Vainio H. 1988. Use of computerized data listings and activity profiles of genetic and related effects in the review of 195 compounds. Mutat Res 205: 295–312.

Wild D. 1978. Cytogenetic effects in the mouse of 17 chemical mutagens and carcinogens evaluated by the micronucleus test. Mutat Res 56:319–327.

Ye J, Zhang X, Young HA, Mao Y, Shi X. 1995. Chromium (VI)-induced nuclear factor-κB activation in intact cells via free radical reactions. Carcinogenesis 16:2401–2420.

Ye J, Wang S, Leonard SS, Sun Y, Butterworth L, Antonini J, Ding M, Rojanasakul Y, Vallyathan V, Castranova V, Shi X. 1999. Role of reactive oxygen species and p53 in chromium (VI)-induced apoptosis. J Biol Chem 274:34974–34980.

Zhang J, Li X. 1986. Chromium contamination in the city of JinZhou. JinZhou Health and Anti-Epidemic Station, JinZhou, China. MRID No. 47325701.

Zhitkovich A. 2005. Importance of chromium-DNA adducts in mutagenicity and toxicity of chromium (VI). Chem Res Toxicol 18:3–11.

Zhitkovich A, Voltkun V, Kluz T, Costa M. 1998. Utilization of DNA-protein cross-links as a biomarker of chromium exposure. Environ Health Perspect Suppl 106:1–7.

Zhitkovich A, Shrager S, Messer J. 2000. Reductive metabolism of Cr (VI) by cysteine leads to the formation of binary and ternary Cr-DNA adducts in the absence of oxidative DNA damage. Chem Res Toxicol 13:1114–1124.

Zhitkovich A, Quievryn G, Messer J, Motylevich Z. 2002. Reductive activation with cysteine represents a chromium (III)-dependent pathway in the induction of genotoxicity by carcinogenic (VI). Environ Health Perspect Suppl 110:729–731.

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