

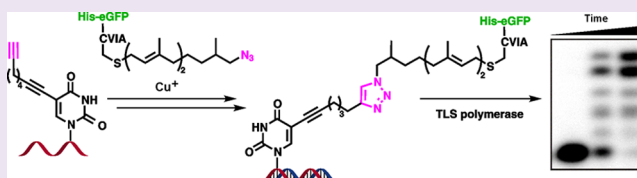
Synthesis of Site-Specific DNA–Protein Conjugates and Their Effects on DNA Replication

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Supporting Information

ABSTRACT: DNA–protein cross-links (DPCs) are bulky, helix-distorting DNA lesions that form in the genome upon exposure to common antitumor drugs, environmental/occupational toxins, ionizing radiation, and endogenous free-radical-generating systems. As a result of their considerable size and their pronounced effects on DNA–protein interactions, DPCs can interfere with DNA replication, transcription, and repair, potentially leading to mutagenesis, genotoxicity, and cytotoxicity. However, the biological consequences of these ubiquitous lesions are not fully understood due to the difficulty of generating DNA substrates containing structurally defined, site-specific DPCs. In the present study, site-specific cross-links between the two biomolecules were generated by copper-catalyzed [3 + 2] Huisgen cycloaddition (click reaction) between an alkyne group from 5-(octa-1,7-diynyl)-uracil in DNA and an azide group within engineered proteins/polypeptides. The resulting DPC substrates were subjected to *in vitro* primer extension in the presence of human lesion bypass DNA polymerases η , κ , ν , and ι . We found that DPC lesions to the green fluorescent protein and a 23-mer peptide completely blocked DNA replication, while the cross-link to a 10-mer peptide was bypassed. These results indicate that the polymerases cannot read through the larger DPC lesions and further suggest that proteolytic degradation may be required to remove the replication block imposed by bulky DPC adducts.



DNA–protein cross-links (DPCs) are among the most abundant and the least understood DNA lesions present in the human genome. These bulky lesions are created when cellular proteins become covalently captured on DNA strands in the presence of free radicals, anticancer drugs, transition metals, or physical agents such as UV light and ionizing radiation.¹ Our previous mass spectrometry based proteomics studies have discovered that many cellular proteins, including DNA polymerases, histone proteins, transcription factors, and DNA repair proteins, can become cross-linked to DNA in cells treated with antitumor nitrogen mustards, 1,2,3,4-diepoxybutane, and cisplatin.^{2–6} Some examples of the participating proteins include HSP 90, tubulins, DNA helicases, PCNA, Fen-1, KU 70, Ku 86, ref-1, PARP, and DNA polymerase δ .^{2–6} DNA–protein cross-linking is nonrandom, with specific amino acid side chains (typically cysteine, lysine, or arginine) participating in cross-linking.^{2,5,6} However, acrolein, crotonaldehyde, and 4-hydroxynonenal can form Schiff base cross-links between DNA and the N-terminal α -amine of the protein.⁷

Despite their ubiquitous nature, the biological consequences of DPC formation have not been fully elucidated, probably a result of their inherent structural complexity and the limited availability of structurally defined DPC substrates. It has been hypothesized that covalent DNA–protein conjugates induced by reactive oxygen species may play a role in the etiology of neurodegenerative and cardiovascular diseases due to their deleterious effects on DNA replication, transcription, repair,

and chromatin remodeling.^{8,9} Indeed, our recent experiments employing epoxide-functionalized protein reagents that selectively induce DPCs have provided the first direct evidence for the ability of DNA–protein cross-links to induce toxicity and mutations in human cells.¹⁰ However, because of the structural complexity of DPC lesions and the difficulty of generating site-specific, chemically defined DPC substrates, there is very limited information and no consensus on how cells respond to this class of DNA lesions. This lack of insight hinders our ability to fully understand the molecular basis of the therapeutic and adverse effects associated with a major class of anticancer agents and may limit insight into a fundamental cause of age-related disorders.

Because of their unusually bulky size and their disruptive effects on key DNA–protein interactions, DPCs are hypothesized to block the majority of DNA transactions.⁹ It has been proposed that large DPCs completely block the progression of replicative DNA polymerases along DNA strands.¹¹ However, the protein component of DPCs may be proteolytically cleaved to peptides, and the resulting smaller DNA–peptide lesions may be bypassed by translesion synthesis (TLS) polymerases, which are recruited to blocked replication forks to carry out DNA polymerization across damaged DNA.^{12,13} Translesion

Received: March 7, 2014

Accepted: June 11, 2014

Published: June 11, 2014

DNA synthesis is a key DNA damage tolerance mechanism that enables cells to overcome replication blocks caused by bulky DNA lesions unsurpassable for replicative DNA polymerases.^{12–14} In humans, there are several known lesion bypass polymerases: hpol η , hpol ι , hpol κ , Rev 1 belonging to the Y-family of polymerases, a newly discovered A family polymerase ν (POLN or pol ν), and hpol ζ belonging to the B-family of human polymerases.^{15–19} Due to an increased size of their active sites and for some of them, the lack of 3' \rightarrow 5' exonuclease proofreading activity, TLS polymerases exhibit low catalytic efficiency and are relatively error-prone.²⁰

Only a few previous studies have directly investigated polymerase bypass of DPC adducts. *E. coli* Pol I and HIV-1 reverse transcriptase were completely blocked by a DPC lesion containing histone H1 cross-linked to DNA via *trans*-[PtCl₂(E-iminoether)₂] or cisplatin.²¹ Furthermore, peptides linked to the minor groove of DNA at the N² position of guanine (via γ -hydroxypropano-dG) prevented primer extension catalyzed by A family lesion bypass polymerase ν .²² In contrast, structurally identical DNA–peptide cross-links placed in the major groove of DNA at the N⁶-dA were efficiently and accurately bypassed by Pol ν .²² Therefore, lesion localization (e.g., within the minor groove or in the major groove of DNA) and size may affect their biological consequences. However, these earlier experiments were conducted with model DPCs containing small peptides, whereas the bulk of DPC lesions in cells involve proteins of 50 kDa or larger.^{1,3,5,6,9,23}

A major limitation in the field is the paucity of DNA substrates containing site-specific, homogeneous, and structurally defined DNA–protein conjugates. Five main strategies have been previously employed to generate DPC-containing DNA repair substrates.^{24–26} Lloyd et al.²⁵ and Sancar et al.^{27,28} used a semi-enzymatic approach to cross-link T4 pyrimidine dimer glycosylase/AP lyase (T4-pdg) to abasic sites of DNA in the presence of sodium borohydride. A similar methodology has been used to covalently attach oxoguanine glycosylase (Ogg) protein to DNA strands containing 8-oxo-dG.²⁹ DNA methyltransferase (Dnmt) has been trapped on DNA containing 5-fluorodeoxycytosine.³⁰ Other approaches involve the use of oxanine (Ox) that spontaneously reacts with amino groups of proteins to give a pyrimidine ring-open structure,^{31,32} disulfide cross-linking,³³ and the Schiff base formation between acrolein-induced γ -HOPdG adducts and lysine residues of proteins and peptides, which can be reduced to a stable amino linker in the presence of NaCNBH₃.^{34,35} These previous methodologies have several limitations such as poor reaction efficiency and low yields,³¹ limited choices of protein reagents (e.g., specific DNA modifying proteins),³⁰ and insufficient site specificity in respect to the cross-linking site within the protein.^{31,35,36}

Our laboratory has been developing novel methodologies to generate synthetic DPCs structurally analogous to DPC adducts found in cells. We recently reported the use of a reductive amination strategy to create a DPC between an N7-deaza-G in DNA and basic lysine or arginine side chains of proteins and peptides.³⁶ The resulting model DPC substrates were site-specific within DNA but involved multiple cross-linking sites within the protein.³⁶

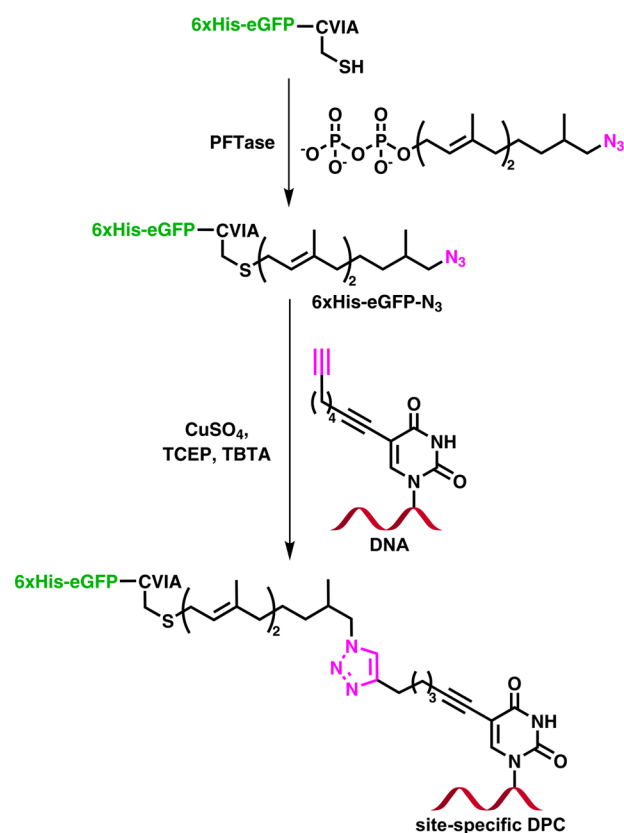
In the present work, a bioorthogonal approach employing copper-catalyzed [3 + 2] Huisgen cycloaddition (click reaction) between azide-functionalized proteins and alkyne-containing DNA was used to generate structurally defined DPC conjugates. The azide groups were incorporated via synthetic

methods for short peptides and enzymatically for a larger protein, while alkyne-containing DNA was generated by solid phase synthesis. The resulting cross-links are site-specific with regard to both protein and DNA. Synthetic DNA–protein conjugates were subjected to *in vitro* DNA replication experiments in order to evaluate the ability of human DNA polymerases to bypass these bulky lesions.

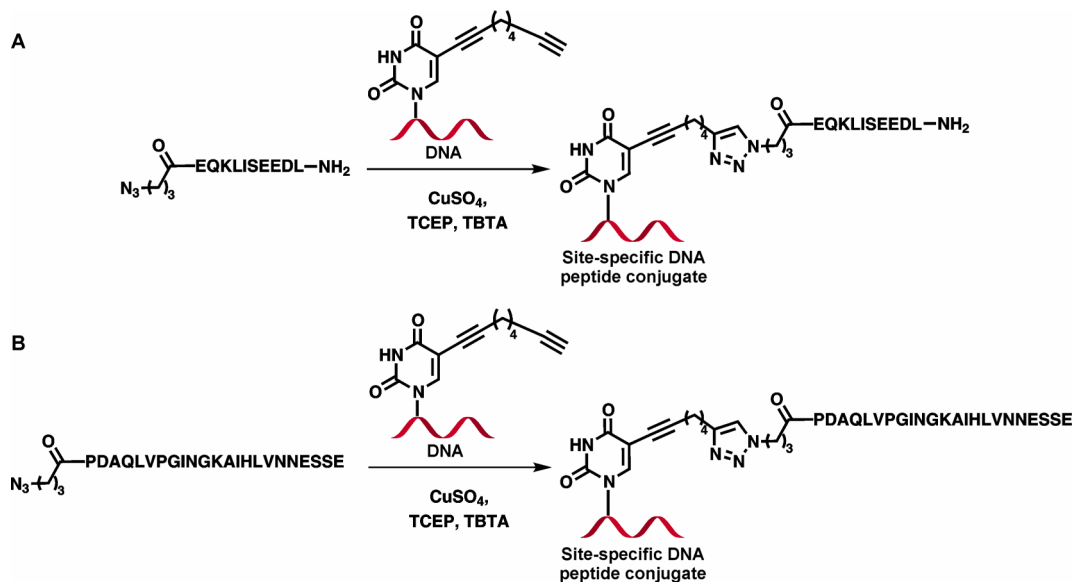
RESULTS AND DISCUSSION

Site-Specific DNA–Protein Cross-Linking Using Alkyne–Azide Cycloaddition (Click) Reaction. The availability of structurally defined DNA–protein and DNA–peptide conjugates is essential for any structural and biological studies of these bulky lesions. In the present work, site-specific DPCs were generated via 1,3-dipolar cycloaddition between azide-containing proteins/peptides and alkyne-functionalized oligodeoxynucleotides in the presence of copper ([3 + 2] Huisgen cycloaddition) to give a 1,2,3-triazole (Schemes 1 and 2).³⁷ To prepare azide-functionalized green fluorescent protein (6 \times His-eGFP-N₃), a previously described eGFP construct bearing an N-terminal His-tag and a C-terminal CVIA sequence was employed.³⁷ The latter sequence allows the cysteine residue within CVIA to be enzymatically prenylated by protein

Scheme 1. Generation of Site-Specific DNA–Protein Conjugates by Copper-Catalyzed [3 + 2] Huisgen Cycloaddition (Click Reaction) between an Alkyne Group from 5-(Octa-1,7-diynyl)-uracil in DNA and an Azide Group within Modified Green Fluorescent Protein (6 \times His-eGFP)^a



^aThe azide group was introduced by enzymatic prenylation of eGFP protein containing a C-terminal CVIA sequence with protein farnesyltransferase (PFTase) using an azide-containing farnesyl diphosphate substrate analogue.

Scheme 2. Synthesis of Site-Specific DNA–Peptide Cross-Links by Copper-Catalyzed Azide–Alkyne Cycloaddition Reaction^a

^aSynthetic 10-mer and 23-mer peptides were prepared via solid phase peptide synthesis and appended with an N-terminal 4-azidobutanoic acid group.

farnesyltransferase (PFTase) using an azide-containing farnesyl diphosphate substrate analogue (Scheme 1).^{37–39} We have previously used the PFTase method to prepare azide-modified proteins that were subsequently linked to the 5'-ends of alkyne-functionalized oligodeoxynucleotides via the Cu-catalyzed click reaction³⁷ or the Cu-free variation,⁴⁰ but internal DNA–protein cross-links have not been previously prepared. We elected to use the Cu-catalyzed reaction in the present study since it generates a less bulky linkage between the protein and DNA. Synthetic 10-mer and 23-mer peptides were prepared via solid phase peptide synthesis and appended with an N-terminal 4-azidobutanoic acid group for subsequent Cu-catalyzed click reaction (Scheme 2). Synthetic DNA oligomers containing C8-(octa-1,7-diynyl)-uracil (C8-alkyne-dU) were prepared by solid phase synthesis starting with commercial phosphoramidites (Glen Research). The resulting biomolecules were purified by HPLC and characterized by mass spectrometry.

Our initial bioconjugation experiments were conducted using 6×His-eGFP-N₃ protein and a ³²P-end-labeled 23-mer oligodeoxynucleotide containing site-specific C8-alkyne-dU (X) (5'-AGG GTT TTC CCA GXC ACG ACG TT-3'). Following cycloaddition reaction in the presence of Cu^I, denaturing SDS-PAGE of the reaction mixture revealed the appearance of a new slowly moving band (Lane 2 in Figure 1A), which was not present in the DNA control (Lane 1 in Figure 1A). The high molecular weight band disappeared when the reaction mixture was incubated with proteinase K, confirming that it corresponds to a covalent DNA–protein conjugate (Lane 3 in Figure 1A). The cross-linking yield was estimated as ~70% based on densitometry analysis (Figure 1A, Lane 2).

In a separate experiment, unlabeled DNA 23-mer (5'-AGG GTT TTC CCA GXC ACG ACG TT-3') was conjugated to 6×His-eGFP-N₃, and the reaction mixture was separated by SDS-PAGE, followed by protein visualization by Simply Blue stain (Figure 1B). A new band at ~35 kDa was observed upon analysis of reaction mixtures (Lane 2 in Figure 1B), which is consistent with the conjugate of 23-mer oligodeoxynucleotide

(7.1 kDa) and 6×His 6His-eGFP-N₃ (28.4 kDa). This band was not observed in protein only control (Lane 1 in Figure 1B) or in control reactions conducted in the absence of Cu (Lane 3 in Figure 1B). To examine the influence of DNA–polypeptide molar ratios on the efficiency of DPC formation, the cycloaddition reaction was repeated in the presence of increasing molar equivalents of GFP, followed by gel electrophoretic analysis (Figure 1C). We found that the DPC yields improved with increasing protein concentration, reaching a maximum yield of DPCs when a 6-fold molar excess of GFP was employed (Lane 4 in Figure 1C). These results indicate that site-specific DNA–protein cross-links can be generated in good yields using copper-mediated 1,3-dipolar cycloaddition between azide-containing proteins and alkyne-functionalized DNA.

DNA–peptide cross-links were similarly generated by cycloaddition reactions between C8-alkyne-dU-containing DNA (7.1 kDa) and synthetic azide-containing peptides (N₃(CH₂)₃CO-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-NH₂, 1.3 kDa or N₃(CH₂)₃CO-Pro-Asp-Ala-Gln-Leu-Val-Pro-Gly-Ile-Asn-Gly-Lys-Ala-Ile-His-Leu-Val-Asn-Asn-Glu-Ser-Ser-Glu, 2.5 kDa). As shown for the 10-mer peptide reaction, the presence of the DNA–peptide conjugates (8.4 kDa) was detected by denaturing PAGE (Figure 1D). A new, low mobility band (Lane 2 in Figure 1D) corresponding to oligonucleotide–polypeptide conjugate was found only when the reaction was conducted in the presence of Cu (Lane 3 in Figure 1D) and disappeared upon incubation with proteinase K (Lane 4 in Figure 1D). A 200-fold molar excess of peptide to DNA was required to achieve optimal yields of DNA–peptide conjugates (81.5% yield, Supplementary Figure S-4). The cycloaddition reaction with 23-mer peptide was conducted analogously (78% yield, Supplementary Figure S-5).

Mass Spectrometry Characterization of DNA–Protein and DNA–Peptide Conjugates. To confirm the formation of covalent DNA–protein and DNA–peptide cross-links, the purified conjugates were characterized by tandem mass spectrometry. In order to simplify MS analysis of DNA–

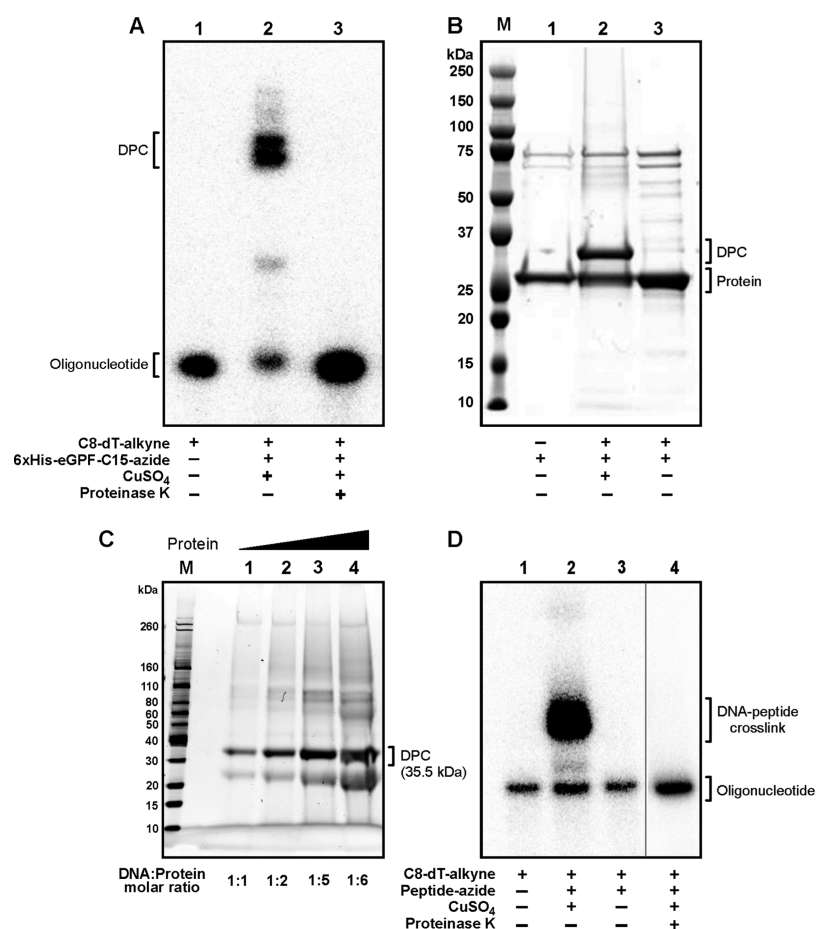


Figure 1. Generation of site-specific DNA–protein cross-links (DPCs) by Cu-catalyzed azide–alkyne cycloaddition. (A) SDS-PAGE analysis of DPCs generated by using 6xHis-eGFP-N₃ protein and ³²P-end-labeled DNA 23-mer (5'-AGG GTT TTC CCA G C8-alkyne-dUC ACG ACG TT-3', where C8-alkyne-dU is 5-(octa-1,7-diynyl)-uracil). Lane 1: Alkyne containing DNA. Lane 2: Reaction mixture following cycloaddition between C8-alkyne-dU-containing DNA and 6xHis-eGFP-N₃ protein. Lane 3: Proteinase K digested reaction from lane 2. (B) The same reaction as in panel A conducted with unlabeled DNA and separated by 12% SDS-PAGE. Proteins were visualized via SimplyBlue staining. Lane M: protein marker. Lane 1: 6xHis-eGFP-N₃. Lane 2: reaction mixture following Cu-catalyzed cycloaddition between 6xHis-eGFP-N₃ protein and alkyne containing DNA. Lane 3: Reaction mixture following cycloaddition conducted in the absence of Cu. (C) The yields of cycloaddition-induced DPCs increase with increased protein:DNA molar ratios. The reaction was conducted as in panel B, but the molar ratio of DNA:6xHis-eGFP-N₃ was varied between 1:1 and 1:6. (D) Denaturing PAGE analysis of DNA–peptide conjugates generated using 10-mer peptide (N₃(CH₂)₃COEQKLISEEDLNH₂) and alkyne containing DNA 23-mer. Lane 1: C8-alkyne-dU containing 23-mer alone. Lane 2: Reaction mixture following Cu-catalyzed cycloaddition between C8-alkyne-dU-containing DNA 23-mer and peptide-N₃. Lane 3: The same reaction as in Lane 2 conducted in the absence of Cu. Lane 4: Proteinase K digested reaction from Lane 2.

peptide cross-links (Scheme 2A and Figure 2A), the DNA component of the cross-link was digested to nucleosides. NanoHPLC-nanospray-MS/MS analysis allowed for the detection of doubly charged peptide species at m/z 823.40, which corresponds to the decapeptide EQKLISEEDL containing a triazole cross-link to deoxyuridine. The doubly charged peptide was subjected to HCD fragmentation within an Orbitrap Velos instrument, and the resulting fragments were analyzed in the accurate mass mode. Both b - and y -series fragment ions were detected (Figure 2A), and the MS/MS fragmentation under HCD conditions was consistent with the predicted conjugate structure (Scheme 2A).

In order to characterize the conjugates between 23-mer DNA oligomer and GFP protein (Scheme 1), DNA was digested to nucleotides, while the protein was cleaved to peptides with trypsin. Following SDS-PAGE purification, gel bands containing DPCs were excised and subjected to in-gel digestion with phosphodiesterase I (PDE I) and trypsin, and the resulting peptide–nucleotide conjugates were analyzed by nanoHPLC-

nanospray-HRMS/MS using an Orbitrap Velos mass spectrometer. The mass spectral data were processed using Thermo Proteome Discoverer 1.3 software (ThermoScientific, San Jose, CA) to identify the cross-linking site(s). A doubly charged ion at m/z 532.77 was observed corresponding to the tetrapeptide CVIA containing a covalent cross-link to dUMP (theoretical mass = 1064.52, Figure 2B). MS/MS fragmentation of m/z 532.77 ions under CID conditions gave rise to a series of b and y fragments, including a singly charged b_2 ion at m/z 862.39 and a doubly charged b_3 fragment ion at m/z 488.24 (Figure 2B). Since the cysteine residue within the sequence CVIA is known to be the site of enzymatic prenylation, these observations are consistent with the predicted site of modification. Taken together, these results are consistent with cycloaddition reaction taking place at the specific cysteine residue of the protein containing the azido modification.

Polymerase Bypass of Synthetic DNA–Protein and DNA–Peptide Conjugates. The model DNA–protein and DNA–peptide conjugates generated by click reaction (Schemes

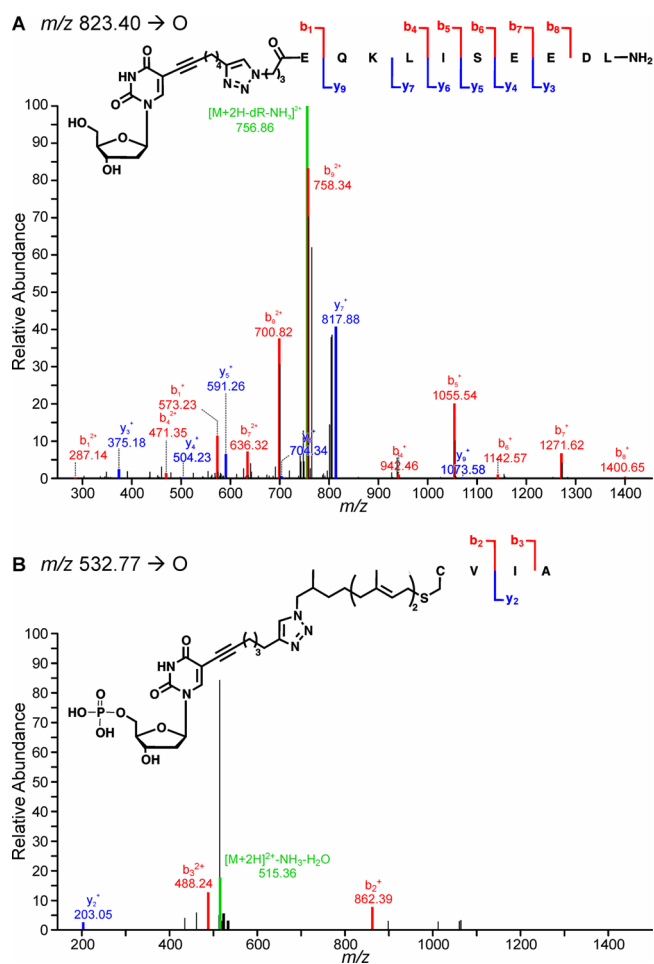


Figure 2. Mass spectrometry characterization of DNA–peptide and DNA–protein conjugates. (A) NanoLC–nanospray–MS/MS characterization of DNA–peptide conjugates generated using 10–mer peptide ($N_3(\text{CH}_2)_3\text{COEQKLISEEDLNH}_2$) and C8–alkyne–dU-containing DNA 20–mer. Following gel purification as shown in Figure 1, the DNA component of the cross-link was digested with phosphodiesterases and alkaline phosphatase, and the resulting peptide–nucleoside conjugate (m/z 823.40, doubly charged) was sequenced by nanoLC–nanospray–MS/MS on an Orbitrap Velos mass spectrometer. (B) NanoLC–nanospray–MS/MS spectrum of eGFP tryptic peptide, CVIA, cross-linked to 5-(octa-1,7-diyne)-2'-deoxyuridine monophosphate. DPCs were generated by Cu-catalyzed cycloaddition between 6 \times His–eGFP– N_3 and C8–alkyne–dU containing DNA 20–mer, and DPCs were isolated by 12% SDS–PAGE as shown in Figure 1. DNA component of the DPCs was digested with phosphodiesterase I, and the resulting protein–nucleotide conjugate (m/z 532.77, doubly charged) was subjected to tryptic digestion followed by MS/MS analysis on an Orbitrap Velos mass spectrometer.

1 and 2) resemble DNA–protein cross-links induced by bis-alkylating agents³ and reactive α,β -unsaturated carbonyls.⁷ Many bis-electrophiles, including nitrogen mustards, platinum compounds, and diepoxides, form DPCs by alkylating cysteine thiols within proteins.^{2,4–6} On the other hand, acrolein, crotonaldehyde, and 4-hydroxynonenal form Schiff base cross-links between DNA and the N-terminal α -amine of the peptide.⁷ Although the linker length within our model DNA–protein conjugates is longer than that observed for cross-links generated physiologically, we anticipate that the linker length will play a relatively minor role in determining the route of lesion processing. It is more likely that the nature of the

protein/peptide and the attachment site within DNA will determine the cellular fate of DNA–protein cross-links.

The model DPC-containing DNA substrates were subjected to several rounds of purification prior to their use in biochemical assays. The reaction mixtures were initially desalted to remove reagents and salts. DPCs were isolated by SDS–PAGE (DNA–protein conjugates) or 15% or 20% (w/v) denaturing polyacrylamide gel containing 7 M urea (DNA–peptide conjugates) and extracted from the gel using a gel elution kit or a freeze–thaw method. DPC purity was confirmed by analysis of purified material via either SDS–PAGE or denaturing PAGE (Lane 3 in Supplementary Figure S-2). To confirm the removal of excess protein, purified DPCs were fractionated by SDS–PAGE and stained with SimplyBlue stain. Purified DPCs were labeled with ^{32}P ATP, and the absence of unreacted oligonucleotides was verified by denaturing PAGE, followed by phosphorimaging. Depending on the purity of DPC substrates obtained from initial isolation, additional gel purifications were carried out. Only conjugates whose purity was greater than 96% were employed in DNA polymerase assays.

To elucidate the influence of DNA–protein and DNA–peptide cross-links on DNA replication, template–primer complexes containing site-specific cross-links to 6 \times His–eGFP– N_3 protein, 23–mer peptide (PDAQLVPGINGKAIHLVNNESSE), 10–mer peptide (EQKLISEEDL), and unmodified dT (negative control) were subjected to primer extension in the presence of human translesion synthesis (TLS) polymerases κ , η , and ι . Two types of experiments were conducted: standing start, with the primer extending to the -1 position from the DPC lesion (Scheme 3B), and running start, with the primer ending four nucleotides upstream from the adduct site on the 18–mer template 5'-TCA TXG AAT CCT TCC CCC-3', where X = unmodified dT or synthetic DPC lesion (Scheme 3C).

In standing start experiments with control template (Figure 3), both hpol κ (a 10:1 molar ratio of polymerase to primer–template duplex) and hpol η (a 4:1 ratio of polymerase to primer–template) completely extended the primer opposite the control template to form 18–mer products (X = dT, Figure 3). hPol ι generated mainly a single nucleotide addition product, probably due to its known low processivity as compared to other Y-family polymerases (Supplementary Figure S-6).^{41–44} The presence of 6 \times His–eGFP–dU at position X completely blocked primer extension by all three human lesion bypass polymerases (Figure 3A,D and Supplementary Figure S-6A). Similar results were obtained for the 23–mer peptide conjugate (Figure 3B,E and Supplementary Figure S-6B). In contrast, all three polymerases were capable of bypassing the smaller DPC containing a 10–mer peptide, albeit with differing efficiency (Figure 3C and Supplementary Figure S-6C). hPol κ extension products included the complete 18–mer and multiple incomplete extension products (Figure 3C). Interestingly, nucleotide incorporation opposite the lesion was more efficient than the addition of subsequent nucleotides, resulting in accumulation of the +1 product (Figure 3C). In the case of hpol ι , the efficiency of primer extension was significantly lower than the substrate bearing a native dT, but nearly complete conversion of a 13–mer to a 14–mer product was observed in 180 min (Supplementary Figure S-6C).

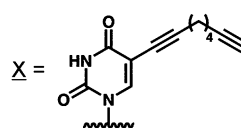
For running start experiments, the 18–mer template (5'-TCA TXG AAT CCT TCC CCC-3', where X = dT or DPC adduct containing 10–mer peptide (EQKLISEEDL), 23–mer peptide

Scheme 3. Sequences of DNA Oligomers Used for Conjugation Reactions with Proteins and Peptides (A) and DNA Substrates Employed in Standing Start (B) and Running Start Primer Extension Experiments (C)

A. DNA templates used for DPC synthesis

5'-AGG GTT TTC CCA GXC ACG ACG TT-3' (23mer template)

5'-TCA TXG AAT CCT TCC CCC-3' (18mer template)



B. Primer template duplex for standing start experiments

5'-TCA TXG AAT CCT TCC CCC-3' (18mer template)

3'-CTTA GGA AGG GGG-5' (13mer primer)

X = dT (positive control)

EQKLISEEDL (10mer peptide)

PDAQLVPGINGKAIHLVNNESSE (23mer peptide)

6xHis eGFP

C. Primer template duplex for running start experiments

5'-TCA TXG AAT CCT TCC CCC-3' (18mer template)

3'-GGA AGG GGG-5' (9mer primer)

X = dT (positive control)

EQKLISEEDL (10mer peptide)

PDAQLVPGINGKAIHLVNNESSE (23mer peptide)

6xHis eGFP

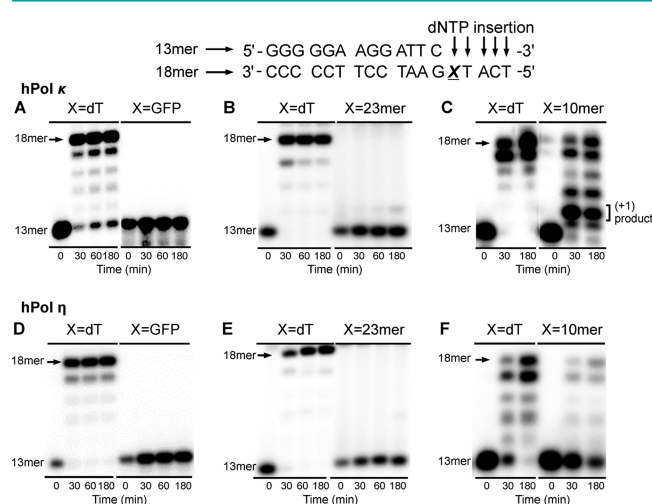


Figure 3. Extension of ^{32}P -labeled primers containing unmodified dT or DNA–protein and DNA–peptide conjugates of increased size adduct by human lesion bypass polymerases hPol κ (A–C) and hPol η (D–F) under standing start conditions. 13-Mer primers were annealed with 18-mer templates containing unmodified dT or covalent cross-links to 6 \times His-eGFP, 23-mer peptide, or 10-mer peptide (Scheme 3B). The resulting primer–template complexes (40 nM) were incubated in the presence of hPol κ (400 nM) or hPol η (160 nM). The polymerase reactions were started by the addition of the four dNTPs (500 μM) and quenched at the indicated time points. The quenched samples were separated by 20% (w/v) denaturing polyacrylamide gel electrophoresis and visualized by phosphorimaging analysis.

(PDAQLVPGINGKAIHLVNNESSE), or 6 \times His eGFP was annealed to a 9-mer (-4) primer (Scheme 3C). Complete primer extension by hpol η and hpol κ was observed for the control substrate (Figure 4), while hpol ι produced a +1 (14-mer) product (Supplementary Figure S-6D). As was the case for our standing start experiments, hpol κ , η , and ι were completely blocked by the cross-links containing 6 \times His-eGFP-dU and the 23-mer peptide (Figure 4A,B,D,E, and Supplementary Figure S-6D,E), whereas the presence of a 10-mer cross-link at position X led to varied amounts of extended products with hpol κ , η and ι (Figure 4C,F and Supplementary Figure S-6F). Low amounts of fully extended products (18-mers) were observed in the experiment with hpol η , suggesting that hpol η bypass of DNA–peptide conjugates is inefficient (Figure 4F). In contrast, hpol ι has shown a robust primer

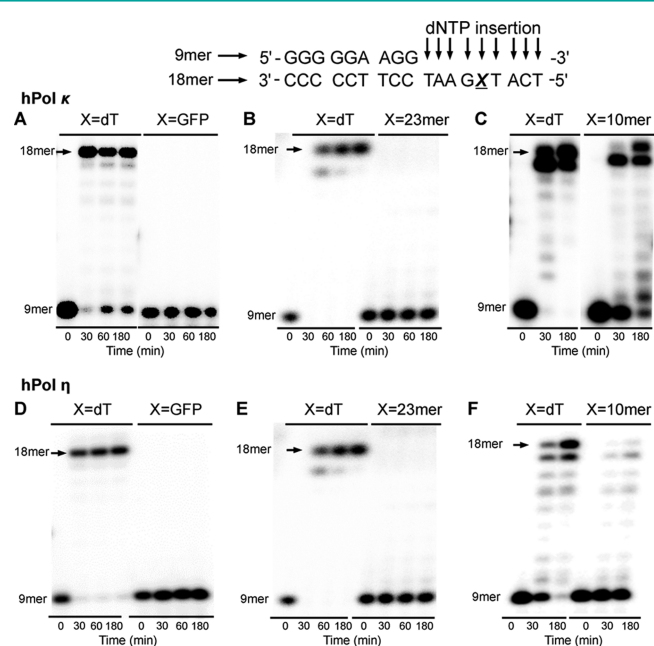


Figure 4. Extension of ^{32}P -labeled primers containing unmodified dT or DNA–protein and DNA–peptide conjugates of increased size adduct by human lesion bypass polymerases hPol κ (A–C) and hPol η (D–F) under running start conditions. The ^{32}P -end-labeled 9-mer primers were annealed to the 18-mer templates containing unmodified dT, GFP, 23-mer peptide, or 10-mer peptide (Scheme 3C). The resulting primer–template complexes (40 nM) were incubated at 37 $^{\circ}\text{C}$ in the presence of hPol κ (400 nM) and hPol η (160 nM). Reactions were started by the addition of all four dNTPs (500 μM) and quenched at indicated time points. The extension products were resolved by 20% (w/v) denaturing PAGE and visualized by phosphorimaging analysis.

extension activity (Supplementary Figure S-6F), suggesting that it may coordinate with other human polymerases to allow for efficient bypass of small DNA–peptide cross-links via polymerase switching.⁴⁵

Our observation of complete polymerase blockage by DNA–protein conjugates (Figures 3A,D and 4A,D) is consistent with an earlier finding of Kuo and collaborators, who reported that 5-azacytidine induced methyltransferase-DNA adducts block DNA replication *in vivo*.¹¹ In contrast, our finding that C⁵-thymine cross-links to a 23-mer peptide block human lesion bypass polymerases κ and η (Figures 3B,E and 4B,E) contradict

earlier reports that pol κ efficiently bypasses γ -HOPdG mediated DNA–peptide cross-links connected to the N² position of guanine in DNA,⁴⁶ while pol ν is able to catalyze replication past γ -HOPdA mediated DNA–peptide cross-links to the N⁶ position of adenine.²² This may be due to structural differences between the DPCs examined in these studies and also due to the differences in peptide size, since previous reports^{22,46} were limited to peptide 4-mers and 12-mers and did not examine the effects of larger peptide lesions on DNA replication. Indeed, our results presented in Figures 3C,F and 4C,F indicate that smaller cross-links to a peptide 10-mer can be bypassed by pol κ and pol η .

Conclusions. Any investigation of the biological effects of DPC lesions in cells is dependent on the availability of structurally defined DNA substrates containing site-specific DPC lesions. In the present study, site-specific cross-links between DNA oligomers and polypeptides of increasing size (10-mer, 23-mer, and 28.4 kDa protein) were generated using copper-catalyzed [3 + 2] Huisgen cycloaddition (click reaction) between an alkyne group from C8-alkyne-dU in DNA and an azide group within engineered proteins/polypeptides. Our optimized reaction conditions and purification strategy generates structurally defined, site-specific DNA–protein and DNA–peptide conjugates in high yield and with excellent purity.

Polymerase bypass experiments conducted with model DPC substrates incorporating peptide 10-mer, peptide 23-mer, and a 28.4 kDa protein have shown that while the two larger lesions blocked all human polymerases tested, the DPC to a 10-mer peptide can be bypassed by polymerases η , κ , and ι . These results suggest that large DPCs generated in cells may require proteolytic processing in order to be tolerated. Our ongoing studies will identify the proteolytic mechanisms involved and elucidate the effects of proteasomal inhibitors on toxicity of common antitumor drugs that are known to form DPCs.

METHODS

Copper-Catalyzed Cycloaddition Reaction between 6×His-eGFP-N₃ and Alkyne-Containing DNA. HPLC-pure DNA oligodeoxynucleotides (5′-AGG GTT TTC CCA GXC ACG ACG TT-3′ or 5′-TCA TXG AAT CCT TCC CCC-3′, 1 nmol), where X = C8-alkyne-dU, were mixed with 6×His-eGFP-N₃ (6 nmol), 2 μ L of tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 5 mM stock in DMSO/*t*-BuOH 1:4), 20 μ L of tris(2-carboxyethyl)phosphine (TCEP, 5 mM stock in H₂O), and 20 μ L of CuSO₄ (5 mM stock in H₂O) in 50 mM phosphate buffer (pH 7.5), in a final reaction volume of 100 μ L. The reaction was allowed to proceed for 1.5–2 h at RT upon mixing with a rotatory shaker. Following desalting on Micro biospin-6 columns, aliquots of the reaction mixtures were withdrawn and resolved by 12% SDS-PAGE. To visualize DPC formation, NuPAGE Novex 12% Bis-Tris gels (Life Technologies, Grand Island, NY) were run at a constant voltage of 130 V for 1 h in 1× NuPAGE MOPS SDS running buffer. The reaction mixtures obtained from DNA–protein cross-linking reactions were reconstituted in NuPAGE SDS sample buffer and heated at 70 °C for 10 min prior to loading on the gel. The unreacted protein and DNA–protein conjugates were visualized by staining with SimplyBlue SafeStain. Proteinase K digestion (6 units, at 37 °C for 48 h) was conducted to confirm the presence of protein in slowly moving DNA bands. The reaction yields were quantified by ImageJ software.

Mass Spectrometry Analysis of DNA–Protein Cross-Links. DNA–protein cross-links containing 6×His-eGFP protein conjugated to synthetic oligodeoxynucleotide 23-mer at position X (5′-AGG GTT TTC CCA GXC ACG ACG TT-3′) were purified by 12% SDS-PAGE and stained with SimplyBlue SafeStain. Gel bands were cut into slices and subjected to reduction with 300 mM DTT (10 μ L), followed by

alkylation with iodoacetamide (10 μ L in 25 mM NH₄HCO₃, pH 7.9). Gel pieces were dehydrated with CH₃CN, dried under vacuum, reconstituted in 25 mM NH₄HCO₃ (pH 7.9) (75 μ L), and incubated with PDE I (120 mU) at 37 °C overnight to digest the DNA portion of the cross-link. The resulting 6×His-eGFP-nucleotide conjugates were subjected to tryptic digestion using MS grade Trypsin Gold at 37 °C for 20 h and desalted using C18 ZipTips (Millipore, Billerica, MA). Samples were dissolved in 0.1% acetic acid (25 μ L), and 5–8 μ L of this solution was used for MS analysis.

NanoLC-nanospray-MS/MS was conducted using an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA) in line with a NanoLC-Ultra 2D HPLC system (Eksigent, Dublin, CA). Chromatography was performed using a hand packed Luna C18, capillary column (75 μ m i.d., 10 cm packed bed, 15 μ m orifice, 5 μ m particle size). The HPLC mobile phases used were 0.1% formic acid in H₂O (A) and 0.1% formic acid in CH₃CN (B). Peptide mixtures (5 μ L) were injected using a 5 μ L loop and loaded onto the column with a 1 μ L/min flow of 2% B for 5.5 min, at which point the injection valve was switched to the load position, and the flow was reduced to 0.3 μ L/min. The following linear gradient profile was then used: 2% to 70% B over 60 min, then to 95% B over 1 min, kept at 95% B for a further 5 min, and decreased to 2% B in 1 min. Finally, the flow rate was increased to 1 μ L/min and kept at 2% B for 4 min. Mass spectrometry analyses were performed using a FTMS mass analyzer with a resolution of 60,000 and a scan range of 300–2000. Peptide MS/MS spectra were collected using data-dependent scanning in which one full scan mass spectrum was followed by 8 MS/MS spectra using an isolation width of 2.5 *m/z*, 35% normalized CID collision energy, 1 repeat count, and 30 s repeat duration with an exclusion mass width of 5 ppm. Spectral data were analyzed using Proteome Discoverer 1.3 software (Thermo Scientific, San Jose, CA) that linked raw data extraction, database searching, and probability scoring. The raw data were directly uploaded, without any format conversion, to search against the protein FASTA database. Search parameters included trypsin specificity and up to 2 missed cleavage sites.

Polymerase Bypass Assay. Oligodeoxynucleotide primers (5′-GGG GGA AGG ATT C-3′ and 5′-GGG GGA AGG-3′, 100 pmol) were radiolabeled in the presence of T4 PNK (20 unit) and γ -³²P ATP (30 μ Ci) at 37 °C for 60 min in 1× PNK buffer (total volume = 20 μ L). The solutions were heated at 65 °C for 10 min to inactivate the enzyme and passed through Illustra Microspin G25 columns (GE Healthcare, Pittsburgh, PA) to remove excess γ -³²P ATP. 5′-³²P-labeled primers (50 pmol) were mixed with 2 equiv of HPLC-pure template strands (5′-TCA TXG AAT CCT TCC CCC-3′ where X = the click reaction generated covalent cross-link from the C-5 position of dU to the C-terminus of 6×His-eGFP, and N-terminus of 23-mer peptide (PDAQLVPGINGKAIHLVNNESSE), or 10-mer peptide (EQKLISEEDL)) in 10 mM Tris buffer (pH 7) containing 50 mM NaCl. Control template strands contained unmodified dT at position X. The strands were annealed by heating at 90 °C for 10 min and cooling slowly overnight to afford the desired radiolabeled template–primer duplexes (Scheme 3).

Primer–template duplexes (40 nM in the final reaction volume of 40 μ L) were incubated with human recombinant DNA polymerases (final concentrations: 160 nM hPol η , 400 nM hPol κ , 80 nM hPol ι) at 37 °C in the presence of a buffered solution containing 50 mM Tris (pH 7.5), 50 mM NaCl, 5 mM DTT, 100 μ g/ μ L BSA, 10% glycerol (v/v), and 5 mM MgCl₂. Primer extension reactions were initiated by adding 0.5 mM solutions of all four dNTPs. Aliquots of the reaction mixtures (4 μ L) were withdrawn at preselected time intervals (0–180 min) and quenched by the addition of 18 μ L of a solution containing 95% formamide (v/v), 10 mM EDTA, 0.03% bromophenol blue (w/v), and 0.03% xylene cyanol (w/v). Samples were loaded on to a 20% denaturing polyacrylamide gel containing 7 M urea and run at 80 W for 2.5 h. The extension products were visualized using a Typhoon FLA 7000 instrument in the phosphorimaging mode.

■ ASSOCIATED CONTENT

■ Supporting Information

Additional experimental procedures including the preparation of DNA oligomers, proteins, and peptides, gel electrophoresis, and mass spectrometry. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. P. Villalta (Masonic Cancer Center Mass Spectrometry Facility, University of Minnesota) for his assistance with mass spectrometry experiments and R. Carlson (Masonic Cancer Center, University of Minnesota) for preparing the figures for this manuscript. The recombinant polymerase ι was generously provided by Professor F. P. Guengerich (Vanderbilt University). This research was supported by a grant from the National Cancer Institute (CA100670) and a Brainstorm Award from the University of Minnesota Masonic Cancer Center.

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