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Influence of Substrate Complexity on the Diastereoselective Formation of Spiroiminodihydantoin and Guanidinohydantoin from Chromate Oxidation

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Abstract

Chromate is a human carcinogen with a poorly defined mechanism of DNA damage. *In vitro* and prokaryotic studies have shown that DNA damage may occur via the formation of the hydantoin lesions guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp) from further oxidation of 8-oxo-7,8-dihydroguanine (8oxoG). The unusual structure of these lesions coupled with their enhanced mutagenicity make them attractive for study with regard to their role in chromate-induced cancer. We have studied the formation of Gh versus Sp and their associated diastereomers following oxidation by model Cr(V) complexes and from *in situ* chromate reduction by ascorbate and glutathione. Identification of the two optically assigned diastereomers of Sp (R-Sp, S-Sp) as well as the two diastereomers of Gh, (Gh1 and Gh2; not yet optically assigned) were carried out using increasingly sterically hindered substrates (nucleoside → ssDNA → dsDNA). Lesion formation and diastereomeric preference was found to be highly oxidant- and substrate-dependent. The Ir(IV) positive control showed a shift from near equal levels of Gh and Sp, and near equal levels of all four diastereomers in the nucleoside, to all Gh formation in dsDNA, with a 5-fold enhancement in Gh2 over Gh1. The two model Cr(V) complexes used in this study, Cr(V)-salen and Cr(V)-ehba, showed opposite trends going from nucleoside to dsDNA with Cr(V)-salen giving enhanced Sp formation (with mainly R-Sp formed) and the Cr(V)-ehba having an oxidation profile nearly identical to that of Ir(IV). The two chromate reduction systems, Cr⁶⁺/ascorbate and Cr⁶⁺/glutathione, designed to model the intracellular reduction of chromate, showed lower levels of oxidation in all substrates. Notable in this group was the shift in the formation of the lesions to essentially all Sp for the Cr⁶⁺/ascorbate system with the most sterically hindered substrate, dsDNA. These results, when coupled with the known diastereomeric preference for excision of hydantoin lesions by the hNEIL1 enzyme, show the importance of defining both levels of lesion formation and diastereomeric preference of formation with regard to their potential impact on chromate carcinogenesis.

Introduction

Chromate was one of the first identified carcinogens when it was linked to formation of nasal tumors in Scottish chrome pigment workers in 1890 (1). Numerous subsequent epidemiological studies demonstrated a link between occupational exposure to this metal and the formation of lung cancer (for a review of chromate toxicology studies see ref. 2). Despite early discovery and numerous exposure studies, the molecular mechanism linking chromate exposure to DNA damage, the progression to mutation, and ultimately cancer formation remains poorly

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Supporting Information Available: Experimental data and procedures for assignment of R-Sp and SSp diastereomers using circular dichroism.

understood. The unique unidirectional uptake of tetrahedral chromate through nonselective ion channels has been shown to result in high levels of chromate in exposed cells (3) and a variety of gross morphological changes in DNA structure. While many types of DNA lesions have been postulated to occur from chromate exposure, including DNA adducts (4), DNA-protein crosslinks (5), double-strand breaks (6), and oxidation of DNA bases (7), little structural data on the nature of these lesions, their mutagenic effects and their cellular prevalence have been forthcoming.

The intracellular reduction of chromate is known to generate oxidant stress, forming a variety of highly oxidizing species including high valent Cr(V) and Cr(IV) complexes (8) as well as free radicals (9). The two electron oxidation product of guanine, 8-oxo-7,8-dihydroguanine (8oxoG)¹, was long-hailed as a primary marker of oxidative stress (10), and its formation has been observed under certain conditions by the intracellular reduction of chromate (11). However, the 8oxoG lesion is only mildly mutagenic (12) and more easily oxidized than any of the four canonical DNA bases (13). The relative ease of 8oxoG oxidation has led to the recent discovery of further oxidized products of guanine, with the lesions of spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh) among them (14), Figure 1. Both of these lesions contain a chiral center at position C4, resulting in loss of the normal planar configuration of unmodified nucleic acid bases. Sp has been found in *E. coli* after treatment with chromate (15), and both Sp and Gh have been identified as substrates for the base excision repair (BER) enzymes NEIL1 and NEIL2 (16). A number of studies have found these two lesion to be far more mutagenic than the parent lesion, giving high rates of G→C and G→T transversion mutations (17–19). As G→C and G→T transversion mutations are prevalent in lung tumors of chromate workers (20), these results have implications for the biological relevance of the Sp and Gh lesions with regard to cancer formation by chromate.

Recently, differences in repair enzyme recognition between the two diastereomers of Sp suggest that diastereo-selectivity may play a role in mutation formation (21). Computational studies suggest that the R-Sp diastereomer disrupts helix stacking of neighboring base pairs less than the S-Sp diastereomer, while experimental data has shown R-Sp to be less thermally destabilizing than S-Sp (22). The repair enzyme hNEIL1 has also been shown to differentiate between the two diastereomers, forming stronger interactions with S-Sp in conformational studies and showing different rates of excision (23,24). Two diastereomers of Gh also exist but they have not been as widely studied as the Sp diastereomers because they are generally more refractory to isolation and identification.

The goal of this study was to identify the relative levels of formation of Sp and Gh following exposure to oxidizing chromium complexes as well as to determine whether there is diastereoselectivity in their formation as substrate complexity increases from nucleoside to duplex DNA. The previous studies showing different repair responses and mutation formation among the different diastereomers suggest that understanding the preferential formation of the hydantoin isomers may be critical in understanding the mechanism of chromate carcinogenesis. Several oxidizing systems were used in this study, including the classical iridium(IV) system pioneered by Cynthia Burrows group (25) as well as model high valent chromium(V) complexes (26) and *in situ* chromate/ascorbate and chromate/glutathione reduction systems designed to correlate with cellular chromate reduction profiles. Lesion formation was analyzed by LC-ESI-MS/MS for differences between oxidation products generated from sterically unhindered nucleosides, single-stranded oligonucleotides, and the more sterically hindered double-stranded oligonucleotide substrate. This study was designed to determine the importance of stereospecific oxidant interactions with DNA and to show how steric constraints imposed by the DNA duplex may influence lesion formation and diastereomeric selectivity. Our results have shown that as substrate complexity increases from nucleoside to duplex DNA, an oxidant-specific shift occurs in both the relative levels of formation of Gh versus Sp as well

as changes in the predominating diastereomer of each lesion. This work has potential significance in understanding the fundamental mechanism of chromate carcinogenicity, and the diastereoselectivity shown in these systems suggests that the predominant R-Sp isomer formed over that of the S-Sp isomer under “normal” background oxidation processes may influence DNA repair rates following chromate oxidation.

Experimental Procedures

Synthesis of Spiroiminodihydantoin (Sp) and Guanidinohydantoin (Gh) Standards

8oxoG and sodium iridium(IV) chloride hexahydrate were purchased from Sigma-Aldrich (St. Louis, MO). The nucleoside was oxidized as described previously (27). Briefly, iridium(IV) chloride was added to 8oxoG at a 1:2 molar ratio in 10 mM potassium phosphate buffer, pH 7.4, to form Sp. The solution was incubated for 45 minutes in a mineral oil bath at 60 °C. To preferentially form Gh, the solution was unbuffered and chilled at 4 °C. Excess oxidant was removed via filtration through 20–24 mg of Sephadex A-25 anion exchange beads with 4–100 μ L water washes. Gh was then purified by HPLC using an Agilent 1100 HPLC and a Zorbax SB-C18 column, dimensions 9.4 mm $\times$ 250 mm. A gradient of 0.5% acetonitrile/minute was used with a flow rate of 1.8 mL/min. Sp purification was accomplished using a gradient from 0% to 50% acetonitrile over 15 minutes. Collected fractions were lyophilized to dryness and reconstituted in Nanopure water. Concentrations were determined by UV-Vis spectroscopy at 230 nm using $\epsilon(\text{Sp}) = 4900 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon(\text{Gh}) = 3000 \text{ M}^{-1}\text{cm}^{-1}$ (14).

Oxidation of 8-oxo-7,8-dihydroguanine nucleoside

Potassium dichromate, ascorbic acid, and reduced glutathione were purchased from Sigma Aldrich (St. Louis, MO). Cr(V)-ehba was synthesized as previously published (28). Cr(V)-salen was formed by 1:2 reaction of Cr(III)-salen and iodosylbenzene in dry acetonitrile at room temperature for 15 minutes. Oxidations of 50 μ L of 100 μ M 8oxoG were carried out in a 10 mM tris buffer (pH 7.4) at 37 °C. Oxidants iridium(IV) chloride, Cr(V)-salen, and Cr(V)-ehba were used at a 1:8 8oxoG:oxidant ratio. Cr⁶⁺/ascorbate and Cr⁶⁺/glutathione were used at 1:4:40 and 1:10:1 ratios of 8oxoG:Cr⁶⁺: reductant, respectively. All oxidations were incubated for 1 hour with the exception of the Cr⁶⁺/glutathione system, which was incubated for 6 hours because of a slower reduction process. Nucleoside oxidations were purified with Sephadex columns as described for synthesis of standards, lyophilized, and reconstituted in water. Samples were analyzed by LC-ESIMS/MS without further processing.

Oxidation of the single-stranded 8oxoG containing oligonucleotide

A 22-mer oligonucleotide of sequence 5'-ACC AGC AGC GXC CGC ACC AGT G-3', where X is 8oxoG, was purchased from TriLink BioTechnologies (San Diego, CA). The oligonucleotide was purified by HPLC with a Dionex column as previously published (26). Fractions containing oligonucleotide were collected in microcentrifuge tubes and lyophilized to approximately one-quarter volume. The DNA was ethanol precipitated and the oligonucleotide pellet was dried at room temperature before reconstituting in water. Concentrations were then determined UV-Vis spectroscopy and calculated from A₂₆₀ using the extinction coefficient provided for the specific sequence. Oxidations were carried out in 50 μ L volumes as detailed above, and products were desalted through the use of Micro Bio-Spin 6 columns purchased from Bio-Rad (Hercules, CA).

Oxidation of the double-stranded 8oxoG containing oligonucleotide

The complement oligonucleotide of sequence 5'-TCA CTG GTG CGG CCG CTG CTG G-3' was purchased from TriLink BioTechnologies (San Diego, CA) and was HPLC purified, ethanol precipitated, and quantified as for the 8oxoG-containing single stranded

oligonucleotide. The 8oxoG containing oligonucleotide was annealed with 20% excess complement using a Thermacycler in oxidation buffer. Double-stranded oligonucleotides were then oxidized and purified using identical conditions as described in the nucleoside section.

DNA Hydrolysis

Phosphodiesterase I and phosphodiesterase II were purchased from Worthington Biochemicals (Lakewood, NJ); calf intestinal alkaline phosphatase was purchased from Promega (Madison, WI); butylated hydroxytoluene and deferoxamine were purchased from Sigma Aldrich (St. Louis, MO). Oxidized oligonucleotides were hydrolyzed prior to analysis as described previously (27), with a few minor adjustments. Antioxidants butylated hydroxytoluene and deferoxamine were added to the digestion mixture at final concentrations of 0.5 mM and 1.5 mM, respectively, to reduce artifactual oxidation due to the digestion conditions (29). Double-stranded oligonucleotides were heated at 95 °C for 5 minutes to denature DNA strands. Samples were then ice-chilled for 3 minutes, with the unbuffered addition of 0.10 units of phosphodiesterase II within the first 30 seconds. A 1-hour incubation at 37 °C followed with a repeat addition of enzyme and incubation. 10X sodium-free phosphodiesterase I buffer (100 mM tris-HCl, 100 mM NH₄Cl, and 100 mM MgCl₂) and 0.5 units of phosphodiesterase I were added and incubated for 45 minutes, with an additional repeat of enzyme and incubation time. Lastly, phosphates were removed with the addition of 5 units of calf intestinal alkaline phosphatase and 45 minute incubation, followed by one repeat of enzyme addition and incubation time. Samples were filtered with Microcon YM-30 centrifugal filters for 25 minutes at 2200 × g to remove enzymes and any undigested DNA. Samples were stored at -80 °C until analyzed.

LC-ESI-MS/MS analysis method

Samples were analyzed by selected reaction monitoring (SRM) using a 1200 series Agilent HPLC coupled to an Agilent 6300 XCT series LC/MSD Trap with electrospray ionization. Separations were achieved using a Thermo Scientific Hypercarb column with 5 μm particle size and dimensions of 100 mm × 2.1 mm. A mobile phase of 100% A and 0% B was ramped to 10% B at 6 minutes with a flow rate of 0.2 mL/minute, where mobile phase A is 0.1% aqueous acetic acid and mobile phase B is 100% MeOH. The mass spectrometer was run at a dry temperature of 350 °C, nebulizer pressure of 40 psi, and dry gas flow rate of 8 L/min. Other pertinent MS parameters are given in Table 1. The buffer front in the first 2.5 minutes of each run was diverted to waste, after which Gh nucleoside m/z of 274 was collected with an isolation window of 2.0 amu, fragmented, and monitored for the free base at m/z 158. After 6 minutes Sp was detected through a 2.0 amu isolation window of m/z 300 with fragmentation and monitoring of the free base at m/z 184. Quantification of Sp and Gh was conducted through the use of two standard addition points to create a curve ranging 1 to 2 orders of magnitude for each oxidant type and DNA substrate. Average baseline levels of lesion found in the unoxidized control samples were then subtracted from levels found in oxidized samples.

Results

Rationale for oxidant systems

Oxidation by Ir(IV) was chosen as a positive control because of its effective formation of Sp and Gh from 8oxoG, and a well-defined mechanism of action (14,17,25). The high-valent chromium complexes, Cr(V)-salen and Cr(V)-ehba, were used in this study as they have been proposed to be models for the highly oxidizing reduction intermediates of chromate formed during intracellular reduction (26). More importantly, it has been shown that these model complexes do not produce oxygen radicals during their reduction to the final stable Cr(III) oxidation state (3). Endogenous reductants including ascorbate and glutathione have been identified as the primary reductants of intracellular chromate (30,31) and act as pro-oxidants

with regard to cellular toxicity (32,33). Both ascorbate and glutathione are known to reduce Cr(VI) to the more highly oxidizing Cr(IV) and Cr(V) oxidation states, as well as producing a variety of radicals species under certain metal/reductant ratios (8,34). These systems have been included in this work to approximate cellular oxidation systems, although no attempt to optimize lesion formation based on varying metal/reductant ratios was made.

Mass spectrometry detection method

We have developed a LC-ESI-MS/MS method as the primary approach to lesion analysis throughout this study. The Sp diastereomers observed with this approach were assigned based on optical methods from previously published protocols (35,36) as validated in our own laboratory (see supporting information). Because optical assignments for the Gh diastereomers have not yet been made, the diastereomers called Gh1 and Gh2 in this study were assigned based solely on their elution profile from the HPLC. Selected reaction monitoring (SRM) was utilized, that allowed for more selective and more sensitive measurements of the lesions of interest. Sodium and potassium salts were substituted in enzyme buffer solutions whenever possible throughout the experimental procedures, allowing only the molecular ion, (M+H) of 274 and 300 for Gh and Sp) to be trapped and the ensuing fragmented free base of each to be selectively monitored at m/z of 158 and 184 for Gh and Sp respectively. These parameters gave detection limits for Gh and Sp of respectively 200 fmol and 400 fmol from a 250–1000 pmol 8oxoG nucleoside sample. The presence of higher salt concentrations from the oligonucleotide digests decreased the detection limits for single-stranded and double-stranded DNA to approximately 300 fmol for Gh and 700 fmol for Sp in a sample with an original concentration of 500–1300 pmol 8oxoG. A typical chromatogram for this system showing the formation of all four diastereomers is given in Figure 2.

Oxidation of the 8oxoG nucleoside and hydantoin lesion formation

The 8oxoG nucleoside was reacted with each of the oxidants and analyzed for Sp and Gh formation by SRM using LC-ESIMS/MS. Oxidations were carried out in triplicate, and standard addition curves over an order of magnitude of lesion formation were used for quantification. Because of the unique matrix of each oxidation system, a standard addition curve was created for each set of conditions. Levels of Sp and Gh found in the unoxidized standard (background) were subtracted from levels found in oxidized samples, allowing for comparison of lesion formed only by the oxidative conditions listed above.

Gh was the predominant lesion under all oxidizing conditions except Ir(IV) (Figure 3). Despite little expected steric influence from the nucleoside substrate, Gh2 was formed preferentially over Gh1. The formation of Gh1 was approximately 75% of Gh2 formation in all but the Cr⁶⁺/ascorbate system, where Gh1 formation was half that of Gh2. In contrast, background levels of oxidation showed opposite diastereomeric preference, with the amount of Gh1 exceeding the amount of Gh2. While the pattern of Gh formation was fairly consistent among all oxidative systems, the efficiency of formation varied between oxidants. Cr⁶⁺ with ascorbate was the least effective at forming Gh, followed by Cr⁶⁺/glutathione. Ir(IV) and Cr(V)-ehba were slightly more effective, forming Gh at very similar levels, and Cr(V)-salen formed more than triple the amount of Gh formed under any other condition.

Sp formation at the nucleoside level was less consistent. Nearly equal formation of diastereomers was seen in the background oxidation and with oxidants Ir(IV) and Cr⁶⁺/glutathione. Oxidation by Cr(V)-salen showed a clear preference for the formation of R-Sp, while Cr(V)-ehba and Cr⁶⁺/ascorbate favored formation of S-Sp. The Cr⁶⁺/ascorbate system was clearly the least-effective at forming Sp, followed by similar levels of formation by Cr(V)-ehba, Cr⁶⁺/ascorbate and Cr⁶⁺/glutathione, with Ir(IV) forming twice as much Sp as the previous systems and Cr(V)-salen forming the most Sp.

Oxidation of single-stranded oligonucleotides containing an 8oxoG moiety

A 22-mer oligonucleotide containing an 8oxoG was oxidized and digested to its respective nucleosides prior to LC-ESI-MS/MS analysis. Samples were run in triplicate with standard additions in each oxidation matrix for quantification. Baseline lesion levels were quantified and subtracted from that observed in oxidized samples prior to comparison. As seen in Figure 4, the single-stranded oxidations differ substantially from the nucleoside oxidations. Gh diastereomeric preference was consistent across the set of oxidants, but now showed preference of Gh1 as opposed to Gh2 preference in the nucleoside oxidations. Gh was no longer the predominant lesion under all conditions. Instead, Sp showed preferential formation with Cr(V)-salen and Cr⁶⁺/ascorbate, as well as in the unoxidized/background sample. Gh was still formed over Sp by Ir(IV), Cr(V)-ehba, and Cr⁶⁺/glutathione, and showed a dramatic increase in the cases of Ir(IV) and Cr(V)-ehba. Efficiency of Gh formation increased in the order Cr⁶⁺/ascorbate < Cr⁶⁺/glutathione ~ Cr(V)-salen < Cr(V)-ehba < Ir(IV).

Sp formation in single-stranded oligonucleotides showed a significant change in the magnitude of diastereomeric preference. S-Sp was on average formed twice as often as R-Sp by Cr(V)-ehba, Cr⁶⁺/glutathione, and Cr⁶⁺/ascorbate, as well as in the background sample. R-Sp was formed preferentially in Ir(IV) and Cr(V)-salen, amounting to 3 and 2.5 times S-Sp respectively. Overall formation of Sp increased in the order Cr⁶⁺/glutathione ~ Cr⁶⁺/ascorbate < Ir(IV) < Cr(V)-salen < Cr(V)-ehba.

Oxidation of double-stranded oligonucleotides containing an 8oxoG moiety

A 22-mer oligonucleotide containing an 8oxoG was annealed to its complement containing cytosine opposite the 8-oxoG moiety, oxidized, and digested to its respective nucleosides prior to LC-ESI-MS/MS analysis. Samples were run in triplicate with standard additions in each oxidation matrix for quantification. Background lesion levels were quantified and subtracted from that in oxidized samples prior to comparison.

There were notable differences between lesion formation in the double-stranded oligonucleotide and the previous, less sterically hindered, substrates (Figure 5). Only Gh was observed to form from Ir(IV) oxidation and Cr(V)-ehba oxidation, while Sp was formed at greater levels than Gh under all other condition including in the background samples. The diastereomers of Gh did not separate well in all samples (presumably from adverse buffer effects in the electrospray source), and values given for diastereomeric preference by Ir(IV), Cr(V)-salen, and Cr(V)-ehba are from a single sample in which we were able to get reasonable separations. Under these conditions, Gh1 was formed less than Gh2, particularly from Ir(IV) oxidation. Gh was not detected in the Cr⁶⁺/ascorbate system and was only minimally formed by Cr⁶⁺/glutathione, with the Gh1 diastereomer being preferred.

Sp formation was seen in some but not all double-stranded samples; Sp was not detected as a result of Ir(IV) nor Cr(V)-ehba oxidation. In Cr(V)-salen, Cr⁶⁺/ascorbate, Cr⁶⁺/glutathione, and background samples, R-Sp was formed preferentially over S-Sp. Cr(V)-salen exhibited the largest amount of Sp formation as well as the greatest difference of formation between diastereomers, with almost 10 times as much R-Sp as S-Sp being formed.

Overall Gh and Sp formation with increasing substrate complexity are compared in Figure 6 and Figure 7, respectively. Background levels of Sp and Gh were fairly consistent, regardless of substrate complexity. Gh formation was also approximately the same across all substrates for Cr(V)-ehba. However, there was a clear decrease in Gh formation with substrate complexity for oxidation with Cr(V)-salen, Cr⁶⁺/ascorbate, and Cr⁶⁺/glutathione systems. Ir(IV) was the only oxidant to show an increase in Gh formation from oxidation of nucleosides to single-stranded oligomers to double-stranded oligomers. Sp formation was also observed to decrease

with complexity for oxidation with Ir(IV), Cr(V)-salen, and Cr⁶⁺/glutathione. Cr(V)-ehba showed an increase in Sp formation from the nucleoside to single-stranded DNA, but no Sp was detected for the double-stranded oligonucleotide. Sp formation increased with substrate complexity only for oxidation with Cr⁶⁺ and ascorbate.

Discussion

Although chromate exposure has been linked to cancer for over a century, the mechanism leading from exposure to DNA damage and downstream events leading to carcinogenesis remains incomplete. Oxidized DNA lesions have been identified as a result of many types of oxidative stress, including chromate exposure. The study of 8-oxo-7,8-dihydroguanine (8oxoG) as a primary oxidized base lesion and marker of oxidant stress predominated until the relatively recent identification of a number of further oxidized lesions including spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh). These lesions have been shown to form as a result of further oxidation of 8oxoG by both metals and endogenous radicals, but many aspects of their formation and biological importance require further study. This work examined Sp and Gh formation using an array of oxidants, including the well-defined Ir(IV) system, chromate reduction with bio-available ascorbate or glutathione, and model high-valent Cr(V) complexes. This work was also designed to study the impact of substrate complexity on lesion formation and diastereomeric preference from oxidation of nucleosides to single-stranded and doublestranded oligonucleotides.

Of the oxidative systems used, Ir(IV), Cr(V)-salen, and Cr(V)-ehba are clearly more efficient at producing Sp and Gh. Oxidizing reactions involving chromate and the endogenous reductants ascorbate or glutathione are ratio-dependent, and were chosen for their cellular relevance as opposed to their oxidative efficiency. Despite this fact, the Cr⁶⁺/ascorbate and Cr⁶⁺/glutathione systems still produced an appreciable amount of Sp and Gh from the available 8oxoG under these conditions.

The 8oxoG nucleoside presented the fewest steric constraints to oxidation, with free rotation possible about the chiral glycosidic bond allowing different oxidant interactions without neighboring bases to impose spatial/rotational constraints. As a result, the Sp diastereomers are formed in equal amounts with Ir(IV) and Cr⁶⁺/glutathione oxidation. However, diastereoselectivity is observed with all other oxidants and for Gh formation, suggesting that either the nucleoside itself influences formation or that the interaction of a given oxidant with the base exhibits some level of stereoselectivity. The variable preference for Sp based on oxidant supports the latter rationale, while the consistent Gh2 preference over Gh1 supports the former. Previous work has shown that the model Cr(V) complexes, Cr(V)-salen and Cr(V)-ehba, form Sp via a two-electron oxo-atom transfer mechanism (37) which differs from the one-electron oxidation mechanism determined for Ir(IV) (14). Based on the above results it is appealing to hypothesize that the Cr⁶⁺/ascorbate system undergoes a similar two-electron mechanism while the Cr⁶⁺/glutathione system involves a one-electron oxidation similar to that of Ir(IV).

A single-stranded 22-mer oligonucleotide containing 8oxoG was used as an oxidation substrate to define product outcomes given more moderate steric restrictions. Diastereomeric selectivity for Gh is consistent across the set as in the nucleoside oxidations, but shows a slight preference for Gh1 instead of Gh2. The preference for Sp is again oxidant dependent, but the magnitude of preference was increased greatly. S-Sp is preferred approximately two times over R-Sp with Cr(V)-ehba, Cr⁶⁺/ascorbate, and Cr⁶⁺/glutathione oxidation, while R-Sp is preferentially formed almost four times over S-Sp with Ir(IV) and Cr(V)-salen oxidation. The marked differences between oxidants suggested that the mechanism by which Sp was formed was highly oxidant-dependent, with all interacting in a stereoselective manner.

A double-stranded oligonucleotide containing 8oxoG was used to represent the most sterically-restrictive substrate, as well as the one with the most biological relevance. Oxidation of this substrate had a wide range of results, all being skewed toward preference of one lesion or the other. Only Gh was formed by Ir(IV) and Cr(V)-ehba, with particularly high levels resulting from Ir(IV) oxidation. Sp was more abundant in Cr(V)-salen oxidation, with the amount of R-Sp being nearly ten-fold the amount of S-Sp formed. Formation of Sp over Gh was also seen with Cr⁶⁺/ascorbate and Cr⁶⁺/glutathione oxidation, with R-Sp being produced in greater amounts than S-Sp in both situations.

Several studies have been done examining the importance of Sp diastereomers, both experimentally and computationally. Both diastereomers were found to be highly mutagenic, but they formed different ratios of G→C and G→T mutations (18). One was also cleaved out of DNA by hNEIL1 at twice the rate of the other (24). Computational studies showed that S-Sp formed more favorable interactions in the hNEIL1 active site (23) and, while the authors were unsure of absolute configuration in the experimental work, they state the conclusion that S-Sp being the more highly repaired lesion is an appealing one. The preference for formation of R-Sp in a double-stranded substrate, sometimes at levels ten times that of S-Sp formation, suggested that the less duplex DNA distorting R-Sp diastereomer was the thermodynamically favored product. This result would also fit with the computational findings that R-Sp was less disruptive to base-pair stacking and van der Waals forces than S-Sp (21). While R-Sp was preferentially formed, it was thought to be the diastereomer that was less easily repaired, which would suggest that this diastereoselectivity may cause enhanced mutagenic effects via chromate oxidation.

Of the identified further-oxidized lesions of 8oxoG, Gh has had relatively little study due to difficulty in isolation and isomer identification. However, these studies show it to be formed readily from 8oxoG in double-stranded oligonucleotides, with Ir(IV) and Cr(V)-ehba forming Gh in much greater amounts than Sp in the double-stranded oligonucleotide. Some diastereomeric preference was observed, but it was often slight. Gh was also created at a constant level between substrates by Cr(V)-ehba, and oxidation with Ir(IV) showed a distinct increase in Gh formation from the nucleoside level to the double-stranded substrate. This increase may result from the fact that the flexible guanidinium group of Gh was more readily accommodated within the double-stranded DNA and thus less sterically restrictive with respect to diastereomer formation than Sp. Gh has been shown to be as mutagenic as Sp and nearly eight times as easy to bypass by DNA polymerases (18). For these reasons, Gh formation could arguably play a more important role in chromate-induced mutation than Sp.

Conclusions

While Sp and Gh have been examined as lesions arising from a number of different oxidants, little or no work has been done to characterize the formation of these lesion with respect to their relative preference of formation, much less their diastereoselectivity. This study yields important insight into a possible mechanism of chromate mutagenicity, emphasizes the importance of the less-disruptive (and potentially less readily repaired) R-Sp diastereomer and suggest that the lesser studied Gh diastereomers may also have an important role in chromate-induced cancer. The detection of these lesions in eukaryotic cells and tissues, with attention to diastereomeric preference, is the next logical step, and will help to define the prevalence and impact of these lesions as well as their potential for cancer initiation from chromate exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

8oxoG	8-oxo-7,8-dihydroguanine
Sp	spiroiminodihydantoin
Gh	guanidinohydantoin
BER	base excision repair
SRM	selected reaction monitoring
Cr(V)-ehba	bis(2-ethyl-2-hydroxybutyrate)oxochromate(V)
LC-ESI-MS/MS	high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry
Cr(salen)	N,N'-ethylene-bis-(salicylideneamino)-oxochromium(V)

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S-Gh

R-Gh

S-Sp

R-Sp

Figure 1.
Free base models of the Gh and Sp enantiomers.

Figure 2.

Typical LC-ESI-MS/MS SRM chromatogram. Samples were separated on a Thermo Scientific Hypercarb column with dimensions 100 × 2.1 mm. The first 2.5 minutes are diverted to waste, followed by Gh monitoring (m/z 274→158) between 2.5 and 7 minutes followed by Sp monitoring (m/z 300→184) from 7 to 20 minutes.

Figure 3.

Formation of Sp and Gh diastereomers by the oxidation of the 8oxoG nucleoside. Background oxidation has been subtracted from all oxidation results and error bars are sample standard deviations of at least N=3.

Figure 4. Formation of Sp and Gh diastereomers by the oxidation of an 8oxoG-containing single stranded oligonucleotide. Background oxidation has been subtracted and error bars are sample standard deviations from a minimum of N=3 replicates.

Figure 5.

Formation of Sp and Gh diastereomers by the oxidation of an 8oxoG-containing double-stranded oligonucleotide (paired opposite cytosine). Background oxidation has been subtracted and error bars are sample standard deviations from a minimum of N=3 replicates except where noted in results section.

Figure 6. Total Gh formation by oxidant and substrate complexity. Oxidized samples have had background lesion levels subtracted prior to comparison.

Figure 7.
Total Sp formation by oxidant and substrate complexity. Oxidized samples have had background lesion levels subtracted prior to comparison.

Table 1

ESI-Ion trap MS parameter settings optimized for Sp and Gh analysis.

Parameter	Setting
Capillary	−3650 V
Skimmer	26.33 V
Cap exit	50.00 V
Oct 1 DC	7.46 V
Oct 2 DC	1.47 V
Trap Drive	32.14 V
Oct RF	179.17 Vpp
Lens 1	−3.00 V
Lens 2	−100 V
Fragmentation amplitude	0.83
SmartFrag	Off
Averages	2
Rolling averages	2