

# Chromium(III) tris(picollinate) is mutagenic at the hypoxanthine (guanine) phosphoribosyltransferase locus in Chinese hamster ovary cells

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## Abstract

Chromium trispicollinate (CrPic) is a popular dietary supplement that is not regulated by the Food and Drug Administration. We are using this compound as a bio-available model to explore the role of Cr(III) in Cr(VI)-induced cancers. The ability of CrPic to cause mutations at the hypoxanthine (guanine) phosphoribosyltransferase (*hprt*) locus of CHO AA8 cells has been measured after a 48 h exposure. The highest dose tested was 80  $\mu\text{g}/\text{cm}^2$  CrPic, which, if fully soluble, would be equivalent to 1 mM or 0.44 mg/ml CrPic, and would correspond to 1 mM Cr(III) or 52  $\mu\text{g}/\text{ml}$  Cr(III). This exposure resulted in  $68 \pm 16\%$  cell survival based on 48 h cell counts, and  $24 \pm 11\%$  survival by 7-day colony formation. Exposure of CHO cells to CrPic produced a statistically significant increase in 6-thioguanine (6-TG)-resistant cells over the dose range tested. The 80  $\mu\text{g}/\text{cm}^2$  CrPic exposure resulted in an average induced mutation frequency (MF) of 58 per  $10^6$  surviving cells, or an average 40-fold increase in *hprt* mutants relative to untreated cells. An equivalent dose of 3 mM Pic was highly cytotoxic and did not yield *hprt* mutants. The dose range of 0.375–1.5 mM Pic produced a slight increase in *hprt* mutants, but the increase was not statistically significant. An equivalent dose of 1 mM chromic chloride yielded an induced MF of 9 per  $10^6$  surviving cells, or a 10-fold increase in mutants with cell survivals of >100%. The coordination of Cr(III) with picollinic acid may make the metal more genotoxic than other forms of Cr(III). In light of the current results and the known ability of Cr(III) and CrPic to accumulate in tissues, as well as the growing evidence of Cr(III) involvement in Cr(VI)-induced cancers, we caution against ingestion of large doses of CrPic for extended periods. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Chromium picollinate; Mutation frequency; Chinese hamster ovary cells; Hypoxanthine (guanine) phosphoribosyltransferase

## 1. Introduction

Chromium(III) tris(picollinate) (CrPic) is a bio-available form of Cr(III) that is a popular dietary supplement. Chromium picollinate provides a bio-available form of Cr(III) that we are using to investigate the involvement of Cr(III) in Cr(VI)-induced cancers. We

have previously shown that CrPic causes chromosomal aberrations in Chinese hamster ovary (CHO) cells [1]. It has also been shown to cause single strand breaks in plasmid DNA in vitro in the presence of ascorbic acid or hydrogen peroxide, presumably by a Fenton mechanism in which reactive oxygen species are produced as chromium cycles between oxidation states of Cr(II), Cr(III), and Cr(IV) [2]. There are many mechanistic aspects of CrPic genotoxicity that remain to be understood, including what individual contributions the metal center and the picollinate ligand make to

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cytotoxicity and DNA damage in cells, and whether or not the observed DNA damage is mutagenic. In the current work, we have measured the ability of CrPic to cause mutations in CHO cells under the same conditions that were found to cause chromosomal aberrations [1]. We have found that the CrPic complex, and to a lesser extent chromic chloride ( $\text{CrCl}_3$ ) are mutagenic at the hypoxanthine (guanine) phosphoribosyltransferase (*hprt*) locus of CHO cells after 48 h exposure.

Experiments were carried out in CHO fibroblasts because this was the cell line used to measure chromosomal aberrations [1]. Also, *hprt* mutations have been quantified in this line for many organic carcinogens [3] as well as the inorganic human carcinogens chromium(VI) [4], cadmium [5] and platinum [6], and the possible animal carcinogen, lead [7]. This allows for comparison of our results to those obtained for other genotoxins.

## 2. Materials and methods

### 2.1. Chemicals and reagents

CrPic was synthesized as the monohydrate by literature methods [8]. Its crystal structure has been determined [9]. Picolinic acid (CAS no. 98-98-6, Aldrich, Milwaukee, WI) and chromium(III) chloride hexahydrate (CAS no. 10060-12-5, Fisher, Houston, TX) were used as supplied. Treatment chemicals consisted of particulate suspensions of CrPic in acetone, and aqueous solutions of  $\text{CrCl}_3$  and Pic. Stock solutions of picolinic acid were adjusted to pH 6.0 with NaOH. Doses for particulate CrPic were based on the  $55 \text{ cm}^2$  surface area of a 100 mM cell culture dish. For example,  $200 \mu\text{l}$  of a stock solution of 22 mg/ml particulate CrPic in acetone was added to one dish giving  $4400 \mu\text{g}$  of CrPic per  $55 \text{ cm}^2$  for a dose of  $80 \mu\text{g}/\text{cm}^2$  CrPic. With a molecular weight of 436.32, this dose corresponds to 1 mM complex in 10 ml of medium. Most of the CrPic was solubilized during the exposure time. After 48 h exposure no particulate material was evident by visual examination of culture plates; however, microscopic examination did show some particulates remaining. The possibility of particulate CrPic uptake by endocytosis cannot be ruled out, although analysis of cells by transmission electron microscopy does not show evidence of intracellular particles (Manygoats

et al., submitted for publication). Chromium(VI), from  $\text{K}_2\text{Cr}_2\text{O}_7$ , (CAS no. 7778-50-9, Mallinckrodt Baker, Inc., Paris, KY) was used as a positive control.

### 2.2. Cell culture

Chinese hamster ovary AA8 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells at passage three were thawed from cryopreservation, cultured in  $\alpha$ MEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), antibiotic/antimycotic (penicillin, streptomycin and amphotericin B) (Sigma) and 1 mM glutamine (Gibco-BRL, Rockville, MD). Cells were maintained at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$ /air incubator calibrated with a Fryrite analyzer (Bacharach Co., Pittsburgh, PA). Cells were determined to be mycoplasma free by PCR analysis (Charles River, Wilmington, MA) on a quarterly basis.

### 2.3. Cytotoxicity and mutagenicity measurements

The HPRT assay was carried out following published procedures [10] with minor modifications. Briefly,  $3.5 \times 10^5$  cells were seeded in 100 mM plates, allowed to adhere over  $\sim 20$  h, and treated with test chemical for 48 h. Cell survival was determined by colony-forming ability. On day 2, cells were trypsinized, quantified on a Z1 Coulter Particle Counter (Beckman Coulter, Inc., Miami, FL) and re-seeded at 200 cells per 60 mM dish in quadruplicate. After 7–8 days, the dishes were stained with crystal violet and the colony number was counted. Cell survival was calculated as per cent colonies in treated dishes relative to untreated controls. From the same cells harvested after 48 h treatments, approximately  $1 \times 10^6$  cells were re-seeded in 100 mM dishes and that amount was re-seeded every 2 days until day 10 post-treatment. After this 8-day expression time, cells were seeded again for colony-forming ability as described above, and  $2.5 \times 10^5$  cells were seeded in quadruplicate in 100 mM dishes and incubated with  $11 \mu\text{g}/\text{ml}$  of 6-thioguanine (6-TG) for 7–8 days for mutant selection. The plating efficiency was  $86 \pm 9\%$ . The doubling time for untreated cells was 13 h. Data are expressed as mutants per  $10^6$  surviving cells, calculated from the observed 6-TG-resistant colonies and the 10-day clonogenic values. Experiments were

repeated five to nine times. Statistical significance for mutants per  $10^6$  surviving cells was determined by a paired, two-tailed *t*-test. Multiple comparisons among group means were accomplished by paired analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons test. Differences were considered significant at  $P < 0.05$ .

### 3. Results

#### 3.1. Cytotoxicity

The survival of CHO cells following 48 h exposure to  $\text{CrCl}_3$ , CrPic, or Pic is shown in Fig. 1. Chromic chloride did not produce a decrease in cell survival for 48 h treatments up to 1 mM Cr, by 48 h cell counts or 7-day colony formation. Higher concentrations of  $\text{CrCl}_3$  were not tested. At concentrations above 1 mM  $\text{CrCl}_3$ , the low pH of the stock solution resulted in precipitation of proteins in the cell culture medium (data not shown). Raising the pH of stock  $\text{CrCl}_3$  solutions produces oligomerization and precipitation of Cr(III) hydroxide. Chromic chloride was deemed a more relevant comparison to CrPic than chromic hydroxide because  $\text{CrCl}_3$  is the starting material for the synthesis of CrPic, it is another popular form

of chromium dietary supplement, and there is more data in the literature regarding its cytotoxicity and genotoxicity. The  $80 \mu\text{g}/\text{cm}^2$  dose, which would be equivalent to 1 mM Cr(III) if the complex were fully soluble, produced a cell survival of  $68 \pm 16\%$  for 48 h cell counts and  $24 \pm 11\%$  by 7-day colony formation. Free picolinate was more cytotoxic than the corresponding CrPic complex. For the 3 mM Pic dose, equivalent to the  $80 \mu\text{g}/\text{cm}^2$  CrPic dose, the 48 h cell count was lowered to 27%, and cell survival by colony formation was only 6%. This difference in cell survival between CrPic and free Pic suggests that either the CrPic complex is not fully dissociated intracellularly, or that the extent of absorption of picolinate is different for free Pic and Cr-coordinated Pic.

#### 3.2. Mutagenicity

The hypoxanthine (guanine) phosphoribosyltransferase (*hprt*) gene codes for the HPRT enzyme that is involved in purine recycling. This enzyme catalyzes the reaction of either hypoxanthine or guanine with 5-phospho- $\alpha$ -D-ribose-1-pyrophosphate (PRPP) to produce either inosine 5'-monophosphate or guanosine 5'-monophosphate, respectively. The *hprt* gene is located on the X chromosome of mammalian cells. The modified purine, 6-TG, also serves as a substrate

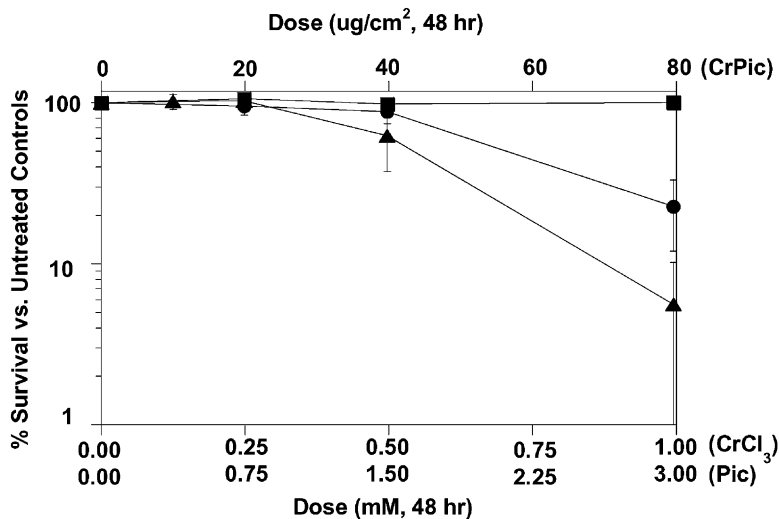


Fig. 1. Cytotoxicity of chromic chloride (■), chromium picolinate (●) and picolinic acid (▲) in CHO-AA8 cells after 48 h exposures. Cells were treated and assayed for 7–8 days colony formation as described in the text. Data represent mean  $\pm$  standard deviation for 7–10 independent experiments.

for the HPRT enzyme. In the presence of a functional enzyme, 6-TG is phosphorylated and subsequently incorporated into DNA, resulting in cell death [11]. Therefore, incubating treated cells with 6-TG allows

for selection of cells carrying mutations to the *hprt* gene. Cells that have lost the functional HPRT enzyme will survive in the presence of 6-TG, and cells with functional HPRT enzyme will die. This HPRT assay

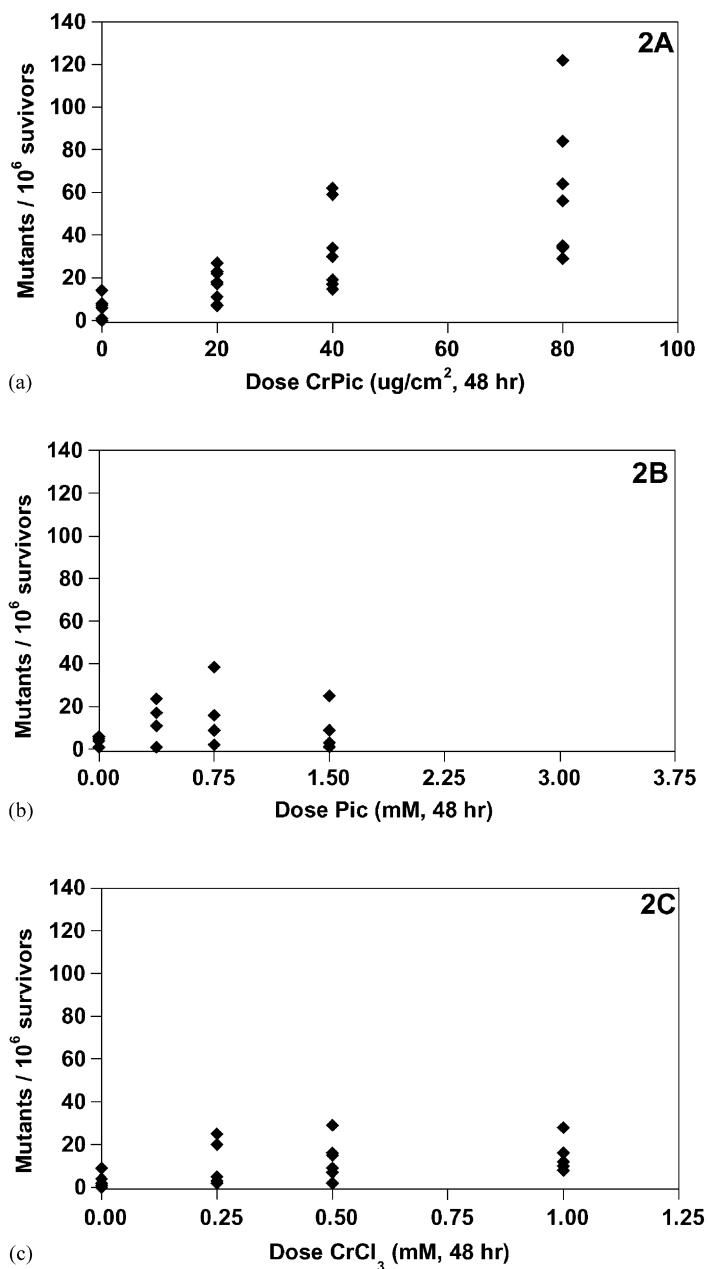


Fig. 2. 6-Thioguanine-resistant cells obtained after 48 h exposure of CHO-AA8 cells to (A) chromium picolinate, (B) picolinic acid, and (C) chromic chloride. Methods are described in the text. Each data point refers to an individual experiment.

Table 1

Cell survival and mutation induction in CHO-AA8 cells treated with chromium picolinate, picolinic acid, and chromic chloride

Treatment <sup>a</sup>	48 h cell counts <sup>b</sup> (%)	Survival at 10 days <sup>c</sup> (%)	Average induced mutation frequency <sup>d</sup>	Average mutant increase above background <sup>e</sup>	<i>P</i> values for mutants per 10 <sup>6</sup> survivors <sup>f</sup>
Untreated	100	100	–	–	–
20 µg/cm <sup>2</sup> CrPic	106 ± 15	99 ± 20	14.6	11.7	<0.001
40 µg/cm <sup>2</sup> CrPic	100 ± 18	100 ± 15	31.0	20.5	<0.001
80 µg/cm <sup>2</sup> CrPic	68 ± 16	93 ± 6	57.9	39.9	<0.001
0.375 mM Pic	101 ± 12	106 ± 16	6.3	4.1	NS
0.750 mM Pic	91 ± 17	103 ± 17	10.0	5.7	NS
1.50 mM Pic	64 ± 9	103 ± 10	11.6	4.2	NS
0.25 mM CrCl <sub>3</sub>	107 ± 17	101 ± 3	6.7	5.5	NS
0.50 mM CrCl <sub>3</sub>	112 ± 20	105 ± 13	9.3	10.3	<0.001
1.00 mM CrCl <sub>3</sub>	118 ± 25	104 ± 8	8.8	10.4	<0.001

<sup>a</sup> 48 h exposure time.<sup>b</sup> Surviving cells immediately after 48 h exposure.<sup>c</sup> Colony formation measured after 2-day treatment and 8-day expression time. Colony formation after 2-day exposures is shown in Fig. 1.<sup>d</sup> Treatment MF – control MF per 10<sup>6</sup> viable cells.<sup>e</sup> Treatment MF/control MF.<sup>f</sup> Data for mutants per 10<sup>6</sup> surviving cells are shown in Fig. 2.

selects for any mutation that produces a non-functional enzyme, including base substitutions and major deletions.

In the HPRT assay, the untreated cells displayed 4 ± 3 spontaneous mutations (range 0–9). The positive control dose of 30 µM Cr(VI) resulted in 71 ± 17 and 51 ± 13% survival by 48 h cell count and 7-day colony formation, respectively. The average induced mutation frequency (treatment MF – control MF) was 22 per 10<sup>6</sup> surviving cells, which yielded a 15-fold increase in 6-TG-resistant cells (treatment MF/control MF).

CHO cells treated with CrPic for 48 h produced *hprt* mutants (Fig. 2A) with a positive dose response for doses of 20, 40, and 80 µg/cm<sup>2</sup>. The highest dose of CrPic tested yielded an average induced MF of 58 per 10<sup>6</sup> surviving cells, and an average 40-fold increase in *hprt* mutants relative to untreated cells (Table 1). Equivalent doses of Pic (3 mM) produced no mutations (Fig. 2B); however, this dose was highly cytotoxic. A low yet statistically insignificant level of mutations was observed for doses of 0.375–1.5 mM Pic (Fig. 2B, Table 1). The 0.50 and 1.00 mM doses of CrCl<sub>3</sub>, equivalent to the 40 and 80 µg/cm<sup>2</sup> doses of CrPic, produced a statistically significant induced MF of 9 per 10<sup>6</sup> surviving cells, or a 10-fold increase in mutants (Fig. 2C, Table 1), but there was no positive dose response between the 0.50 and 1.00 mM CrCl<sub>3</sub> treatments.

#### 4. Discussion

Chromium picolinate was more mutagenic than equivalent extracellular doses of chromic chloride or free picolinate ligand. This is consistent with our previous observation that CrPic caused chromosomal aberrations [1]. For example, chromosomal aberrations were 4-fold higher for the 40 µg/cm<sup>2</sup> dose of CrPic than the equivalent dose of 1.5 mM Pic [1]. For *hprt* mutations, the 40 µg/cm<sup>2</sup> CrPic dose was also 3-fold more mutagenic than the 1.5 mM Pic dose based on induced mutation frequency (Table 1). Chromosomal aberrations were not detected for CrCl<sub>3</sub>; however, a low, but significant increase in 6-TG-resistant mutants was observed here (Table 1). Based on induced MF (Table 1), CrPic was almost 7-fold more mutagenic than CrCl<sub>3</sub> at the 1 mM Cr(III) dose, and 3-fold more mutagenic at the 0.50 mM Cr(III) dose. The observation of CrPic-induced mutations is more relevant for potential cancer causing ability than chromosomal aberrations because although chromosome damage has been correlated with tumor formation, falsely positive chromosomal aberrations may be observed in vitro as a result of high cytotoxicity [12].

It has been recommended that the acceptable dose and cytotoxicity ranges for genotoxicity testing be lowered to minimize the observation of false positive results. A new limit of 40% cytotoxicity (60%

survival) based on cell counts rather than the previous 50% ceiling was suggested, as well as lowering of the highest dose tested to 1–2 mg/ml from the previously accepted limit of 5 mg/ml or 10 mM, whichever was lower [13]. In the current study, mutations were observed after treatments with 20, 40, and 80  $\mu\text{g}/\text{cm}^2$  CrPic. This corresponds to 0.11, 0.22, and 0.44 mg/ml CrPic. Cytotoxicity for these doses based on cell counts after 48 h exposure were 0–36% (Table 1). Therefore, the test doses used in this study and our previous work [1] were well within the recently recommended guidelines.

Literature reports of Cr(III)-induced mutations are inconsistent. Trivalent Cr as the insoluble  $\text{Cr}_2\text{O}_3$  produced a 10-fold increase in 6-TG-resistant mutants in Chinese hamster V-79 lung cells after 18 h exposure [14]. The same compound produced a 4-fold increase in 6-TG-resistant mutants in human foreskin fibroblasts after 48 h exposure at 0.25 mM, and anhydrous  $\text{CrCl}_3$  produced 5-fold induction of mutations at 0.75 mM, whereas doses of up to 1 mM of soluble  $\text{CrCl}_3$  were not mutagenic in V79 cells [15]. The soluble Cr(III) complex,  $\text{Cr}(\text{CH}_3\text{COO})_3$ , did not cause *hprt* mutations in Chinese hamster V79/4-K1 cells after 24 h exposures of up to 200  $\mu\text{g}/\text{ml}$  (0.87 mM) compound [16]. These data may reflect a greater mutagenicity of insoluble Cr(III) compared to soluble Cr(III), which is consistent without our results with particulate CrPic and soluble  $\text{CrCl}_3$ . However, an alternative explanation may be the Fenton chemistry that could occur more readily with CrPic than with  $\text{CrCl}_3$  [2].

The observation of low, but significant mutations in  $\text{CrCl}_3$ -treated cells could be due to direct or indirect pathways. Chromic chloride has been shown to covalently bond to DNA in liver and kidney of rats treated with  $\text{CrCl}_3$  [17]. It has also been shown to be mutagenic through increases in DNA polymerase processivity and Cr-induced polymerase bypass of oxidative DNA damage in vitro [18]. The ability of Cr(III) to interfere with DNA polymerase may explain the observation of weak *hprt* mutations (Table 1) in the absence of chromosomal aberration [1].

The comparison of the genotoxicity of CrPic to that of  $\text{CrCl}_3$  is limited by differences in solubility, uptake and cytotoxicity. In this study,  $\text{CrCl}_3$  was not cytotoxic or substantially mutagenic over the dose range tested. However, discussion of mutant frequency in

terms of extracellular concentration may still be a useful, albeit incomplete, comparison. If a person ingests an equal amount of  $\text{CrCl}_3$  or CrPic in a dietary supplement, the  $\text{CrCl}_3$  may be less harmful. It may be due to differences in absorption or differences in molecular interactions with DNA, the mechanisms have yet to be clarified; however, there is a difference in genotoxic endpoints.

Chromium(VI) as  $\text{K}_2\text{Cr}_2\text{O}_7$  was used in this study as a positive control. The *hprt* mutations produced by Cr(VI) complexes in CHO cells have been reported. A 24 h exposure of CHO-K1 cells to three Cr(VI) compounds, 4  $\mu\text{M}$   $\text{K}_2\text{Cr}_2\text{O}_7$ , 6  $\mu\text{M}$   $\text{CrO}_3$ , or 8  $\mu\text{M}$   $\text{PbCrO}_4$  solubilized in NaOH, resulted in ~50% clonogenic cell survival and 7-, 5-, and 10-fold induction of mutations, respectively [4]. In V79/AP4 cells, a 1 h exposure to 32  $\mu\text{g}/\text{ml}$   $\text{K}_2\text{Cr}_2\text{O}_7$  produced an induction MF of 22 per  $10^6$  viable cells, or a 4-fold increase above background [19]. In the current study, a 30  $\mu\text{M}$  dose of Cr(VI), equivalent to 4.4  $\mu\text{g}/\text{ml}$   $\text{K}_2\text{Cr}_2\text{O}_7$ , produced 51% clonogenic survival, an induced MF of 22 per  $10^6$  surviving cells, or a 15-fold increase in mutations. Thus, with a larger dose of  $\text{K}_2\text{Cr}_2\text{O}_7$  used, Cr(VI) was less mutagenic in this current study. This likely reflects differences in cell culture medium that would affect Cr(VI) metabolism. The  $\alpha\text{MEM}$  used in this study has 78-fold more ascorbate and 5-fold more cysteine than the McCoy's 5A medium of the previous study [4], which for an equivalent exposure to Cr(VI) would increase the extent of extracellular reduction of Cr(VI), and thus, decreasing the amount of total Cr absorbed, increasing the cell survival, and decreasing mutations. Since the ultimate genotoxic agent is not Cr(VI) per se, but a metabolite such as Cr(V), Cr(IV), Cr(III), free radicals, or some combination, the extent to which one can compare Cr(VI) mutation frequencies under different metabolizing conditions is limited without measurement of specific DNA lesions that may be altered when metabolism conditions change.

Ultimately, we are using CrPic as a bio-available model to explore the role of Cr(III) in Cr(VI)-induced cancers. However, a simple comparison of induced mutation frequency for Cr(VI) and CrPic is of limited value. The metabolism of Cr(VI) is known to produce a range of DNA lesions, including adducts, cross-links, strand breaks and oxidized bases [20]. The extent to which Cr(III) contributes to a subset of the observed lesions and mutations is not yet clear.

The CrPic complex may have a cellular chemistry that is different from CrCl<sub>3</sub> and Cr(VI). Thus, comparison of the types of DNA damage produced and the nature of mutations will be more important than the amount of mutations. Such studies are currently in progress.

The toxic metals cadmium, platinum, and lead have also been shown to cause *hprt* mutations in Chinese hamster ovary cells. Cadmium acetate at a dose of 1 μM for 24 h resulted in ~15% clonogenic survival, and an average of 28 mutants versus 1.2 in untreated cells, or 23-fold mutant induction in CHO-K1 cells [5]. *Cis*-platin, or *cis*-Pt(NH<sub>3</sub>)<sub>2</sub>Cl<sub>2</sub>, was the most mutagenic of a series of Pt(II) complexes over 16–24 h exposures [6]. A 3 μM dose of *cis*-platin produced ~20% clonogenic survival with 140 mutants per 10<sup>6</sup> surviving cells, with 0–15 spontaneous mutants observed [6]. A 1.5 mM dose of lead acetate for 24 h produced ~75% clonogenic survival and an average of 53 mutants relative to 0.65 mutants in untreated cells, or an average 82-fold induction of mutations in CHO-K1 cells [7]. The results for CrPic are closest to those for lead, with the 80 μg/cm<sup>2</sup> dose (1 mM Cr(III)) producing a 40-fold increase in mutations after 48 h exposure. It should be noted that this dose represents the exposure concentration, not the intracellular concentration of CrPic, which has not been measured.

Although cadmium, platinum, lead and Cr(VI) have been reported to be mutagenic, their mechanisms of action are quite different. Cadmium and lead may act through epigenetic pathways, causing oxidative stress or inhibition of DNA repair [21,22]. Platinum is a direct acting genotoxin, producing ApG and GpG intrastrand cross-links [23]. As discussed above, Cr(VI) produces a range of DNA lesions that have not yet been directly linked to specific mutations.

Determination of the specific types of CrPic-induced DNA lesions responsible for the observed mutations is currently under investigation. DNA strand breaks have been observed with CrPic and ascorbate in vitro [2]; however, neither Cr-DNA adducts or DNA strand breaks appear to be major DNA lesions in CHO cells (Stearns et al., in preparation). Fenton chemistry may still occur with this complex in cells, with oxidized bases such as 8-oxo-deoxyguanosine being a more significant lesion than single strand breaks.

Chromium picolinate is a widely used dietary supplement that is not regulated by the Food and Drug Administration. The doses used in this study were

higher than doses one would ingest from a few pills. However, it is known that Cr(III) can accumulate in the body [24]. Ingestion of CrPic supplements was found to produce serum levels of chromium [25] that were equivalent to serum levels measured in workers occupationally exposed to chromium [26], and to produce urinary chromium levels higher than those in people environmentally exposed to chromium [27]. Chromium has also been shown to accumulate in rat liver, kidney, spleen, lung, gastrocnemius, and heart after ingestion of CrPic in the diet [28]. There is also growing evidence of the involvement of Cr(III) in Cr(VI)-induced cancers [29,30]. The current results suggest that the structure and coordination chemistry of CrPic may make it more genotoxic than other forms of Cr(III). Therefore, in light of these and previous results [1], we caution against taking excessive doses of chromium picolinate for long periods of time.

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