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# Molecular analysis of *hprt* mutations induced by chromium picolinate in CHO AA8 cells

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## Abstract

Chromium picolinate (CrPic) is a popular dietary supplement, marketed to the public for weight loss, bodybuilding, and control of blood sugar. Recommendations for long-term use at high dosages have led to questions regarding its safety. Previous studies have reported that CrPic can cause chromosomal aberrations and mutations. The purpose of the current work was to compare the mutagenicity of CrPic as a suspension in acetone versus a solution in DMSO, and to characterize the *hprt* mutations induced by CrPic in CHO AA8 cells. Treatments of 2% acetone or 2% DMSO alone produced no significant increase in 6-thioguanine (6-TG)-resistant mutants after 48 h exposures. Mutants resistant to 6-TG were generated by exposing cells for 48 h to 80  $\mu\text{g}/\text{cm}^2$  CrPic in acetone or to 1.0 mM CrPic in DMSO. CrPic in acetone produced an average induced mutation frequency (MF) of 56 per  $10^6$  surviving cells relative to acetone solvent. CrPic in acetone was 3.5-fold more mutagenic than CrPic in DMSO, which produced an MF of 16.2. Characterization of 61 total mutations in 48 mutants generated from exposure to CrPic in acetone showed that base substitutions comprised 33% of the mutations, with transversions being predominant; deletions made up 62% of the mutations, with one-exon deletions predominating; and 1–4 bp insertions made up 5% of the characterized mutations. CrPic induced a statistically greater number of deletions and a statistically smaller number of base substitutions than have been measured in spontaneously generated mutants. These data confirm previous studies showing that CrPic is mutagenic, and support the contention that further study is needed to verify the safety of CrPic for human consumption.

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**Keywords:** Chromium picolinate; *hprt*; Mutational spectrum; Genotoxicity; Chinese hamster ovary

## 1. Introduction

Chromium picolinate (CrPic) is a popular dietary supplement, originally advertised to the public as an aid for weight loss and bodybuilding. The use of CrPic as an anabolic agent was originally patented in 1992 [1]. Sales of chromium supplements in the US were reported to be

second only to those of calcium supplements in 1999 [2], with sales of chromium-based supplements exceeding \$500 million in that year [3]. It is estimated that 10 million Americans take some form of chromium supplement [2], with CrPic being the most popular.

In spite of its marketing success, clinical studies have not supported the efficacy of CrPic for weight loss or muscle building. A recent meta-analysis [4] found only a minor effect, if any, of CrPic on weight loss relative to placebo in 10 randomized, double-blind, placebo-controlled trials [5–14]. The mounting evidence against CrPic's efficacy for weight loss has recently led

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to a shift in focus to another market, namely type II diabetes.

Several studies have shown significant effects for CrPic on serum parameters related to diabetes, with responses proportional to the doses ingested. At a dose of 200  $\mu\text{g}$  CrPic/day for 2 months CrPic supplementation had no effect on glucose control, LDL or HDL levels; however, triglyceride levels were decreased in 28 non-insulin-dependent diabetics [15]. Supplementation with 600  $\mu\text{g}$  CrPic/day decreased fasting glucose levels by 40% in three subjects with steroid-induced diabetes [16]. In the most widely publicized study, 180 subjects were either given placebo, 200 or 1000  $\mu\text{g}$  Cr/day as CrPic for 4 months [17]. Both fasting and 2 h glucose levels, as well as total plasma cholesterol were lowered most significantly for the 1000  $\mu\text{g}$  group. Fasting and 2 h insulin levels decreased significantly with both CrPic doses.

The recommended use of CrPic to treat type II diabetes raises several safety issues. A dose of 1000  $\mu\text{g}$  Cr as CrPic is 18–77 times higher than average daily dietary Cr intakes [18–20]. A recent patent recommends even higher doses of CrPic: 1000–10,000  $\mu\text{g}$  Cr/day, for diabetics [21]. The Cr(III) in CrPic is more bioavailable than dietary Cr, and this metal can accumulate in humans [22,23]. Coordination of Cr by picolinate may alter the redox chemistry of Cr(III) [24], making it more toxic than other forms of Cr(III). Diabetics using CrPic would be ingesting large amounts of the supplement over their lifetimes; therefore, the potential for toxic effects from long-term exposures to high doses of CrPic needs to be considered.

There are a growing number of studies characterizing the cytotoxicity and genotoxicity of CrPic. The first study to question its safety showed that CrPic caused chromosomal aberrations after 24 h exposures in Chinese hamster ovary (CHO) AA8 cells, whereas equivalent doses of chromic chloride and chromium nicotinate were inactive, and free picolinate was clastogenic only above the 1.5 mM concentration [25]. The same doses of CrPic were mutagenic at the *hprt* locus in CHO cells after 48 h exposure, whereas equivalent doses of free picolinate and chromic chloride were not mutagenic [26]. CrPic was also shown to cause mitochondrial damage and apoptosis in the CHO AA8 cell line [27]. Studies funded by the supplement manufacturer did not find evidence of *hprt* mutations [28] or chromosomal aberrations [29]; however, the mutagenicity of CrPic has been independently confirmed in *Drosophila melanogaster* [30] and in the mouse-lymphoma assay [31].

Observations that CrPic can cause direct DNA damage have supported reports of its mutagenicity. An *in vitro* study showed that CrPic caused single strand breaks

in isolated plasmid DNA in the presence of ascorbate plus dioxygen, dithiothreitol plus dioxygen, or hydrogen peroxide, presumably by a Fenton-type mechanism where CrPic cycles between the Cr(III), Cr(II) and Cr(IV) oxidation states to generate free radicals [24]. Analysis of CrPic-exposed cells by the alkaline comet assay have shown DNA strand breaks that increased with post-treatment exposure to formamidopyrimidine glycosylase (FPG) and decreased with post-treatment exposure to methyl methanesulfonate (MMS), suggesting the presence of DNA strand breaks, oxidative damage and DNA crosslinks (Lencinas et al., in preparation).

The purpose of the current study was to characterize the *hprt* mutations induced by CrPic in CHO AA8 cells. Results showed that the mutation spectrum was significantly different than that reported for spontaneous mutations in CHO K1-BH4 cells [32]. Determination of the molecular spectrum of mutations will assist in the elucidation of the mechanism underlying the observed CrPic-induced mutations, will be helpful for both predicting and confirming the types of DNA lesions induced by this dietary supplement, and will contribute to evaluation of the safety of this compound for human ingestion.

## 2. Materials and methods

### 2.1. Chemicals

Chromium trispicolinate (CAS No. 14639-25-9) was synthesized as previously described [27]. Elemental analysis of CrPic was carried out by inductively coupled plasma optical emission spectroscopy (ICP-OES). Expected % Cr for monohydrate  $\text{Cr}(\text{Pic})_3 \cdot \text{H}_2\text{O}$  (MW 436.32)  $\text{CrC}_{18}\text{H}_{14}\text{O}_7\text{N}_3$  11.9%; expected % Cr for anhydrous  $\text{Cr}(\text{Pic})_3$  (MW 418.31)  $\text{CrC}_{18}\text{H}_{12}\text{O}_6\text{N}_3$  12.4%; found  $12.5 \pm 0.6\%$ . The solubility of CrPic in DMSO is  $\sim 50$  mM. The solubility in aqueous solution is  $\sim 0.6$  mM at ambient temperature and  $\sim 3$  mM at  $37^\circ\text{C}$  (data not shown). CrPic was prepared as a 50.0 mM stock solution in DMSO by sonication, or was prepared as an acetone slurry of 22.0 mg CrPic/mL by stirring suspensions overnight with a 0.5 in. stir bar in a sealed 20 mL liquid scintillation vial. Treatments consisted of 200  $\mu\text{L}$  stock solution or solvent in 10.0 mL medium in a 100 mm dish with a 55  $\text{cm}^2$  growth area.

### 2.2. Cell culture

CHO AA8 cells (American Type Culture Collection, Manassas, VA) were cultured as adherent monolayers as previously described [26]. Cells were treated with 200  $\mu\text{M}$  hypoxanthine, 0.04  $\mu\text{M}$  aminopterin, and 17.5  $\mu\text{M}$  thymidine (HAT; Sigma, St. Louis, MO) for 3 days to eliminate background mutations and recovered for 2 days in medium supplemented with 200  $\mu\text{M}$  hypoxanthine and 17.5  $\mu\text{M}$  thymidine (HT; Sigma).

The recovered cells were subcloned, in 350,000-cell aliquots, into 100-mm dishes and allowed to adhere for 18–20 h.

### 2.3. Generation and measurement of *hprt* mutations induced by chromium picolinate

The mutation frequency was determined for CHO AA8 cells exposed to CrPic as a solution in DMSO and compared to that for cells exposed to an equivalent dose of CrPic as a suspension in acetone. For these studies, HAT-treated and HT-recovered cells were treated with 1.00 mM CrPic in DMSO or with 80  $\mu\text{g}/\text{cm}^2$  CrPic in acetone for 48 h. Previous studies under identical culturing conditions found some residual particulate CrPic in the extracellular medium, but found no intracellular particulate CrPic by transmission electron microscopy [27]. Control dishes consisted of cells treated with 200  $\mu\text{L}$  (2%) of DMSO or acetone, as well as dishes with no treatment.

For sequencing studies, cells were HAT-treated then divided into thirty-five 100-mm dishes and cultured as independent populations. Cells were recovered for 2 days with 200  $\mu\text{M}$  hypoxanthine and 17.5  $\mu\text{M}$  thymidine. Each 100-mm dish was treated with 80  $\mu\text{g}/\text{cm}^2$  CrPic, as a suspension in acetone, for 48 h. The cells were then subcloned and grown for 9 days to allow for protein turnover. Mutants resistant to 6-thioguanine (6-TG; Sigma) were selected with  $\alpha$ -MEM supplemented with 11  $\mu\text{g}/\text{mL}$  6-TG. Up to four clones were picked from each dish. Mutants were expanded in  $\alpha$ -MEM with 11  $\mu\text{g}/\text{mL}$  6-TG from 1 colony to  $(4\text{--}12) \times 10^6$  cells. Cells were frozen as pellets at  $-20^\circ\text{C}$  in 500-cell and 2,000,000-cell aliquots prior to analysis.

### 2.4. Mutation characterization

Methods for characterization of *hprt* mutants in CHO cells have been detailed previously [33] and are briefly described here. The *hprt* mRNA from these mutants was copied into complementary DNA (cDNA) and amplified, using single-tube reverse transcription (RT) and polymerase chain reaction (PCR) reactions (RT-PCR), followed by a second, nested, PCR (RT-PCR–PCR), and this amplified cDNA was sequenced. Mutants that did not yield amplification products were subjected to multiplex RT-PCR–PCRs with primers specific for *hprt* mRNA along with those specific for elongation-factor 2 (*EF-2*) mRNA as an internal positive reaction control, and wild-type AA8 cells multiplexed in parallel as a further positive control.

Mutants with exons skipped in their cDNA sequences and those that produced no cDNA amplicons were further characterized by multiplex PCR of *hprt* exons, with *EF-2* exon-specific primers as an internal positive PCR control. Exons that were skipped in a mutant's cDNA sequence but amplified by genomic PCR were amplified individually and sequenced.

#### 2.4.1. RT-PCR

About 500 cells were lysed and the cellular mRNA reverse-transcribed using a RobusT RT-PCR kit (Finnzymes Oy-MJ

Research, Waltham, MA) with 200 nM *hprt*-specific primers [33], 1 U/ $\mu\text{L}$  Prime RNase Inhibitor<sup>TM</sup> (Eppendorf, Hamburg, Germany), and 0.1% Nonidet P-40 (Roche Molecular Biochemicals, Mannheim, Germany). Reverse transcription was carried out for 1 h at  $50^\circ\text{C}$  and followed by 36 cycles of PCR with a primer-annealing temperature of  $60.5^\circ\text{C}$ . Less stringent RT-PCRs of mutants producing no amplicons were reverse-transcribed at  $48^\circ\text{C}$  and amplified at  $59^\circ\text{C}$ . Multiplex RT-PCRs of repeated failures had the same reaction conditions, except 200 nM of each *EF-2*-specific primer [33] was included in the reaction as a positive control, and the RT and PCR were at 48 and  $59^\circ\text{C}$ , respectively. All RT-PCRs included negative controls.

#### 2.4.2. Nested PCR

The second, internal, amplification reaction was performed, with 6.7% sucrose, 100  $\mu\text{M}$  xylene cyanol, 400 nM of each internal *hprt*-specific primer [33], and 10% RT-PCR reaction as template; the annealing temperature was  $61^\circ\text{C}$ . Mutants yielding more than one band were reamplified at annealing temperatures up to  $65.1^\circ\text{C}$ , while those with faint single bands were reamplified at annealing temperatures as low as  $59^\circ\text{C}$ . Multiplex nested amplifications differed by having 300 nM of each *hprt*-specific inner primer [33], 500 nM of each *EF-2*-specific inner primer [33] as an internal control, and a primer-annealing temperature of either 59 or  $61^\circ\text{C}$ . All nested PCRs included negative controls. Products were visualized on 1.5% agarose gels.

#### 2.4.3. cDNA sequencing

Single-band (amplicon) reactions were purified and sequenced at the University of Arizona DNA Sequencing Service using 2–6 *hprt* cDNA-specific sequencing primers [33], as necessary, to provide unequivocal DNA sequence. Sequences were aligned with SeqMan<sup>TM</sup> (DNASTAR, Inc., Madison, WI) and miscalls corrected. The mutant consensus sequences were compared to wild-type AA8 consensus sequence, using MegAlign<sup>TM</sup> (DNASTAR, Inc.) to identify sequence changes.

#### 2.4.4. Genomic PCR

Wild-type CHO AA8 and mutant genomic DNAs were isolated from  $2 \times 10^6$ -cell aliquots using ZR Genomic DNA Kit<sup>TM</sup> columns (Zymo Research, Orange, CA) and quantified. Exons 1, 2, 4, 5, 6, 7 and 8 together, and 9 were amplified from these genomic DNAs, with a positive internal PCR control using *EF-2*-specific genomic primers [33]. Exons 1, 2, 6, and 7/8 were multiplexed in a single PCR reaction, exons 5 and 9 were multiplexed in a second reaction, and exon 4 was not amplified with any other *hprt* exon. Exon 3 was amplified alone, without *EF-2*-specific primers. Negative results for exon 3 were reamplified to ensure the results were repeatable. Additionally, in each set of exon 3 amplifications there were other mutants whose exon 3 did amplify, which suggested the negative results were not due to reaction failure. Although amplification of exons 3–5 produced a spurious artifact band, the amplification of the target DNA appeared to be unaffected. Amplicon bands were visual-

Table 1

Cell survival and *hprt* mutation induction in CHO AA8 cells exposed to chromium picolinate in acetone or DMSO solvents

Dose, 48 h	% survival 48 h ± S.E.M.	% survival 9 days ± S.E.M.	Mutants per 10 <sup>6</sup> surviving cells ± S.E.M.	<i>p</i> -Value	Average induced mutation frequency	Average mutation increase above background
Untreated	(100)	(100)	4.4 ± 1.2	–	–	–
2% acetone	94.2 ± 3.8	95 ± 3	5.2 ± 1.2	n.s. <sup>a</sup>	2.6 <sup>b</sup>	1.2 <sup>c</sup>
80 µg/cm <sup>2</sup> CrPic in 2% acetone	16.5 ± 0.5	75 ± 1	61.6 ± 6.8	<0.05 <sup>d</sup>	56.4 <sup>e</sup>	11.8 <sup>f</sup>
2% DMSO	56.5 ± 3.4	97 ± 2	6.5 ± 2.7	n.s. <sup>a</sup>	3.9 <sup>b</sup>	1.5 <sup>c</sup>
1.0 mM CrPic in 2% DMSO	35.5 ± 3.9	92 ± 2	22.7 ± 4.3	<0.01 <sup>d</sup>	16.2 <sup>e</sup>	3.5 <sup>f</sup>

<sup>a</sup> Differences in mutants per 10<sup>6</sup> surviving cells were not significant relative to untreated cells.

<sup>b</sup> Treatment MF – untreated MF.

<sup>c</sup> Treatment MF/untreated MF.

<sup>d</sup> Differences in mutants per 10<sup>6</sup> surviving cells were statistically significant by Wilcoxon–Mann–Whitney rank sum test relative to solvent treatment.

<sup>e</sup> Treatment MF – solvent MF.

<sup>f</sup> Treatment MF/solvent MF.

ized on 2.5% Synergel<sup>TM</sup> (Diversified Biotech, Boston, MA), 1% agarose gels.

#### 2.4.5. Single-exon amplification and sequencing

Single-exon PCR reactions were performed on mutant genomic DNAs with the following concentrations of each primer: 0.075 µM for exon 1, 0.5 µM for exon 2, 1.0 µM for exon 3, 1.5 µM for exon 4, 2.33 µM for exon 5, 1.0 µM for exon 6, 0.5 µM for exons 7/8, and 0.6 for exon 9 [33]. Single-exon amplifications included wild-type AA8 genomic DNA and H<sub>2</sub>O run in parallel as positive and negative controls, respectively.

An aliquot of each reaction was visualized on 2.5% Synergel<sup>TM</sup>, 1% agarose gels, while the remainder was purified using DNA Clean & Concentrator<sup>TM</sup>-5 columns (Zymo Research), and quantified. The amplicons were sequenced in both directions with the appropriate primers [33] by the University of Arizona DNA Sequencing Service. Changes in the mutants' exon sequences were identified by comparisons to published CHO *hprt* exon sequences [32] using MegAlign<sup>TM</sup> (DNASTAR, Inc.).

#### 2.4.6. Statistics

Statistical significance for induced mutation frequencies was evaluated by the Wilcoxon–Mann–Whitney rank sum test. The significance of differences among the mutation spectra was determined by performing the R × C contingency table log-likelihood ratio test [34]. When differences were significant, the tables were subdivided [35] to investigate the nature of the differences. Comparisons were considered significant at  $p \leq 0.05$ .

### 3. Results

A previous study, funded by the supplement manufacturer, reported that CrPic was not mutagenic at the

*hprt* locus in CHO K1 cells when treated for 5 or 48 h with CrPic as a solution in DMSO at doses up to 500 µg/mL CrPic (1.2 mM) [28]. In the current study the mutagenicity of CrPic was compared for solutions of CrPic in DMSO and suspensions of CrPic in acetone after 48-h exposures in CHO AA8 cells. Results were not consistent with those reported in the manufacturer's study.

In the current study, cytotoxicity and mutagenicity of CrPic was measured in CHO AA8 cells after 48 h exposures using either acetone or DMSO as solvents. Under these conditions 2% acetone (200 µL) was found to be nontoxic, producing little decrease in 7-day colony formation relative to untreated cells, whereas exposure to the same volume of DMSO in the absence of CrPic decreased cell survival to 56%, making DMSO 40% more cytotoxic than acetone (Table 1). Cells were also exposed to 80 µg/cm<sup>2</sup> CrPic as a suspension in acetone or to 1.0 mM CrPic as a solution in DMSO. In spite of the increased toxicity of DMSO, cell survival for CrPic in acetone was ~50% lower than that for CrPic in DMSO (Table 1). The cell survival of 16% for exposures to CrPic in acetone in the current study was similar to the previous observation of 24% survival under identical conditions [26]. The cell survival of 35% for exposures to CrPic in DMSO was also similar to the previously observed cell survival of 25% under identical conditions [28]. The colony formation assay carried out after the 48 h treatment and 9-day recovery time showed that cells had recovered to >90% survival for all treatments except that of CrPic in acetone (Table 1).

Mutations at the *hprt* locus were measured by selection for 6-TG-resistant colonies. A low background mutation frequency was observed in untreated cells, and

Table 2  
Chromium picolinate-induced mutations observed in CHO AA8 *hprt* cDNA

Mutant(s)	Mutation	Exon	Predicted protein consequence
Base substitutions			
Transitions			
C97a	T <sub>114</sub> → C	6	Pro <sub>38</sub> (CCT) → Pro (CCC)
C96b <sup>a</sup>	A <sub>475</sub> → G	6	Lys <sub>159</sub> (AAG) → Glu (GAG)
C96b <sup>a</sup>	T <sub>479</sub> → C	6	Val <sub>160</sub> (GTT) → Ala (GCT)
Transversions			
C102b	G <sub>27</sub> → C	1	TG/at → TC/at <sup>b</sup> in exon 1 donor splice site
C99b	G <sub>190</sub> → C	3	Ala <sub>64</sub> (GCC) → Pro (CCC)
C11, C78	A <sub>217</sub> → T	3	Lys <sub>73</sub> (AAA) → stop (TAA)
C60	T <sub>220</sub> → A	3	Phe <sub>74</sub> (TTC) → Ile (ATC)
C110a	A <sub>409</sub> → C	6	Ile <sub>137</sub> (ATT) → Leu (CTT)
C91b	A <sub>415</sub> → C	6	Thr <sub>139</sub> (ACT) → Pro (CCT)
Deletion			
C95d	T <sub>480</sub>	6	Frameshift

<sup>a</sup> Mutant has more than one mutation.

<sup>b</sup> Lower case letters designate intron sequences.

exposures to acetone or DMSO in the absence of CrPic did not produce a statistically significant increase in mutants relative to untreated cells (Table 1), nor were the differences between the solvents significant ( $p=0.80$ ). On the other hand, suspensions of CrPic in acetone and solutions of CrPic in DMSO produced significantly more 6-TG-resistant mutants than their respective solvent controls (Table 1), with almost three-fold more mutants observed for CrPic in acetone than in DMSO ( $p<0.01$ ). Both the average induced MF and the average mutation increase above background were over three-fold higher for CrPic in acetone than in DMSO (Table 1). These data showed that DMSO solvent does affect the mutagenicity of CrPic; however, CrPic was still mutagenic regardless of the form in which it was administered to cells.

The mutation spectrum was characterized in CHO AA8 cells exposed to CrPic in acetone by sequencing *hprt* cDNAs and exons from 6-TG-resistant mutants. Cells were exposed to 80  $\mu\text{g}/\text{cm}^2$  CrPic as an acetone suspension for 48 h. This dose produced an average induced MF of 57.2 (difference from spontaneous) or 56.4 (difference from acetone-treated controls). This was consistent with the value of 58 mutants per  $10^6$  surviving cells reported previously [26]. Mutants were selected with 6-TG after a 9-day recovery time to allow for expression of the 6-TG-resistant (6-TG<sup>r</sup>) mutant phenotype. A total of 136 mutants were picked and expanded for molecular characterization. A number of these were removed from the analysis because they were not amenable to characterization (14 mutants) or were not independent mutants but a sibling clone of another mutant from the same independent population

(74 mutants). Ultimately, 61 total mutations were identified in 48 mutants.

The *hprt* mRNA from 6-TG<sup>r</sup> mutants was copied into complementary DNA (cDNA) and amplified by RT-PCR followed by a nested PCR (RT-PCR-PCR). Thirteen mutants produced more than one amplicon, even with higher RT and PCR temperatures, and were eliminated from the study. Single-amplicon reactions were sequenced. Eleven point mutations were identified in the mutants' cDNAs (Table 2). Of these mutations, three were transitions, seven were transversions and one contained a single-base deletion. There was no dominant base substitution apparent; however, half of the base substitutions occurred at A (A → T twice, A → C twice, A → G once), 3/10 occurred at T (T → A and T → C) and two occurred at G (G → C). Half of the base substitutions occurred in exon 6, 4 of 11 occurred in exon 3, and 1 occurred in exon 1.

The genomic DNAs of mutants that skipped exons in their cDNA sequences and those failing to produce an *hprt*-specific cDNA band were multiplexed with *EF-2* primers as an internal PCR reaction control. Exons that failed to amplify were tabulated as genomic deletions (Table 3). The 16 single and multiexon deletions that included exons 1 and/or 9 resulted in no cDNA being amplified, presumably because no mature *hprt* mRNA was produced by virtue of a loss of 5' promoter sequence and/or the loss of the 3' polyadenylation signal. The remaining 17 deletions of at least one complete exon resulted in the exclusion of those same exons from the corresponding cDNA sequences (Table 3). Six mutants (C22, C34, C82a, C89a, C93a and C95b) had no exon

Table 3  
Chromium picolinate-induced exon deletions and sequence changes detected in genomic DNA

Mutant(s)	Genomic mutation	cDNA phenotype
<b>Base substitutions</b>		
<b>Transversions</b>		
C18 <sup>a</sup> , C22 <sup>a</sup> , C82a <sup>a</sup>	Intron 1: g <sub>[exon 2]–30</sub> → t <sup>b</sup>	Exons 2–3 skipped
C2 <sup>a</sup>	Intron 1: g <sub>[exon 2]–30</sub> → t	Exons 2–4 skipped
C26	Intron 1: g <sub>[exon 2]–30</sub> → t	Exons 2–6 skipped
C22 <sup>a</sup>	Intron 2: g <sub>[exon 2]+42</sub> → c	Exons 2–3 skipped
C18 <sup>a</sup>	Exon 3: A <sub>83</sub> → T	Exons 2–3 skipped
C2 <sup>a</sup>	Exon 3: A <sub>83</sub> → T	Exons 2–4 skipped
C108b <sup>a</sup>	Intron 4: a <sub>[exon 5]–1</sub> → t	Exon 5 skipped
C109a <sup>a</sup>	Intron 8: g <sub>[exon 9]–1</sub> → c	1st 17 bases of exon 9 skipped
<b>Deletions</b>		
C108b <sup>a</sup>	Intron 5: g <sub>[exon 5]+1</sub>	Exon 5 skipped
C108b <sup>a</sup>	Intron 5: c <sub>[exon 5]+42</sub>	Exon 5 skipped
C56 <sup>a</sup>	Intron 8: a <sub>[exon 8]+3</sub> agtaatg <sub>+10</sub>	Exon 8 skipped
C109a <sup>a</sup>	Intron 8: c <sub>[exon 9]–18</sub>	1st 17 bases of exon 9 skipped
C85b, C90a, C98a	Exon 1 deleted	No cDNA amplified
C81a, C86a, C88a, C92a, C104b	Exon 2 deleted	Exon 2 skipped
C108b <sup>a</sup> , C108d, C110b	Exon 4 deleted	Exon 4 skipped
C22 <sup>a</sup> , C34, C82a <sup>a</sup> , C89a, C93a, C95b	Exon 6 deleted	Exon 6 skipped
C105a	Exon 7/8 deleted	Exons 7–8 skipped
C106b	Exon 9 deleted	No cDNA amplified
C107b, C109b	Exons 1–4 deleted	No cDNA amplified
C84a, C94b	Exons 1–5 deleted	No cDNA amplified
C111a	Exons 3–4 deleted	Exons 3–4 skipped
C97b	Exons 4–5 deleted	Exons 4–5 skipped
C97c, C105d	Exons 6–9 deleted	No cDNA amplified
C83b, C87b, C95c, C98c, C107c, C108a	Exons 1–9 deleted	No cDNA amplified
<b>Insertions</b>		
C56 <sup>a</sup>	Intron 7: g inserted after g <sub>[exon 7]+19</sub>	Exon 8 skipped
C109a <sup>a</sup>	Intron 8: c inserted after c <sub>[exon 9]–42</sub>	1st 17 bases of exon 9 skipped
C109a <sup>a</sup>	3' UTR: t inserted after t <sub>[exon 9]+120</sub>	1st 17 bases of exon 9 skipped

<sup>a</sup> Mutant has more than one mutation.

<sup>b</sup> Lower case letters designate intron sequences. The intron in which the mutation occurs is noted, and the location of the mutated nucleotide is given relative to the nearest exon, with negative numbers being 5' of the exon and positive numbers being 3' of the exon.

6 in both the genomic DNA and cDNA and five (C81a, C86a, C88a, C92a and C104b) had exon 2 deleted from both their genomic and cDNAs.

The mutants that skipped exon(s) in their cDNAs yet had those same exon(s) present in their genomic DNAs were presumed to be splicing mutations. The relevant exon(s) of presumed splicing mutants were amplified singly and sequenced. A total of 10 transversions, 4 deletions and 3 insertions were identified (Table 3). Half of the transversions were g → t mutations within intron 1, at the 30th base 5' of exon 2. Two of these, C26 and C82a, skipped exons 2–6 and 2–3, respectively, even though this was the only mutation they possessed flanking the affected exons (Table 3). Two others, C2 and C18, had an A → T transversion within exon 3, although this resulted in skipping exons 2–4 in C22 but exons 2–3 in C18. The last mutant in this group, C22, had a g → c

substitution in intron 2, 42 bases 3' of exon 2. Those two transitions resulted in exons 2–3 being skipped in its cDNA. Such variable cDNA phenotypes involving at least exons 2–3 have been observed in human splicing-error *hprt* mutants, along with mutations within exon 3 whereby exons 2–3 were excluded from the mutant's cDNA sequence [36]. In mutant C109a, a cryptic splice site using bases within exon 9 was used upon loss of the exon 9 donor splice site, with the result that the first 17 bases of exon 9 were skipped in its cDNA. This same splice-site loss and cDNA phenotype has also been documented in human *hprt* mutants [36]. There were three single-base deletions, three insertions of one base, and one deletion of eight bases (Table 3). In all seven instances, multiple mutations were observed in each mutant's sequences; only the loss of the exon 5 acceptor splice site in C108b could easily explain the observed

Table 4  
Comparison of chromium picolinate-induced and spontaneous *hprt* mutants

Mutations	CrPic-induced** 61 mutations	Spontaneous <sup>a</sup> 56 mutations
Base substitutions	20 (33%)**	34 (60.7%)
All transitions	3 (4.9%)**	12 (21.1%)
All transversions	17 (27.9%)	22 (39.3%)
G → T or C → A	5 (8.2%)*	12 (21.1%)
G → C or C → G	4 (6.6%)	4 (7.1%)
Deletions	38 (62%)***	17 (30.4%)
Small (1–52 bp)	5 (8.2%)	4 (7.1%)
One exon	19 (31.1%)*	9 (16.1%)
Multiexon	8 (13.1%)	3 (5.4%)
Whole-gene	6 (9.8%)*	1 (1.8%)
Insertions	3 (5%)	5 (8.9%)
Small (1–4 bp)	3 (4.9%)	2 (3.6%)
Large (at least 86 bp)	0 (0%)*	3 (5.4%)

<sup>a</sup> Ref. [32].

\* Significantly different than spontaneous,  $p < 0.05$ .

\*\* Significantly different than spontaneous,  $p < 0.01$

\*\*\* Significantly different than spontaneous,  $p < 0.001$ .

cDNA phenotype. However, the cumulative effect of the multiple mutations may be responsible for the resultant cDNA sequence changes.

Mutations induced by CrPic differed significantly from those previously observed to arise spontaneously in the *hprt* gene [32] ( $p < 0.01$ ; Table 4). The published spontaneous spectrum was determined in CHO K1-BH4 cells, which Xu et al. also compared to spontaneous mutations in the hamster V79 lung fibroblast line [32]. These published values were used to determine that the two spontaneous spectra were not significantly different ( $p > 0.1$ ), which suggested that the *hprt* mutation spectrum generated spontaneously is independent of cell type. A statistical comparison of the CrPic-induced spectrum and the K1-BH4 spontaneous spectrum, using the same categories as in the comparison of the K1-BH4 and V79 spectra [32], demonstrated that the overall CrPic spectrum was significantly different than the overall spontaneous spectrum ( $p < 0.01$ ). Specifically, there were significantly fewer point mutations ( $p < 0.01$ ) and more genomic deletions ( $p < 0.001$ ) in the CrPic-treated cells. Of the 61 mutations identified after exposure to CrPic, 33% (20/61) consisted of point mutations, while 62% (38/61) of the CrPic-induced mutations were deletions (Table 4). This differed from the spectrum reported for spontaneous mutants, which identified 61% base substitutions and 30% deletions [32]. There was not a significant difference between small deletions of 52 bases or fewer between the two spectra. There were significantly more single-exon and whole-gene deletions in the CrPic

spectrum ( $p < 0.05$ ). CrPic also induced 13.1% (8/61) multiexon deletions compared to 5.4% (3/56) generated spontaneously, although the difference was not statistically significant. Nor was there a significant difference in the amount of insertions observed, with 4.9% for CrPic exposure (3/61) and 8.9% in spontaneously generated mutations (5/56) (Table 4).

#### 4. Discussion

This study compared the effect of DMSO and acetone solvents on the mutation frequency of CrPic-induced 6-TG-resistant mutants in CHO AA8 cells after 48 h exposures, and characterized the mutation spectrum generated from CrPic exposure in acetone suspension. Results for the mutagenicity of CrPic in DMSO solution differed from those recently reported in a study funded by the supplement manufacturer [28].

The manufacturer's study tested CrPic in DMSO solution at concentrations up to 500  $\mu\text{g/mL}$  CrPic (1.2 mM) for 5 and 48 h exposures and found no significant increase in mutation frequency relative to solvent control [28]. Although the magnitude of effect at 48 h differs from the current study, these results are not necessarily unexpected in light of the current understanding of CrPic toxicity. First, it is well known that trivalent Cr complexes are taken up slowly by cells because there is no known active transport [37]. Therefore, shorter exposure times should produce lower levels of toxicity. This interpretation is consistent with the reported cytotoxicity for CrPic in DMSO as measured by clonogenic survival. The manufacturer's study found 78% survival after 5 h exposure compared to 25% survival for the 48 h exposure in the absence of S9 fraction [28]. Second, CrPic has been proposed to undergo redox-activated production of reactive oxygen species as a result of the aromatic picolinate ligands shifting the  $\text{Cr}^{3+}/\text{Cr}^{2+}$  redox potential to a physiologically obtainable range [24]. DMSO has long been known to be an effective radical scavenger [38]. Therefore, it would be predicted that a compound whose mechanism of action may include free radical production would be less toxic in the presence of a radical scavenger. Individual chemicals must always be evaluated in terms of their known mechanism of action when choosing testing conditions. It is for that reason that the guidelines for genotoxicity testing cited in the manufacturer's study, specifically for solvents and exposure times, are simply guidelines and not fixed regulatory requirements.

CrPic has been proposed to cause oxidative damage as an extension of its proposed redox chemistry; however, this has not been consistently observed. CrPic caused an increase in thiobarbituric acid reactive species (TBARS),

cytochrome *c* reduction, 2,3- and 2,5-dihydroxybenzoic acids and DNA fragmentation in cultured J774A.1 macrophages exposed to 200  $\mu$ M CrPic for 24 h [39,40]. It also caused strand breaks in isolated plasmid DNA when reacted with ascorbate, H<sub>2</sub>O<sub>2</sub> or dithiothreitol [24]. Rats injected daily with 5  $\mu$ g CrPic showed increased 8-oxo-2'-deoxyguanosine (8-oxodG) in urine after 31 days, and increased TBARS, conjugated dienes, and 8-oxodG in liver and kidney after 60 days [41]. Conversely, doses of 400  $\mu$ g Cr/day for 8 weeks did not produce oxidative damage in eight individuals as measured by antibodies titers to an oxidized DNA base, 5-hydroxymethyl-2'-deoxyuridine (HMdU) [42]. Nor did a 1 h exposure of 200  $\mu$ M CrPic provide evidence of ROS by oxidation of 2',7'-dichlorofluorescein in human lung A549 cells [43].

The major signals for oxidative damage within mutation spectra are proposed to be base substitutions of G  $\rightarrow$  T or C  $\rightarrow$  A (a G  $\rightarrow$  T mutation on the complementary strand) and G  $\rightarrow$  C or the complementary C  $\rightarrow$  G, which arise from the presence of 8-oxodG [44,45] and its further oxidized products spiroiminodihydroantoin and guanidinohydroantoin [46–50]. The observed mutation spectrum induced by CrPic is at least partially consistent with the oxidative damage hypothesis. The combined category of G  $\rightarrow$  T, C  $\rightarrow$  A, G  $\rightarrow$  C, or C  $\rightarrow$  G base substitutions showed 9/61 (15%) and 16/56 (29%) such point mutations for CrPic and spontaneous mutations, respectively (Table 4), and differences were not significant ( $p=0.076$ ). However, for G  $\rightarrow$  T or C  $\rightarrow$  A transversions alone, CrPic had significantly less of these point mutations than were observed to arise spontaneously ( $p<0.05$ ). Thus the role of oxidative damage in CrPic-induced mutagenesis is not yet entirely clear. It is possible that the redox cycling of CrPic may produce oxidative damage from high and low valent Cr species rather than classic reactive oxygen species, thus producing a unique mutation spectrum.

For example, in contrast to oxidative damage-related point mutations, CrPic induced significantly more deletions than have been found to arise spontaneously (Table 4). The exact sizes of the genomic deletions were not ascertained, but single-exon genomic deletions can potentially be large deletions, if large quantities of the flanking introns are also deleted. There were considerably more CrPic-induced multiexon genomic deletions than were reported for spontaneous mutations ( $p<0.05$ ), and significantly more whole-gene deletions in the CrPic mutants than the reported spontaneous mutants ( $p<0.05$ ) [32].

The preponderance of large deletions generated by CrPic suggested the presence of DNA damage as

being responsible for the induction of mutations based on a comparison of the CrPic mutation spectrum to other known mutagens. The mutational spectrum of the DNA–DNA interstrand crosslinker mitomycin C included mostly large multigenic deletions at the *tk* locus of mouse-lymphoma L5178Y cells, consistent with its identification as a clastogen [51]. Bleomycin, a radiomimetic that induces DNA double strand breaks, was found to produce large deletions in CHO K1-BH4 cells [52]. Studies are currently in progress to confirm the presence of DNA strand breaks, oxidative damage and DNA crosslinks in CrPic-treated cells, and to evaluate a possible solvent dependence for Cr uptake (Lencinas et al., in preparation).

In summary, CrPic was found to be mutagenic at the *hprt* locus in CHO AA8 cells as either a suspension in acetone or a solution in DMSO, and the induced mutation spectrum differed from that shown to arise spontaneously. Results of this study are consistent with previous reports of CrPic-induced chromosomal aberrations [25] and mutations [26,30,31], and point to the presence of DNA damage in CrPic-exposed cells. These data support the contention that further study is needed to verify the safety of this dietary supplement for long-term high-dose use in humans.

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