Published in final edited form as:

Chem Res Toxicol. 2017 February 20; 30(2): 669–677. doi:10.1021/acs.chemrestox.6b00397.

Mutagenicity of a Model DNA-Peptide Cross-Link in Human Cells: Roles of Translesion Synthesis DNA Polymerases

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Abstract

DNA-protein cross-links are formed upon exposure of cellular DNA to various agents, including antitumor drugs, UV light, transition metals, and reactive oxygen species. They are thought to contribute to cancer, aging, and neurodegenerative diseases. It has been proposed that DNA-protein cross-links formed in cells are subject to proteolytic degradation to the corresponding DNA-peptide cross-links (DpCs). To investigate the effects of DpCs on DNA replication, we have constructed plasmid DNA containing a 10-mer Myc peptide covalently linked to C7 of 7-deaza-dG, a hydrolytically stable mimic of N7-dG lesions. Following transfection in human embryonic kidney cells (HEK 293T), progeny plasmids were recovered and sequenced. Translesion synthesis (TLS) past DpC was 76% compared to unmodified control. The DpC induced 20% targeted G→A and G→T plus 15% semi-targeted mutations, notably at a guanine (G₅) five bases 3' to the lesion site. Proteolytic digestion of the DpC reduced the mutation frequency considerably, indicating that the covalently attached 10-mer peptide was responsible for the observed mutations. TLS efficiency and targeted mutations were reduced upon siRNA knockdown of pol η, pol κ, or pol ζ, indicating that they participate in error-prone bypass of the DpC lesion. However, the semi-targeted mutation at G₅ was only reduced upon knockdown of pol ζ, suggesting its critical role in this type of mutations. Our results indicate that DpCs formed at the N7 position of guanine can induce both targeted and semi-targeted mutations in human cells and that the TLS polymerases play a critical role in their error-prone bypass.

Graphical abstract

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SUPPORTING INFORMATION

Supporting Information, which includes translesion synthesis efficiency and mutations found in progeny from the control 7-deaza-dG and 7-deaza-DHP-dG constructs, is available free of charge via the internet at <http://pubs.acs.org>.

Keywords

TLS; mutagenicity; DNA-peptide cross-link; siRNA knockdown

INTRODUCTION

DNA-protein cross-links (DPCs) are readily formed in cells and tissues exposed to various physical and chemical agents, including UV light, γ -radiation, transition metal ions, and bifunctional chemicals such as α , β -unsaturated aldehydes, nitrogen mustards, mitomycin C, and platinum anticancer drugs.^{1–3} DPCs have been proposed to contribute toward ageing, cancer, and neurodegenerative diseases.^{4–6} Despite their great potential for adverse biological effects, only a limited number of studies explored cellular effects of DPCs. A study of formaldehyde-induced DNA-histone DPC in human cells showed that when cells were treated with an inhibitor of proteasomes, DPC repair was inhibited, suggesting that DPC removal in cells may involve proteolytic degradation of the cross-linked proteins.⁷ More recent studies revealed that specialized proteases such as Wss1 in the yeast cleave the protein constituent of DPC following collision of the replisome/CMG helicase with a DPC located on the DNA leading strand.⁸ Duxin *et al* used a plasmid containing a site-specific DPC to demonstrate that in *Xenopus* egg extracts, DPC repair involves proteolytic degradation and is coupled to DNA replication.⁹ The resulting DNA-peptide cross-links (DpCs) may serve as substrates for nucleotide excision repair (NER).^{7,10,11}

Owing to their bulky size, DPCs are believed to interfere with DNA-protein interactions, replication, and transcription.² We have previously shown that large DPCs completely block DNA replication.^{12,13} However, DpCs generated upon proteolytic processing may be a substrate for translesion synthesis (TLS). Lloyd and coworkers have shown that small DpCs in which the peptide was covalently linked to N^2 position of dG blocked DNA replication, while the same peptide conjugated to N^6 position of dA was bypassed and induced small number of mutations in both *Escherichia coli* and simian kidney cells.^{14,15} It was also established that *E. coli* pol II, pol III, and pol V are inefficient in replicating past these DpCs, whereas pol IV is modestly efficient in their bypass.¹⁵ It was furthermore found that pol κ is highly efficient and accurate in TLS of the dG- N^2 -DpCs.¹⁵ We have shown that proteins and large peptides (>20 amino acids) conjugated to the C-5 position of dT completely block bypass by the TLS polymerases η and κ .¹⁶ However, smaller C5-dT DpC conjugates (<10-mer peptides) were bypassed in an error-prone manner, giving rise to large numbers of deletions and point mutations.^{13,16} In our recent study, 10-mer peptide DpCs conjugated to the C-7 position of deazaguanine were found to be bypassed by human lesion

bypass polymerases η and κ *in vitro*.¹⁷ To our knowledge, there are no reports of the biological outcome of a DpC in human cells.

The most common sites for both DPC and DpC formation in the presence of bis-electrophiles is the N7 position of dG.³ For example, the anticancer drugs phosphoramidate mustard and cyclophosphamide, as well as platinum compounds, predominantly form N7 dG adducts.^{18–20} In a recent study, concentration-dependent formation of cross-links with cysteine thiols of proteins to the N7-position of guanines in DNA was observed in human fibrosarcoma cells upon treatment with cyclophosphamide metabolite, nornitrogen mustard.²¹ Unfortunately, N7 dG adducts are hydrolytically unstable, and as a result, the adducted DNA strands are not only challenging to synthesize but also not stable enough for biological studies in cells. Despite their limited hydrolytic stability, protein-conjugated N7 dG adducts may persist long enough in cells to interact with the repair and replication apparatus before undergoing hydrolytic depurination.²² Indeed, preliminary investigation in our laboratory showed that N7 dG linked proteins in duplex DNA at neutral pH may persist in significant concentration even after 16 h incubation at 37 °C. Even so, to circumvent the limited hydrolytic stability of N7 dG-DpC adducts, we have synthesized a model DpC conjugate in which a 10-mer peptide was linked to the C7 position of 7-deaza-dG.^{24,25} Previous studies have revealed that the presence of 7-deaza group in guanine had minimal effects on DNA structure, and 7-deaza-dG has been used as a model in previous studies of cellular repair of other N7-guanine adducts such as G-G cross-links induced by nitrogen mustards.²⁴ Following incorporation of model DpC conjugate into a plasmid and its replication in human embryonic kidney (HEK) 293T cells, we for the first time examined the influence of a bulky DpC lesion on the efficiency and the fidelity of DNA synthesis in human cells and evaluated the contributions of individual TLS polymerases in its bypass using a siRNA knockdown approach.

EXPERIMENTAL PROCEDURES

Materials

All starting materials, reagents and solvents were of commercial grade and used as such unless otherwise specified. [γ -³²P] ATP was from Du Pont New England Nuclear (Boston, MA). The enzymes were obtained from New England Biolabs (Beverly, MA). Unmodified oligonucleotides were purchased from Midland Certified Reagents (Midland, TX).

siRNAs

Synthetic siRNA duplexes against *POLH* (SI02663619), *POLK* (SI04930884), and negative control siRNA (1027280) were acquired from Qiagen (Valencia, CA), whereas the same for *REV3* was purchased from Integrated DNA Technologies (Coralville, IA). Sequences of all the siRNAs have been reported.²³

Methods

Synthesis of a site-specific DNA-peptide cross-link (DpC)—A site-specific DNA-peptide cross-link (DpC) was generated by a post-synthetic reductive amination strategy reported previously.²⁴ Briefly, synthetic DNA oligonucleotide (5'-AGG GTT TTC CCA

XXTC ACG ACG TT-3', where X=7-deaza-7-(2,3-dihydroxypropan-1-yl)-dG (DHP-deaza-dG), was produced by solid phase synthesis on a ABI 394 DNA synthesizer (Applied Biosystem, CA) according to previously published method,²⁵ purified by semi-preparative off-line HPLC, and characterized by HPLC-ESI⁻-MS.

The HPLC-purified 23-mer oligodeoxynucleotide containing a site-specific DHP-deaza-dG (500 pmol in 17.5 μ L water) was oxidized in the presence of 50 mM NaIO₄ (35 μ L) in 15 mM sodium phosphate buffer (pH 5.4, 35 μ L) for 6 h at 4 °C in the dark, converting diol on the DHP-deaza-dG to an aldehyde group. Na₂SO₃ (35 μ L of 50 mM solution) was added to quench excess NaIO₄. The aldehyde-containing oligodeoxynucleotide was incubated with 100-fold molar excess of the peptide Ac-EQKLISEEDL-CONH₂ in the presence of 25 mM NaCNBH₃ (12.5 μ L) at 37 °C overnight (16–18 h) after adjusting the pH to 7 with 0.1 M NaOH. The DNA-peptide conjugate was purified by electrophoresis on a 20% polyacrylamide gel containing 7M urea (denaturing PAGE) followed by Sep-Pac C18 SPE desalting.

Proteolysis of DpC 23-mer

The proteolysis reaction was carried out by incubating 15.0 pmol of DpC 23-mer substrate with 3.2 units of Proteinase K (New England Biolabs, Ipswich, MA) at 37 °C for 48 h in a Tris.HCl buffer (30mM Tris.HCl (7.6), 2mM DTT). The completion of reaction was confirmed by denaturing PAGE, which showed complete conversion of the DpC 23-mer substrate into a faster-running band, although it eluted slightly more slowly compared to an unmodified 23-mer.

Construction and characterization of a pMS2 vector containing a single DpC and its replication in HEK 293T cells

DpC-containing single-stranded vector, pMS2, carrying neomycin and ampicillin resistance genes, was constructed according to previous reports.^{26,27} Control plasmids containing a dG, 7-deaza-dG, or 7-(2,3-dihydroxyprop-1-yl)-7-deaza-dG were also generated in a similar manner. The HEK 293T cells were grown to ~90% confluency and transfected with 50 ng of construct in 6 μ L of Lipofectamine cationic lipid reagent (Invitrogen, Carlsbad, CA). After transfection with modified or unmodified pMS2, the cells were allowed to grow at 37 °C in 5% CO₂ for 48 h and the plasmid DNA was collected and purified.²⁸ It was used to transform *E. coli* DH10B, and transformants were analyzed by oligonucleotide hybridization followed by DNA sequence analysis.^{27,29} For the hybridization, left (5'-GGTACCAGCGATAGG-3') and right (5'-CGTTATCGCTTGCA-3') probes were used to select plasmids containing the insert, whereas a 15-mer wild type probe 5'-TTCCCAGTCACGACG-3' was used to identify the error-free progeny. Transformants that failed to bind with both left and right probes were omitted. DNA sequencing was performed on all transformants that did not hybridize with the wild-type probe but were positive to left and right probes.

Determination of TLS efficiency

Lesion-containing or control pMS2 constructs were mixed with equal amounts of a single-stranded pMS2 DNA construct containing a 23-nucleotide sequence different from the DpC

(or control) DNA sequence.³⁰ The mixed DNA was used to transfect HEK 293T cells and processed as described above. Oligonucleotide probes for the complementary sequences for both the wild type and the mutant plasmid were used to analyze the progeny. The mutant DNA was used as an internal control and it gave equal number of progeny as the control construct. The percentages of the colonies originating from the lesion-containing plasmid relative to the unmodified plasmid, as determined by oligonucleotide hybridization, provided the TLS efficiency. The mutant progeny analysis was performed in the same manner as described for the progeny from the DpC construct.

Mutational analyses of TLS products from human cells with polymerase knockdowns

Prior to transfection of the control and DpC-containing vectors, synthetic siRNA duplexes were transfected into HEK 293T cells using Lipofectamine. HEK 293T cells were plated in 6-well plates at 50% confluence. Following 24 h incubation, they were transfected with 100 pmoles of siRNA duplex mixed with Lipofectamine, diluted in Opti-MEM (Gibco), per well. One day before transfection of the plasmid, cells were seeded in 24-well plates at 70% confluence. Cells were then co-transfected with another aliquot of siRNA and either control plasmid or lesion-containing plasmid at a ratio of 2:1. After 24 h incubation, progeny plasmids were isolated as described earlier. Oligonucleotide hybridization followed by DNA sequence analyses provided the frequency and types of mutants relative to non-mutant progeny for each polymerase knockdown experiment.

RT-PCR analysis and Western blotting

Total RNA was extracted from the cells 72 h after the first transfection of siRNA duplexes and 100 ng total RNA was used for RT-PCR analysis. Efficiency of siRNA knockdown was determined as described earlier.^{23,31} Reverse transcription and PCR initial activation step were executed for 30 min at 50 °C and 15 min at 95 °C, respectively. Details of RT-PCR showing the inhibition of DNA polymerases and GAPDH mRNA expression were described in detail in ref.²³ RT-PCR products were analyzed on 2% agarose gel run at 100V for 3 h in 1 × TBE buffer.

Western blotting procedure has been reported in our earlier publication.²³ Briefly, cells were washed with cold phosphate-buffered saline and lysed in ice-cold RIPA buffer containing protease inhibitor cocktail. After 1 h incubation on ice, the mixture was centrifuged at 10,000 rpm for 15 min at 4 °C. Western blotting and protein concentration determination were performed on the supernatant. The protein extracts were boiled in loading sample buffer. Proteins were separated on either 5% or 7% SDS-PAGE gels for 2 h and transferred onto PVDF membranes. The membranes were blocked with 5% milk and incubated with antibodies that specifically recognize human DNA polymerases. Human β -actin antibody was used to confirm equal gel loading. Horseradish peroxidase-conjugated goat anti-rabbit and goat-anti mouse were used at 1:5,000 dilutions. The signals were developed using Pierce ECL Western Blotting Substrate and the images were taken using a PhosphorImager.

RESULTS

Synthesis and characterization of a model DpC

Our strategy to synthesize a model DpC cross-link at the 7-position of 7-deaza-dG,²⁴ is summarized in Scheme 1. In this approach, a 23-mer oligonucleotide containing a centrally positioned 2,3-dihydroxypropyl-7-deaza-dG (5'-AGGGTTTTCCCA_XTCACGACGTT-3', where X = (7-deaza-DHP-dG)) was first synthesized. Periodate cleavage of the vicinal diol group of the 7-deaza-DHP-dG generated an aldehyde, which was conjugated to the ε-amino group via Schiff base of lysine of a 10-mer peptide derived from c-Myc protein (Ac-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-CONH₂). C-Myc is a transcription factor that plays a key role in cell cycle progression, apoptosis, and cellular transformation. C-Myc regulates the expression of >15% of all human genes, and is upregulated in many types of cancer.^{32,33} Because of its strong affinity for DNA and the presence of nucleophilic amino acids such as Lys in its structure, c-Myc is anticipated to participate in DNA-protein cross-linking upon exposure to *bis*-electrophiles. The unstable Schiff base was immediately reduced with NaCNBH₃ to form a stable amine linkage. The covalent DpC was purified by denaturing PAGE and characterized by HPLC-ESI-MS as described previously.²⁴

Replication of a plasmid containing a unique DpC in human cells

The 23-mer containing the DpC lesion at the 13th position from the 5' end (5'-AGGGTTTTCCCA_XTCACGACGTT-3') was ligated to an *EcoRV*-digested gapped pMS2 plasmid using the previously described methodology (Scheme 2).^{34,35} Since biological effects of 7-deaza-DHP (used as a precursor in our cross-linking experiments) or its unsubstituted analog, 7-deaza-dG, have not been previously evaluated, we have also made constructs containing 7-deaza-DHP and 7-deaza-dG, in addition to native dG control. Ligation efficiency of the DpC 23-mer on both sides of the gap was ~35%, based on agarose gel electrophoresis. Each construct and an unmodified plasmid DNA (containing a different sequence at the ligation site) were co-transfected into HEK 293T cells. The unmodified DNA was used as an internal control. Cells were incubated for 24 h to allow for one round of replication of the plasmids, which were isolated and used to transform *E. coli* DH10B cells. The percentages of the colonies originating from the lesion-containing plasmid relative to the unmodified pMS2 plasmid, indicating the percentage of TLS, were determined by oligonucleotide hybridization followed by DNA sequencing.^{23,30}

In HEK 293T cells, the TLS frequency for the DpC containing plasmid was $76 \pm 3\%$, as compared to 100% progeny generated from the undamaged DNA (Figure 1). In comparison, plasmids containing 7-deaza-DHP-dG and 7-deaza-dG allowed for $89 \pm 4\%$ and $92 \pm 3\%$ TLS, respectively (Supplementary Figure S1). Sequencing analyses of the progeny plasmids isolated from HEK 293T cells and amplified in *E. coli* has revealed that the DpC lesion is significantly mutagenic, inducing $36 \pm 2\%$ mutations at or near the lesion site (Figure 2A). Over 20% of targeted mutations occurred at the modified G covalently linked to the peptide, including 11% G→A transitions and 9% G→T transversions. In addition, approximately 15% mutations were detected at neighboring bases (semi-targeted mutations, see Figure 2A). Many of those occurred at G₅, located five bases downstream from the DpC site (11% frequency). In comparison, mutation frequencies (MFs) of 7-deaza-DHP-dG and 7-deaza-dG

were $7 \pm 3\%$ and $3 \pm 1\%$, respectively, with most mutations observed up to three bases 5' and five bases 3' to the altered G (Figure 2B & C). Our observation that both 7-deaza-dG and 7-deaza-DHP-dG are weakly mutagenic in human cells (MF 3–5%) and that they reduce TLS efficiency by 4–8% is not surprising, since NMR studies showed that 7-deaza-dG causes significant destabilization in several neighboring base-pairs.³⁶

While both 7-deaza-DHP-dG and 7-deaza-dG were weakly mutagenic, the MF of the DpC was much higher than these altered dGs. To further establish that the cross-linked peptide was responsible for the observed mutations, the DpC 23-mer was subjected to proteolytic digestion by Proteinase K and analyzed by PAGE. As shown in Figure 2E, the proteolyzed 23-mer (lane 2) eluted faster than the starting DpC 23-mer conjugated to a 10-mer peptide (lane 3). However, it eluted slightly more slowly than the unmodified 23-mer (lanes 1 & 4), suggesting that at least one amino acid still remained cross-linked to the 7-deaza-dG. The proteolyzed DpC conjugate was used to make a pMS2 construct, which was replicated in HEK 293T cells and the progeny were analyzed as described before. The TLS of this construct was $88 \pm 3\%$, exhibiting a 12% increase in lesion bypass relative to that of the DpC. Moreover, the total MF decreased from $36 \pm 2\%$ before proteolysis to $11 \pm 2\%$ after proteolysis (Figure 2D), with both targeted and semi-targeted mutations considerably reduced. These results confirm that the majority of polymerase stalling and mutation events observed upon replication of DpC containing plasmid in human cells can be attributed to the peptide lesion.

Which TLS polymerases are involved in bypassing this DpC in human cells?

In order to determine the identities of the polymerases bypassing the DpC in human cells, we employed the duplex siRNA knockdown approach to constrain the expression of specific lesion bypass polymerases. HEK 293T cells were first transfected with appropriate siRNA for 48 h to decrease the expression of the TLS polymerase pol η , pol κ , or pol ζ . Afterward, an additional aliquot of siRNA and a mixture of DpC-containing construct and unmodified plasmid were co-transfected into the cells. The magnitude of siRNA knockdown was determined by RT-PCR and by Western blotting analysis,²³ and the knockdown efficiency in each case was found in excess of 70%. As shown in Figure 1, lesion bypass was significantly reduced in cells with reduced expression of TLS polymerases pol η , pol κ , and pol ζ , suggesting that each of these polymerases plays a role in bypassing the DpC lesion. The greatest decrease to $33 \pm 4\%$ TLS (from $76 \pm 3\%$) by a single polymerase was observed when pol ζ was knocked down. Of the several two-polymerase knockdown experiments, simultaneous knockdown of pol κ and pol ζ had the greatest reduction (to $25 \pm 3\%$) in TLS of DpC. By contrast, knockdown of these polymerases had little effect on the TLS efficiency of 7-deaza-DHP-dG and 7-deaza-dG constructs (Figure S1 in SI), suggesting that these altered dG residues can be bypassed by other polymerases, including perhaps the replicative DNA polymerases. When a TLS polymerase was knocked down, the MF and the types of mutations from these altered dGs also varied (Figure S2 in SI).

Mutagenicity of the DpC lesion was also significantly influenced by polymerase knockdowns (Figure 3). Upon knockdown of pol η , total MF dropped by nearly 40%, and combined MF of targeted G→A and G→T mutations was reduced by more than 60% (to

~8%). However, the frequency of semi-targeted mutations of $G_5 \rightarrow A$ was unaffected. In pol κ knockdown cells, total MF was reduced by nearly 60%, and targeted $G \rightarrow A$ transitions were completely eliminated. As in the case of pol η , the semi-targeted $G_5 \rightarrow A$ mutations occurred at approximately the same frequency. This suggests that pol κ plays a role in targeted $G \rightarrow A$ mutations, but neither pol η nor pol κ is essential for the semi-targeted $G_5 \rightarrow A$ mutations. In contrast, pol ζ knockdown lowered the MF by nearly 80% and eliminated the targeted $G \rightarrow T$ transversions (Figure 3), indicating that pol ζ is the most important polymerase for mutagenesis of this DpC lesion, especially for the targeted $G \rightarrow T$ transversions. In addition, $G_5 \rightarrow A$ mutations were reduced by 70% in the absence of pol ζ , suggesting a critical role of pol ζ in semi-targeted mutagenesis by DpC lesions. Of the three two-polymerase knockdown experiments performed, knockdown of pol ζ and another polymerase exhibited a larger reduction in MF than the one (pol η and pol κ) that did not include pol ζ . For example, when pol κ and pol ζ were simultaneously knocked down, total MF was reduced by 85%, whereas simultaneous knockdown of pol η and pol ζ reduced the MF by approximately 75%. In contrast, simultaneous knockdown of pol η and pol κ reduced the MF by only about 50% (Figure 3). We conclude that pol ζ is a critical polymerase for both TLS and mutagenesis by this DpC.

DISCUSSION

The focus of this study was to understand the replication properties of a DpC lesion conjugated to the 7-position of dG. We found that the DpC at the 7-position of 7-deaza-dG is significantly mutagenic in human cells, and that human TLS polymerases are involved in its bypass and mutagenesis. The magnitude of TLS was most influenced in pol ζ knockdown cells, as demonstrated by nearly 60% reduction in TLS relative to negative control HEK 293T cells, whereas in pol κ knockdown cells TLS was reduced by 50% (Figure 1). Therefore, it is not surprising that simultaneous knockdown of pol κ and pol ζ reduced the TLS by 70%. In contrast, knockdown of pol η resulted in reduction of TLS by only 30%.

Mutagenesis was reduced when each of these three TLS polymerases was knocked down (Figure 3). Knockdown of pol η resulted in a major reduction in targeted $G \rightarrow A$, although $G \rightarrow T$ events were also reduced. Compared to the effects of diminished pol η , knockdown of pol κ resulted in a larger reduction of targeted $G \rightarrow T$ as well as a complete elimination of $G \rightarrow A$ mutations. Likewise, targeted $G \rightarrow A$ was reduced significantly and targeted $G \rightarrow T$ was completely eliminated in pol ζ knockdown cells. This suggests not only the characteristic roles of these three polymerases in the major targeted mutations but also that they work cooperatively. However, the semi-targeted $G_5 \rightarrow A$ transitions were significantly reduced only in pol ζ knockdown cells. The two-polymerase knockdowns experiments showed that the greatest reduction in MF (by 85%) occurred when pol κ and pol ζ were knocked down simultaneously (Figure 3). It appears that both G_5 and the peptide-linked 7-deaza-G site block DNA replication requiring recruitment of TLS polymerases. Based on the two-polymerase TLS model proposed by others,^{37,38} it is conceivable that after a TLS polymerase inserts T opposite G_5 , pol ζ is critical for extension from the $G_5:T$ mispair. However, pol ζ does not always continue synthesis past the lesion-containing G, requiring participation of other TLS polymerases. That the semi-targeted mutations at G_5 were due to the presence of the peptide was clearly demonstrated, when proteolytic digestion of the

peptide reduced the G₅→A mutations by 80%. Previously postulated mechanisms for semi-targeted mutations triggered by DNA adducts comprise misaligned primer-template structures,³⁹ similar to the ones suggested for frameshift mutagenesis.^{40,41} A combined crystallographic, primer extension, and molecular modeling study of TLS of a bulky DNA adduct by Dpo4 indicated that the semi-targeted mutations are driven by polymerase–template–primer–dNTP alignments in an arrangement where the bulky adduct is accommodated within the polymerase but not by Watson-Crick base-pairing between the incorrectly aligned template–primer DNA and dNTP,⁴² in a manner analogous to Rev1–induced templating mechanism.⁴³ In contrast to these mechanisms, in the current case the mutation at G₅ must occur before the polymerase actually replicates the lesion-containing G, implicating the long arm of the peptide in the mutational mechanism. We hypothesize that an amino acid from the peptide residue interacts with the G₅, five bases 3' to the lesion, which results in misincorporation of T opposite it. It also seems likely that pol ζ is required for further extension of this mispair at G₅. Currently it is unknown if such semi-targeted mutations are also prevalent with other DpCs. Based on the result of this study, we postulate a model, which attempts to summarize the roles of certain critical TLS polymerases in both targeted and semi-targeted mutagenesis induced by DpC (Scheme 3). Evidently, this scheme lacks the role of other polymerases that may play additional but significant roles in error-prone DpC bypass.

In a prior *in vitro* study of a DpC, which contained the same 10-mer peptide conjugated to C5 position of thymidine in a different oligonucleotide sequence, hpol κ and η were able to incorporate each of the four dNTPs opposite the lesion.¹³ Based on MS quantitation, 98 and 81% of the replication products of this DpC by hpol κ and hpol η, respectively, were erroneous extension products, which included a large fraction of –1 and –2 products.¹³ This study highlights the ability of DpCs to induce error-prone DNA synthesis when replication is carried out by the TLS polymerases. Although the mutational outcome is different, our current cellular study validates this notion. Conversely, our results for cellular mutagenicity of a peptide conjugated to 7-deaza-G in DNA presented herein differ from our earlier primer extension results for the same adduct by human pol η and pol κ.¹⁷ *In vitro* replication past this DpC in the presence of a single TLS polymerase (pol η or pol κ) was extremely inefficient and did not induce any mutations as demonstrated by single nucleotide incorporation experiments under steady-state conditions and HPLC-ESI-MS/MS sequencing of replication products. This differences may be attributed to the use of a different DNA sequence context and the requirement of several polymerases including pol ζ for efficient lesion bypass.

In conclusion, in this study cellular replication past a model DpC lesion structurally analogous to N7-guanine adducts formed in cells upon treatment with *bis*-electrophiles (nitrogen mustards, platinum compounds, dibromoethane, and 1,2,3,4-diepoxybutane)^{21,44–46} shows that the lesion is significantly mutagenic in human cells, inducing both targeted and semi-targeted mutations. A large fraction of the mutations was attributed to the presence of the peptide, and most mutations were induced by the TLS polymerases pol η, pol κ, and pol ζ. This work suggests that there is a high potential for erroneous replication of DpCs and that the TLS polymerases play a critical role in their error-prone bypass.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING

This work was supported by the NIEHS grant ES023350.

ABBREVIATIONS

DPC	DNA-protein cross-link
DpC	DNA-peptide cross-link
HEK	human embryonic kidney
TLS	translesion synthesis
NER	nucleotide excision repair
PAGE	polyacrylamide gel electrophoresis
MF	mutation frequency
7-deaza-DHP-dG	7-deaza-7-(2,3-dihydroxypropyl)-2'-deoxyguanosine

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Figure 1.

Effects of siRNA knockdowns of TLS polymerases on the extent of replicative bypass of DpC. Percent TLS in various polymerase knockdowns was measured relative to an internal control. The data represent the means and standard deviations of results from two independent experiments. HEK 293T cells were treated with negative control siRNA (wt 293), whereas the other single or double polymerase(s) knockdowns are indicated below on the X-axis.

Figure 2.

The types and frequencies of mutations induced in the progeny from the DpC (Panel A), 7-deaza-DHP-dG (Panel B), and 7-deaza-dG (Panel C) constructs in HEK 293T cells. The local DNA sequence is shown on the X-axis. Panel D shows the same after the DpC-23-mer was subjected to proteolytic digestion followed by construct preparation (described in the Materials and Methods). The data represent the average of two independent experiments. Panel E shows the polyacrylamide gel electrophoresis profile of the DpC-23-mer before

(lane 3) and after (lane 2) proteolysis. Lane 1 and 4 show the elution of the unmodified 23-mer.

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Figure 3.

The types and frequencies of mutations induced in the progeny from the DpC construct in HEK 293T cells also transfected with siRNA for single and double polymerase(s) knockdowns (as indicated).

Scheme 1.

Synthesis of a DpC by a post-synthetic reductive amination. A 23-mer oligonucleotide containing a 7-deaza-7-(2,3-dihydroxypropan-1-yl)-dG (at the 13th position indicated by an X) was synthesized. It was oxidized with NaIO₄ in sodium phosphate buffer for 6 h at 4 °C in the dark to generate 7-deaza-7-(2-oxoethyl)-2'-deoxyguanosine. In the subsequent step, the 10-mer peptide (Ac-EQKLISEEDL-CONH₂) derived from the c-Myc protein (EQKLISEEDL) (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) was added, followed by 0.5 M NaCNBH₃ at 37 °C overnight to form the DpC. The predominant product formed a Schiff

base between the amino group of lysine and the aldehyde group of 7-deaza-7-(formylmethan-1-yl)-dG, which was reduced by NaCNBH₃. It was purified by HPLC and characterized by ESI-MS.

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Scheme 2.

General protocol for construction of DpC-containing pMS2 plasmid and its replication in HEK 293T cells. Mutational analyses of the progeny were carried out by oligonucleotide hybridization as described.

Scheme 3.

Postulated pathways of mutagenic TLS of the DpC construct. X denotes the 7-deaza G covalently linked to the peptide on the template. Left side of the scheme suggests the pathways leading to targeted mutations, whereas the same for semi-targeted mutations is proposed on the right. For the latter, we believe that an amino acid from the peptide residue interacts with G₅, five bases 3' to the lesion, which allows dTTP misincorporation opposite

it. It is noteworthy that very few mutants contained both targeted and semi-targeted mutations, which justifies separating the two pathways.

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