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The Non-Bulky DNA Lesions Spiroiminodihydantoin and 5-Guanidinohydantoin Significantly Block Human RNA Polymerase II Elongation *in vitro*

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Abstract

The most common, oxidatively generated lesion in cellular DNA is 8-oxo-7,8-dihydroguanine, which can be oxidized further to yield highly mutagenic spiroiminodihydantoin (Sp) and 5-guanidinohydantoin (Gh) in DNA. In human cell-free extracts, both lesions can be excised by base excision repair and global genomic nucleotide excision repair. However, it is not known if these lesions can be removed by transcription-coupled DNA repair (TCR), a pathway that clears lesions from DNA that impede RNA synthesis. To determine if Sp or Gh impede transcription, which could make them viable substrates for TCR, either an Sp or a Gh lesion was positioned on the transcribed strand of DNA under the control of a promoter that supports transcription by human RNA polymerase II. These constructs were incubated in HeLa nuclear extracts that contained active RNA polymerase II, and the resulting transcripts were resolved by denaturing polyacrylamide gel electrophoresis. The structurally rigid Sp strongly blocks transcription elongation, permitting nominal lesion bypass of $1.6 \pm 0.5\%$. In contrast, the conformationally flexible Gh poses less of a block to human RNAPII, allowing $9 \pm 2\%$ bypass. Furthermore, fractional lesion bypass for Sp and Gh is minimally affected by glycosylase activity found in the HeLa nuclear extract. These data specifically suggest that both Sp and Gh may well be susceptible to TCR since each poses a significant block to human RNA polymerase II progression. A more

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00295. HPLC elution profiles, MALDI-TOF mass spectra, and CD spectra of site-specifically modified 11-mer oligonucleotides containing *R* and *S* diastereomeric Sp and Gh lesions and analysis of the incisions in 147 bp DNA substrates containing *S*-Sp and Gh lesions after incubation in HeLaScribe Nuclear Extract (Figures S1–S3) (PDF)

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Notes

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general principle is also proposed: Conformational flexibility may be an important structural feature of DNA lesions that enhances their transcriptional bypass.

Graphical abstract

INTRODUCTION

Extracellular and intracellular chemical agents as well as various forms of radiation jeopardize the integrity of cellular genomes by inducing damage to DNA.^{1,2} The resulting alterations to the genetic material can include single-strand and double-strand breaks, and chemical modifications to the bases, sugars and phosphate groups.^{3–5} If the damage were to remain in the genome, fundamental cellular processes that rely on the chemical information in DNA, including replication and transcription, would be severely compromised. To prevent DNA damage from accumulating, cells have evolved a host of DNA repair pathways—sometimes referred to as genome maintenance mechanisms—that detect and repair DNA damage.^{6–8} Indeed, compromised genome maintenance can lead to developmental defects, cancer and other adverse consequences to organisms, including humans.

The effect of DNA damage on replication has been well characterized, and mutational spectra for various types of DNA lesions have been reported.^{9–11} In recent years, the relative effects of different lesions on the stalling of bacteriophage, prokaryotic and eukaryotic RNA polymerases have been extensively studied using *in vitro* transcription assays.¹² In the majority of cases, DNA damage poses strong blocks to the progression of transcription complexes, with bypass occurring infrequently in most cases. Indeed, the pausing or stalling of RNA polymerases at the sites of the lesions is the first step in their subsequent removal by a genome maintenance pathway called transcription-coupled DNA repair (TCR).⁷ Furthermore, when lesion bypass does occur during transcription, the nucleotide sequences of the resulting transcripts are often altered, with base misincorporations, deletions and insertions occurring in a process that has been called transcriptional mutagenesis.^{12–16}

As mentioned, most DNA lesions impede the progress of elongating RNA polymerases, but they do so to varying extents. The relative lesion bypass during transcription, which is defined as the fraction of full-length transcripts relative to the sum of extended and unextended transcripts, depends strongly on the chemical nature of the DNA lesion and the particular RNA polymerase being studied. Typically, lesion bypass during transcription

occurs in a manner that depends on the lesion's chemical structure, size and shape¹⁴ that in turn govern how it is accommodated in the enzyme's active site.^{17, 18} In addition, the presence of accessory proteins that interact with the RNA polymerase elongation complex plays a significant role in the bypass of some lesions.^{19, 20}

There is little doubt that reactive oxygen species (ROS), which are typically overproduced by macrophages and neutrophils in chronically inflamed tissues, play a significant role in damaging DNA. Indeed, guanine is the most easily oxidized natural base in DNA²¹ and is the primary target of ROS.¹ Perhaps the best studied oxidized guanine lesion is 8-oxo-7,8-dihydroguanine (8-oxoG), which is ubiquitous in cellular DNA²² and is mutagenic,²³ yielding 1–5% G to T transversion mutations upon replication in wild-type *E. coli*.^{24, 25} Furthermore, 8-oxoG is even more prone to oxidation than the parent guanine,²⁶ and thus a spectrum of deeper oxidation products of guanine have been identified that include diastereomeric spiroiminodihydantoin (Sp), 5-guanidinohydantoin (Gh)^{27–36} and 5-guanidino-4-nitroimidazole (NIm) (Figure 1).^{37, 38} These deeper oxidation products induce mutations following replication, with Sp and Gh being at least one order of magnitude more mutagenic than 8-oxoG.^{24, 25} It is worth noting that Sp and Gh have been detected in DNA in a mouse model of infection-induced colitis, and their cellular levels have been correlated with the progression of this potentially serious condition that can lead to the initiation of colon cancer.³⁹

Little is known about the effects of the deeper oxidation products of guanine on transcription. Earlier experiments demonstrated that in standard, multiple-round transcription experiments, bacteriophage T7 RNA polymerase (T7RNAP) elongation was only mildly inhibited by a site-specifically positioned NIm lesion, while human RNA polymerase II (RNAPII) elongation was strongly inhibited under similar conditions.¹⁷ These results are consistent with the known structural properties of these two polymerases since T7RNAP contains a more open active site than human RNAPII.^{40, 41} It has been proposed that the RNA polymerase bypass of NIm may well be favored because of the lesion's intrinsic torsional flexibility.¹⁷

In the work presented here, the relationship between the structural characteristics of Sp and Gh and their effects on human RNAPII elongation was studied. The Sp and Gh lesions possess one chiral carbon and can exist as a pair of structurally rigid *R* and *S* diastereomers. In aqueous solutions, the *S*-Sp and *R*-Sp diastereomers can be purified and separated from one another,^{27, 42} and for this work, the *S*-Sp isomer was selected. In contrast, the analogous *S*-Gh and *R*-Gh diastereomers are easily interconvertible, making it unfeasible to study their individual characteristics.^{43, 44} Note that like NIm, the Gh lesion exhibits torsional flexibility⁴⁵ around the four C–N bonds in addition to the glycosidic bond; in contrast, the *S*-Sp residue is rigid (Figure 1). Based on prior results obtained by investigating T7RNAP and human RNAPII transcription past the torsion-flexible NIm lesion, it was predicted that the structurally rigid *S*-Sp should pose a strong, if not complete, block to human RNAPII elongation, whereas the flexible Gh lesion should permit a certain degree of transcriptional bypass similar to that observed in the case of NIm.¹⁷ As reported here, this is indeed the case, supporting further the notion that DNA lesions that have a more flexible structure pose less of a barrier to transcription, perhaps making them greater contributors to transcriptional

mutagenesis and less susceptible to clearance by TCR. These results are discussed in the context of other types of DNA lesions and their impact on transcription.

MATERIALS AND METHODS

Synthesis of *S*-Sp- and Gh-Modified DNA Duplexes

The oligodeoxynucleotides containing the diastereomeric *S*-Sp or *R*-Sp lesions were generated by oxidation of guanine in the 5'-CCATCGCTACC-3' with photochemically generated carbonate radical anions at pH 7.5 to 8.0 as described previously^{34–36} (for more details, see the Supporting Information). The *S*-Sp and *R*-Sp adducts, which are stable and do not interconvert into one another, were purified by anion-exchange HPLC techniques.^{30, 46} The stereochemistry assignments of the Sp diastereomers proposed earlier⁴⁷ were revised by Fleming et al.,⁴⁸ and were adopted here as described in Figure S1 (Supporting Information). The oligonucleotides containing single diastereomeric *S*-Gh or *R*-Gh lesions, were prepared by the oxidation of 8-oxoG embedded in the oligonucleotide 5'-CCATC[8-oxoG]CTACC-3' with (NH₄)₂IrCl₆ at pH 6.0 as described elsewhere.^{30, 31} (for more details, see the Supporting Information). According to Burrows and co-workers^{43, 44, 49} these Gh adducts are mixtures of *R*-Gh and *S*-Gh diastereomers that are in equilibrium with one another (Figure S2, Supporting Information) and, to a pH-dependent extent, with their constitutional isomer iminoallontoin.⁴⁹

The *S*-Sp-modified and Gh-modified oligodeoxynucleotides as well as the unmodified control 11-mer 5'-CCATCGCTACC-3' were phosphorylated at the 5'-ends using USB OptiKinase™ (Affymetrix) according to the manufacturer's protocol. Each phosphorylated oligomer was mixed with an equimolar amount of the 90-mer 3'-CACGACATGAGTCCACACCTTAGTTGGGTGTCGACTGTCCCGTCCAGAACCGGTC AACCTATAGGTTTTGTAGAACAACCTTTTTTTTTT-5'-biotin and a 30% molar excess of the 22-mer 5'-GGTAGCGATGGGTGCTGTACTC-3', heated to 80 °C, and cooled slowly overnight to room temperature to facilitate annealing (Figure 2A). The annealed DNA fragments were ligated with T4 DNA Ligase (Affymetrix) at 16 °C for 16 h, resulting in the formation of a 101-mer from the 11-mer containing a DNA lesion and the biotinylated 90-mer. After ligation, the resulting 101-mer oligodeoxynucleotides were purified by denaturing polyacrylamide gel electrophoresis (PAGE), isolated by standard ethanol precipitation, and then annealed to the 96-mer 5'-TTGCGGTAGCGATGGGTGCTGTACTCAGGTGTGGAATCAACCCACAGCTGACAGG GCAGGTCTTGCCAGTTGGGATATCCAAAACATCTTGTTGA-3' that was phosphorylated at its 5'-end using USB OptiKinase™ (Figure 2A). Note that in all cases, the *S*-Sp, Gh or guanine in the 101-mer was situated opposite cytosine in the 96-mer.

Synthesis of DNA Templates for Transcription by Human RNAPII

The DNA duplexes constructed from the 101-mer containing *S*-Sp, Gh or guanine annealed to a complementary 96-mer were used to synthesize DNA templates that could support transcription by human RNAPII (Figure 2B)^{50, 51} In brief, a plasmid containing the cytomegalovirus (CMV) immediate-early promoter/enhancer element that supports human RNAPII transcription was cut with restriction enzyme BbsI (New England BioLabs). The

linearized plasmid and the 101/96-mer duplex were incubated with T4 DNA ligase at 16 °C for 16 h (Figure 2B). The full-length, ligated products were isolated using Streptavidin MagneSphere® Paramagnetic Particles (Promega Corporation) that bound to the biotin tag. The product bound to the paramagnetic particles was digested with BglIII (New England BioLabs) to remove excess portions of the plasmid not needed for *in vitro* transcription. The linear template was then detached from the particles by digestion with EcoRV (New England BioLabs), purified by agarose gel electrophoresis and extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen).

***In vitro* Transcription**

The transcription reactions were performed using human RNAPII present in HeLaScribe® Nuclear Extract in vitro Transcription System (Promega Corporation).^{50–52} Reactions were carried out with 50 fmol of template in transcription buffer supplied with the HeLaScribe® system and supplemented with 400 µM each of ATP, GTP and UTP, 16 µM [α -³²P]CTP (~25 Ci/mmol) (PerkinElmer Inc.) and 8 units of HeLaScribe® Nuclear Extract. The mixture was incubated at 30 °C for 60 min and quenched with HeLa Extract Stop Solution. The samples were extracted with phenol/chloroform and precipitated with ethanol. The nucleic acid pellet was re-suspended in nuclease-free water, and the products were resolved by 7% denaturing PAGE in 8 M urea dissolved in TBE (8.9 mM Tris-borate, 0.2 mM EDTA (pH 8.0)). The resulting gels were dried and analyzed using a Typhoon™ FLA 9000 (GE Healthcare Life Sciences) with densitometric traces generated from the autoradiographs using ImageQuant™ TL (GE Healthcare Life Sciences).

Determination of DNA Repair activity in HeLaScribe® Nuclear Extract

The DNA templates were incubated with HeLaScribe® Nuclear Extract as described in the previous section, except that radioactive [α -³²P]CTP was replaced by CTP.⁵³ After 60 min, transcription was arrested, the reaction mixtures were extracted with phenol/chloroform, and nucleic acids were precipitated with ethanol. The pellets were re-suspended in nuclease-free water, and the nucleic acids were treated with I-PpoI (Promega Corporation). The resulting products were divided into two portions. Nucleic acids in the first portion were radiolabeled at their 5'-ends with [³²P]phosphate via an exchange reaction in the presence of USB OptiKinase™ and [γ -³²P]ATP according to the standard protocol.^{50, 51} The second portion was treated with Fpg (New England BioLabs), and then the resulting nucleic acids were radiolabeled at their 5'-ends with [³²P]phosphate also via the exchange reaction. It is important to note that the exchange reaction radiolabels all nucleic acids present, including RNA.

RESULTS

Characterization of Modified DNA Templates for Human RNAPII Transcription

The use of site-specifically modified DNA templates to study transcription is predicated on two notions. Firstly, the DNA cannot contain single-strand nicks in the backbone that could interfere with transcription and be misconstrued as DNA lesions. Secondly, each DNA template must be homogeneous for damage and not contain any unmodified, normal DNA that would confound the results.

The site-specifically modified templates containing either *S*-Sp or Gh and the undamaged control template were tested for the absence of single-strand nicks in the DNA backbone that could result from incomplete ligation during template preparation. In order to verify the absence of nicks of the double-strand DNA templates, each was digested with I-PpoI and the resulting products were radiolabeled at their 5'-ends with [³²P]phosphate via an exchange reaction.^{50, 51} In the case of the DNA templates used in these studies, I-PpoI digestion should produce single-strand DNA fragments 187 (top strand) and 191 (bottom strand) nucleotides in length when ligation goes to completion (Figure 3A). In contrast, incomplete ligation would result in the presence of fragments that are 110 (from top strand) and 118 nucleotides in length (from bottom strand).

Following I-PpoI analysis of the undamaged control DNA template, no bands were observed that approximated 110 or 118 nucleotides in length, but the expected bands of length 187 and 191 nucleotides were observed (Figure 3B, Lane UM). Similar results were observed for I-PpoI analyses of the DNA templates containing either Gh (Figure 3B, Lane Gh) or *S*-Sp (Figure 3B, Lane *S*-Sp). These results indicate that no detectable single-strand nicks were present in the undamaged control DNA template or in those DNA templates in which either Gh or *S*-Sp was present, showing that ligation was complete within the sensitivity of the assay.

Following the demonstration that no detectable nicks were observed in the DNA, it was essential to test for the presence of the lesions in their respective templates. Both *S*-Sp and Gh are substrates for the base excision repair enzyme formamidopyrimidine-DNA glycosylase (Fpg).⁵⁴ This bifunctional glycosylase excises Sp and Gh residues, thus resulting in the formation of abasic sites. Fpg also has an abasic-lyase activity that subsequently cleaves the backbone of DNA at abasic sites, yielding a nick in the DNA strand at the original position of the damaged purine.^{54, 55} Since Fpg efficiently excises *S*-Sp and Gh, this enzyme was used to confirm the presence of these lesions in the purified DNA templates.

In brief, the digestion products generated by I-PpoI treatment were incubated with Fpg to cleave the oligonucleotides selectively at the sites of the *S*-Sp or Gh lesion when present, thus yielding DNA fragments 67 and 123 nucleotides in length, originating from the bottom strand as shown in Figure 3A, and a 187 nucleotide fragment, as originating from the top strand as shown in Figure 3A. In the absence of DNA damage, only oligonucleotides 191 and 187 bases in length should be observed following the addition of Fpg. The DNA fragments derived from consecutive treatment of DNA templates containing I-PpoI and Fpg were radiolabeled at their 5'-ends with [³²P]phosphate via an exchange reaction,^{50, 51} resolved by denaturing PAGE and analyzed as described above for the I-PpoI protocol. The results are shown in Figure 3C. When the DNA templates contained Gh or *S*-Sp, DNA 191 nucleotides in length was not detected following I-PpoI and Fpg treatment, while DNA fragments 67, 123 and 187 nucleotides in length were observed, indicating that the Gh or *S*-Sp lesions were present (Figure 3C, Lanes Gh and *S*-Sp). As, expected, the control DNA duplexes were not susceptible to cleavage by Fpg; therefore, only the 187 and 191 base oligonucleotides were observed after exposure to Fpg (Figure 3C, Lane UM).

***In vitro* RNAPII Transcription of DNA Templates Containing Either Gh or S-Sp**

After the integrity of the DNA templates was confirmed, *in vitro* transcription reactions were performed to test the impact of Gh or S-Sp on RNA synthesis catalyzed by human RNAPII. The HeLaScribe® Nuclear Extract *in vitro* Transcription System was used as the source of human RNAPII and other essential transcription factors. Unmodified control DNA templates were used as controls.

A DNA template supplied with the HeLaScribe® Nuclear Extract *in vitro* Transcription System was used to demonstrate that the HeLaScribe® Nuclear Extract was indeed transcription-active. This template encodes a run-off transcript 363 nucleotides in length. As shown in Figure 4, Lane 16, an intense band of that size was observed. In the absence of human RNAPII, NTPs, or the control DNA template supplied with the kit, the run-off transcript was not observed (Figure 4, Lanes 13–15).

Transcription of the unmodified, control DNA template should yield a run-off transcript 384 nucleotides in length, which was indeed the case as shown in Figure 4, Lane 4. Such products were not observed when either the HeLaScribe® Extract, NTPs or the DNA template was omitted from the incubation mixture (Figure 4, Lanes 1–3). In contrast to the unmodified control DNA, transcription of the templates containing Gh or S-Sp should result in the formation of a truncated transcript 317 nucleotides in length if these lesions were to block human RNAPII progression. The Gh and S-Sp lesions are indeed strong blocks to transcription since RNA approximating 317 bases in length was formed (Figure 4, Lanes 8 and 12), while the yield of run-off RNA was quite low. Note that no intermediate bands between the 317-mer and 384-mer RNA transcripts were evident. Furthermore, as in the case of the control template, omission of the HeLaScribe® Extract, NTPs, or a DNA template resulted in no observable RNA of the expected lengths (Figure 4, Lanes 5–7 and 9–11).

A quantitative analysis of the autoradiographs indicated that the yield of run-off transcript was in excess of 99% in the case of the unmodified template. In contrast, the yield of run-off transcript using templates with Gh during transcription was $9 \pm 2\%$ and using templates with S-Sp during transcription was $1.6 \pm 0.5\%$ (Table 1). The results reflect the average of three independent experiments. The previously measured value for transcription past NIm of $9 \pm 5\%$ is close to the Gh value, which is likely a consequence of the similarities in their structures and conformational flexibilities.¹⁷

Assessing the potential impact of DNA repair on the results of the transcription assays

In principle, repair mechanisms such as base excision repair (BER) and nucleotide excision repair (NER) could have an impact on the results of our transcription assays. However, our investigations indicate that the HeLaScribe® Nuclear Extracts are not NER-competent (Supporting Information). However, the HeLaScribe® Nuclear Extracts do exhibit some BER activity as described below. Glycosylase-catalyzed removal of the damage without subsequent complete repair can generate nicks or single-base deletions in the DNA templates that could also impede elongation by human RNAPII.^{15, 53} Note, however, that removal of the damage followed by re-synthesis of DNA and subsequent ligation would

complete a cycle of BER, resulting in a DNA template equivalent to the undamaged control DNA that would permit the synthesis of run-off transcripts.

To determine if glycosylase activity in the HeLaScribe[®] Nuclear Extract removes *S*-Sp or Gh, DNA templates containing either of these lesions were incubated with these nuclear extracts. Glycosylase activity without subsequent repair would result in the formation of oligodeoxynucleotides 67, 123 and 187 bases in length following I-PpoI digestion. The absence of glycosylase activity or complete cycles of BER would result in oligodeoxynucleotides 187 and 191 bases in length following I-PpoI digestion. The results are shown in Figure 5. Incubation of the *S*-Sp (Figure 5, Lane 3) or Gh (Figure 5, Lane 4) DNA templates with HeLaScribe[®] Nuclear Extract generates demonstrable but nominal amounts of oligodeoxynucleotides that are 67 and 123 bases in length, results that are consistent with glycosylase-induced strand cleavage at the sites of the lesions.

These incision products are not observed in the case of the unmodified template (Figure 5, Lane 1). Note that the 187/191 fragments associated with the intact templates as illustrated in Figure 3A are not resolved in this experiment and appear after incubation with HeLaScribe[®] Nuclear Extract as a single band following I-PpoI digestion of DNA templates containing either *S*-Sp (Figure 5, Lane 3) Gh (Figure 5, Lane 4) or unmodified guanine (Figure 5, Lane 1). The yields of the glycosylase-induced cleavage products were approximately 10% for the *S*-Sp template and 15% for the Gh template as estimated from the histograms obtained by scanning the autoradiograph in Figure 5. These results indicate that the consequences of glycosylase activity present in the HeLaScribe[®] Nuclear Extract exert a minimal effect on generating single-strand breaks or one-base deletions.^{15, 53} Note that single-strand breaks pose strong but not absolute blocks to RNAPII elongation in HeLa extracts.⁵³ Hence, these repair intermediates could result in the production of truncated transcripts that block human RNAPII progression or could result in altered transcripts when human RNAPII bypasses them. Finally, complete repair of *S*-Sp or Gh by either BER or NER could affect these values by generating repaired DNA templates that would permit transcription bypass, but such events are indeed insignificant (see Supporting Information).

DISCUSSION

Both *S*-Sp and Gh are deeper oxidation products of guanine that are more mutagenic than the well-investigated lesion 8-oxoG from which they arise.^{23–25} It was shown previously that *S*-Sp and Gh are repaired by both NER and BER.⁵⁶ However, the role of other genome maintenance pathways, especially TCR, remains to be elucidated. The data reported here indicate that *S*-Sp and Gh strongly impede human RNAPII elongation *in vitro*. The limited but demonstrable bypass of each of these lesions by human RNAPII suggests that they could contribute to transcriptional mutagenesis⁹² if they were to persist in the human genome, an event that can alter the nucleotide sequence of RNA and compromise its function.^{7, 13, 15} Glycosylase activity contributes only slightly to the formation of intermediate nicks or deletions in the templates that could also be responsible for stalling human RNAPII transcription *in vitro* or contribute to bypass. In contrast, NER activity is completely absent from the *in vitro* system used; hence, there is no contribution from this pathway to the stalling or bypass of human RNAPII in the experiments reported here.

To place the results with *S*-Sp and Gh in perspective, it is of interest to compare the impact of other DNA lesions on the transcriptional activity of human RNAPII. Most DNA lesions studied thus far exert strong blocks to transcription elongation by human RNAPII that allow variable but limited bypass, and the *S*-Sp and Gh lesions behave similarly.^{13, 15} A basic hypothesis of this project is that the structurally rigid *S*-Sp would pose a stronger block to human RNAPII elongation than the conformationally more flexible Gh and NIm lesions, which is the case (Table 1). The extent of full-length, run-off transcript formation is similar, within experimental error, in the cases of the relatively flexible Gh and NIm lesions, and strongly inhibited by the rigid *S*-Sp lesion. By contrast, other lesions such as uracil and a synthetic tetrahydrofuran apurinic/apyrimidinic (AP) site do not block RNAPII,⁵⁷ although natural AP sites derived from the deglycosylation of uracil are known to stall RNAPII *in vitro*^{58, 59} and in human cells.⁶⁰

At the genomic level, the strand-specific TCR mechanism can give rise to an inverse correlation between the expression of a given gene and an asymmetric distribution of mutation densities on the transcribed and non-transcribed strands; this strand bias results in higher mutation load on the non-transcribed strand that can have adverse impact on its accurate replication by DNA polymerases.⁶¹ Since *S*-Sp and Gh can arise from the further oxidation of 8-oxoG, it is of interest to recall its impact on transcription and TCR. Importantly, 8-oxoG induces pausing or stalling of mammalian RNAPII, ranging from 30% to 50% bypass.¹⁹ In contrast, other *in vitro* studies showed that 8-oxoG is readily bypassed by mammalian RNAPII *in vitro*.^{53, 62, 63} Further investigations have shown that the bypass of 8-oxoG by RNAPII depends on base sequence context^{12, 64–66} and the presence of elongation factors such as TFIIIS, elongin or CSB.^{19, 20}

It has been reported that in mammalian cells, the removal of 8-oxoG does not exhibit any significant strand bias between the transcribed and non-transcribed strands of an active transcription unit.^{64, 66} On the other hand, Guo et al.⁶⁷ provided direct evidence that 8-oxoG positioned in the transcribed strand of an actively transcribing gene in human fibroblasts is preferentially repaired more efficiently than in the non-transcribed strand. It has been proposed that such strand bias could occur in human cells by the removal of 8-oxoG by the glycosylase hOGG1 that creates an abasic site that would stall RNAPII, thus creating an OGG1-dependent TCR strand bias.⁶⁰

For comparison, the impact of other DNA lesions, including oxidized bases and DNA cross-links on transcription reported in the literature are summarized in Table 1.

Another product of oxidation of DNA, 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol), does not act as a strong block to rat liver RNAPII⁶⁸ or human RNAPII during transcription.⁵³ However, other studies have reported that human RNAPII partially stalls at thymine glycol lesions²⁰ or bypasses them with approximately 35% efficiency.¹⁹ On the other hand, 5-hydroxymethylcytosine weakly blocks rat liver RNAPII (90% bypass) relative to cytosine and 5-methylcytosine, while its oxidation products 5-formylcytosine and 5-carboxylcytosine induce transient pausing of yeast RNAPII, yielding bypass products with approximately 40% efficiency.^{16, 69} The 5-carboxylcytosine and 5-formylcytosine are endogenous cytosine modifications that are produced during the demethylation of the 5-methylcytosine epigenetic

mark by the demethylation pathway catalyzed by TET dioxygenases^{70–73} Furthermore, it has been shown that the bypass of such lesions catalyzed by rat liver RNAPII is about 40% lower in both cases than for the unmodified template that contains cytosine.⁶⁹

Relatively non-bulky, alkylated bases have also been investigated for their effects on transcription elongation. *N*3-, *O*²- and *O*⁴-ethylthymidine derivatives are bypassed by human RNAPII present in HeLa cell extracts with a 40% to 60% efficiency,⁷⁴ while carboxymethylated *N*3- and *O*⁴-thymine derivatives are bypassed with 20% to 25% efficiency.¹⁸

Crosslinks in DNA have also been investigated for their impact on transcription elongation. The highly mutagenic intranucleotide cross-linked cyclopurine DNA lesions 5',8-cyclo-2'-deoxyadenosine (cdA) and 5',8-cyclo-2'-deoxyguanosine (cdG) are generated by the reactions of hydroxyl radicals with purines in DNA.⁷⁵ Unlike some of the other guanine oxidation products, the S-cdA and S-cdG lesions are very strong blocks of human RNAPII since they are weakly bypassed at less than 1% *in vitro*.⁷⁶ Yeast and calf thymus RNAPII are also inefficient in bypassing these lesions.⁷⁷ Malonaldehyde is a lipid peroxidation product that reacts with guanine in DNA to form an unsaturated six-membered exocyclic ring bonded with *N*²- and *N*3-guanine sites (M₁dG, pyrimido[1,2- α]purin-[10(3*H*)-one). While this lesion is more bulky than the cdA, cdG, Sp, Gh, and NIm lesions, M₁dG is bypassed with moderate efficiency since the yield of full-length transcripts generated by rat RNAPII is 30% to 40%.⁷⁸

Additional cross-linked lesions have been studied. The DNA structure-distorting UV-induced cyclobutane pyrimidine dimers (CPD) and 6–4 photoproducts (6,4 pyrimidine-pyrimidones) are intrastrand cross-linked DNA lesions that act as strong blocks to RNAPII elongation.^{79, 80} The cisplatin-derived DNA lesions are also strong blocks of transcription catalyzed by RNAPII and other RNA polymerases,^{81, 82} although 10% to 25% full-length transcript formation has been reported in the case of some cisplatin derived lesions.^{19, 83} The monofunctional pyriplatin adduct is also a dominantly stalling lesion.⁸⁴

The fact that many lesions in DNA act as impediments to transcription elongation as elucidated above is incontestable. However, the underlying structural features of a DNA lesion that cause it to impede RNA polymerase progression or permit bypass that could result in transcriptional mutagenesis are less clear. Consider NIm, for example. Prior results have shown that NIm blocks human RNAPII and permits approximately 9% bypass.¹⁷ Transcription stopped immediately prior to the adduct, and computer modeling suggests that the majority of the *syn* and *anti* conformations can cause steric collisions between it and amino acids in the transcription complex's active site. In fact, only one collision-free *anti* conformation could be found, and it permitted three hydrogen bonds to form between the lesion and an incoming CTP, which offers an explanation for why full-length transcripts that were produced contained the cytosine at the position opposite the ring-open purine.

In recent years, literature has begun to accumulate that provides crystal structures of eukaryotic RNAPII with DNA templates that contain lesions. These structures reveal the complexity of blocking and bypass effects imposed on the polymerase by different DNA

modifications that emphasize both the uniqueness of each lesion's structural effects as well as important commonalities.^{7, 85, 86} For example, steric effects of a DNA lesion are prominent causes of blockage to transcription elongation⁷ because they inhibit entry of the damaged base at or adjacent to the polymerase's active site and also prevent translocation. In some cases, however, RNA polymerases can bypass a lesion, often by adding adenosine in a manner that echoes the "A rule" for DNA polymerases.⁸⁷ Importantly, the steric effects that thwart RNA polymerase elongation are manifest in all the cross-linked lesion structures, including CPDs,⁸⁸ the cdA⁷⁷ lesions, and the cis-Pt cross-linked adduct,⁸⁹ although the specific entry and translocation blocks, as well as the bypass locations in the polymerase can differ because of the variable chemical structures of these lesions. The strong blocking effects of different cross-links^{73, 75, 76, 78, 79} can be accounted for by the steric restraints of the cross-links.

Another recent, informative structure is that of the 5-carboxycytosine modification positioned in an RNAPII elongation complex.¹⁶ This lesion is significantly bypassed during transcription elongation, although it reduces transcriptional efficiency when compared to cytosine by approximately 40%.⁶⁹ The most fascinating finding of that structural study is the identification of lesion-specific hydrogen bonds between the 5-carboxyl group and a glutamine side chain of the polymerase that is in a DNA recognition loop, termed an "epi" loop; this shifts the incoming nucleotide's position to compromise elongation and stalls translocation in a specific position "above the bridge helix". However, being governed by more fluid hydrogen bonding interactions than the covalent steric constraints of cross-links appears to be consistent with the greater bypass for this case. These structures may offer some molecular insights on the stalling or bypass of the Sp, Gh and Nim lesions that we have studied. The rigid and stable Sp lesion with its two rings strongly covalently angled with respect to one another appears incapable of being housed in the template position of the RNAPII active site; this is reminiscent of the CPD and cdA crosslinks, and would likewise impede translocation if bypassed according to the "A" rule. On the other hand, Gh is inherently flexible because of the three flexible torsion angles (Figure 1) and also exists in equilibrium with its constitutional isomer iminoallantoin in a pH dependent manner at the nucleoside level, as well as in double-stranded DNA.⁴⁹ Therefore, the Gh as well as the NIm¹⁷ lesions, with their multiple hydrogen bonding capabilities and torsional flexibilities,^{17, 45, 90} could engage the RNA polymerase with hydrogen bonds, and thereby impede its progress as in the example of the 5-carboxycytosine. However, disengagement of such hydrogen bonds to permit some elongation could be a plausible mechanism to allow partial bypass that is much less tractable for structurally rigid lesions like cross-links and Sp.

CONCLUSION

The DNA lesions *S*-Sp and Gh that result from the further oxidation of 8-oxoG pose strong impediments to the progression of human RNAPII elongation *in vitro*. In the experimental system used, glycosylase activity generated nicked DNA to a very low extent, which could also contribute nominally to the stalling of human RNAPII *in vitro*. These results suggest that both *S*-Sp and Gh should be subject to TCR in addition to their susceptibilities to NER and BER that have already been documented.⁵⁶ The very limited run-off transcripts that were observed during transcription could be due to bypass of the lesions or the single-strand

breaks formed via glycosylase activity, although the latter strongly block transcription. These results also support the hypothesis that the greater the conformational flexibility of a lesion, the more likely the probability that RNA polymerase bypass can occur, thus limiting the signal that triggers TCR, and potentially resulting in the formation of full-length transcripts that are functionally defective.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BER	base excision repair
NER	nucleotide excision repair
TCR	transcription-coupled DNA repair
RNAPII	RNA polymerase II
T7RNAP	bacteriophage T7 RNA polymerase
Fpg	formamidopyrimidine-DNA glycosylase
8-oxoG	8-oxo-7,8-dihydroguanine
Sp	spiroiminodihydantoin
Gh	5-guanidinohydantoin
NIm	5-guanidino-4-nitroimidazole
thymine glycol	5,6-dihydroxy-5,6-dihydrothymine

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Figure 1.
Structures of the oxidatively generated *S*-Sp, Gh, and NIm lesions.

Figure 2.

(A) Synthesis of site-specifically modified DNA duplexes. 11-mer: 5'-CCATC[X]CTACC, where X = S-Sp, Gh, or G; 90-mer: 5'-biotin-TTTTTTTTTTCAACAAGATGTTTTGGATATCCCAACTGGCCAAGACCTGCCCTGTCAGCTGTGGGTTGATTCCACACCTGAGTACAGCAC; 22-mer: 5'-GGTAGCGATGGGTGCTGTACTC; 96-mer: 5'-TTGCGGTAGCGATGGGTGCTGTACTCAGGTGTGGAATCAACCCACAGCTGACAGG GCAGGTCTTGGCCAGTTGGGATATCCAAAACATCTTGTTGA. (B) Preparation of templates from linearized pCI-neo-G-less vector,⁵⁰ which contains the CMV immediate-

early promoter/enhancer element (yellow) that promotes human RNAPII transcription, and the *S*-Sp/Gh lesions. The DNA template is produced by ligating the linearized pCI-neo-G-less plasmid to the DNA duplex from (A), followed by digestion of the extraneous portions of the plasmid with BglII, and finally the duplex from the paramagnetic beads by digestion with EcoRV. (C) Transcription from the unmodified linear template with hRNAPII results in full-length run-off transcripts 384 nucleotides in length, while in the modified templates transcripts truncated at the site of the lesion are 317 ribonucleotides in length.

Figure 3. Verification of template integrity of unmodified (UM), Gh and *S*-Sp modified linear templates by enzymatic digestion with I-PpoI and Fpg proteins. (A) Representative schematic of enzymatic digests to verify template integrity. I-PpoI digestion is used for verification of complete ligation of the DNA duplex to the CMV promoter fragment. The Fpg assay results in excision of the *S*-Sp and Gh lesions, generating 67 and 123 nucleotide fragments. (B): Representative denaturing gel showing the products of digestion of the linear templates with I-PpoI. (C) Representative denaturing gel showing the products of digestion

first with I-PpoI, followed by treatment with Fpg-protein. Lanes M: oligodeoxynucleotide size markers (5'-[³²P]phosphate-labeled 50 bp Ladder (New England Biolabs)).

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Figure 4. Denaturing PAGE analysis of transcription products of the unmodified, *S*-Sp and Gh linear DNA templates induced by RNAPII after incubation in HeLaScribe® Nuclear Extract (Promega Corporation) for 60 min. Lanes M: oligodeoxynucleotide size markers 5'-[³²P]phosphate-labeled 50 bp Ladder). Lanes 1 – 4: unmodified DNA template. Lanes 5 – 8: Gh template. Lanes 9 – 12: *S*-Sp template. Lanes 13 – 16: control DNA template containing a CMV immediate-early promoter.

Figure 5.

Denaturing PAGE analysis of glycosylase-catalyzed incisions in DNA templates containing *S*-Sp and Gh lesions after incubation in HeLaScribe® Nuclear Extract for 60 min. DNA templates were incubated in HeLaScribe® Nuclear Extract, followed by restriction digestion in the presence of I-PpoI to generate DNA fragments 187 and 191 nucleotides in length. Glycosylase-catalyzed incision during incubation in HeLaScribe® Nuclear Extract resulted in the appearance of DNA 67 and 123 nucleotides in length when a lesion was present on the DNA template (Lanes 1, 3 and 4). Glycosylase-catalyzed incision was carried out to completion in the presence of Fpg (Lanes 2, 5 and 6).

Table 1

Transcriptional Bypass of DNA lesions

lesion	RNA polymerase	bypass, %	refs
Oxidative			
8-oxo-7,8-dihydroguanine (8-oxoG)	hRNAPII	60–100	19, 53, 63
	T7	< 95	63
(<i>S</i>) – spiroiminodihydantoin (<i>S</i> -Sp) *	hRNAPII	1.6 ± 0.5	this work
5-guanidinohydantoin (Gh) *	hRNAPII	9 ± 2	this work
5-guanidino-4-nitroimidazole (NIm) *	hRNAPII	9 ± 5	17
thymine glycol	rat RNAPII	100	68
	hRNAPII	0–35	19
	T7	50	68
5-hydroxymethylcytosine	rat RNAPII	90	16, 69
5-methylcytosine	yeast RNAPII	40	16, 69
5-formylcytosine	yeast RNAPII	40	16, 69
5-carboxylcytosine	rat RNAPII	60	16, 69
Alkylating			
<i>N</i> ³ -ethylthymidine	hRNAPII	40	74
<i>O</i> ² -ethylthymidine	hRNAPII	30–35	74
<i>O</i> ⁴ -ethylthymidine	hRNAPII	60	74
carboxymethylated N3-thymine	hRNAPII	26	91
carboxymethylated O4-thymine	hRNAPII	19	91
Cross-Linking			
(<i>S</i>)-5',8-cyclo-2'-deoxyadenosine (cdA)	yeast, calf	1	77
	hRNAPII	inefficient	76
5',8-cyclo-2'-deoxyguanosine (cdG)	hRNAPII	inefficient	76
M ₁ dG, pyrimido[1,2- α]purin-10(3 <i>H</i>)-one	rat RNAPII	30–40	78
cyclobutane pyrimidine dimer (CPD)	RNAPII	strong	79
6,4-pyrimidine-pyrimidone	RNAPII	strong	80
cisplatin-derived lesion	RNAPII	10–25	81, 82, 84
pyriplatin	yeast RNAPII	predominantly stalling	84

* Standard incubation conditions (60 min) in nuclear extracts from Promega.