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DNA–Protein Cross-Links: Formation, Structural Identities, and Biological Outcomes

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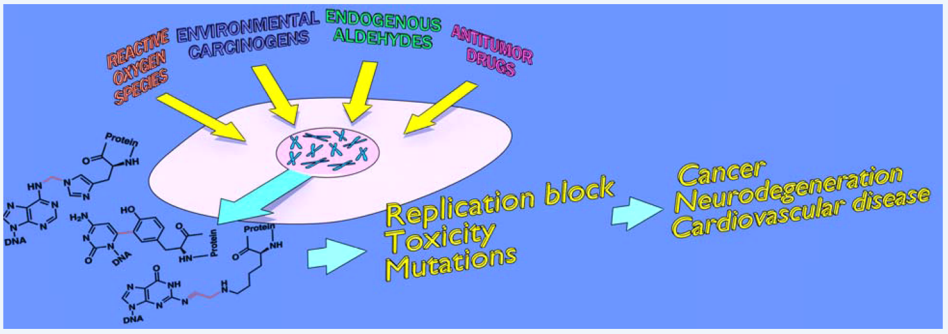

CONSPECTUS: Noncovalent DNA–protein interactions are at the heart of normal cell function. In eukaryotic cells, genomic DNA is wrapped around histone octamers to allow for chromosomal packaging in the nucleus. Binding of regulatory protein factors to DNA directs replication, controls transcription, and mediates cellular responses to DNA damage. Because of their fundamental significance in all cellular processes involving DNA, dynamic DNA–protein interactions are required for cell survival, and their disruption is likely to have serious biological consequences.

DNA–protein cross-links (DPCs) form when cellular proteins become covalently trapped on DNA strands upon exposure to various endogenous, environmental and chemotherapeutic agents. DPCs progressively accumulate in the brain and heart tissues as a result of endogenous exposure to reactive oxygen species and lipid peroxidation products, as well as normal cellular metabolism. A range of structurally diverse DPCs are found following treatment with chemotherapeutic drugs, transition metal ions, and metabolically activated carcinogens. Because of their considerable size and their helix-distorting nature, DPCs interfere with the progression of replication and transcription machineries and hence hamper the faithful expression of genetic information, potentially contributing to mutagenesis and carcinogenesis. Mass spectrometry-based studies have identified hundreds of proteins that can become cross-linked to nuclear DNA in the presence of reactive oxygen species, carcinogen metabolites, and antitumor drugs. While many of these proteins including histones, transcription factors, and repair proteins are known DNA binding partners, other gene products with no documented affinity for DNA also participate in DPC formation. Furthermore, multiple sites within DNA can be targeted for cross-linking including the N7 of guanine, the C-5 methyl group of thymine, and the exocyclic amino groups of guanine, cytosine, and adenine. This structural complexity complicates structural and biological studies of DPC lesions.

Two general strategies have been developed for creating DNA strands containing structurally defined, site-specific DPCs. Enzymatic methodologies that trap DNA modifying proteins on their DNA substrate are site specific and efficient, but do not allow for systematic studies of DPC lesion structure on their biological outcomes. Synthetic methodologies for DPC formation are based on solid phase synthesis of oligonucleotide strands containing protein-reactive unnatural DNA bases. The latter approach allows for a wider range of protein substrates to be conjugated to DNA and affords a greater flexibility for the attachment sites within DNA. In this Account, we outline the chemistry of DPC formation in cells, describe our recent efforts to identify the cross-linked proteins by mass spectrometry, and discuss various methodologies for preparing DNA strands containing structurally defined, site specific DPC lesions. Polymerase bypass experiments conducted with model DPCs indicate that the biological outcomes of these bulky lesions are strongly dependent on the peptide/protein size and the exact cross-linking site within DNA. Future studies are needed to elucidate the mechanisms of DPC repair and their biological outcomes in living cells.

INTRODUCTION

DNA–protein cross-links (DPCs) are bulky DNA lesions formed when proteins become covalently trapped on chromosomal DNA. DPCs can be induced by exposure to various physical and chemical agents including ionizing radiation, UV light, transition metal ions, environmental contaminants, and common anticancer drugs such as nitrogen mustards, mitomycin C, and platinum compounds (**1**–**9** in Figure 1).[1−3] DPCs are also detected in untreated cells, probably a result of endogenous exposure to reactive oxygen species and α,β-unsaturated aldehydes formed as lipid peroxidation byproducts.[1,2] DNA–protein

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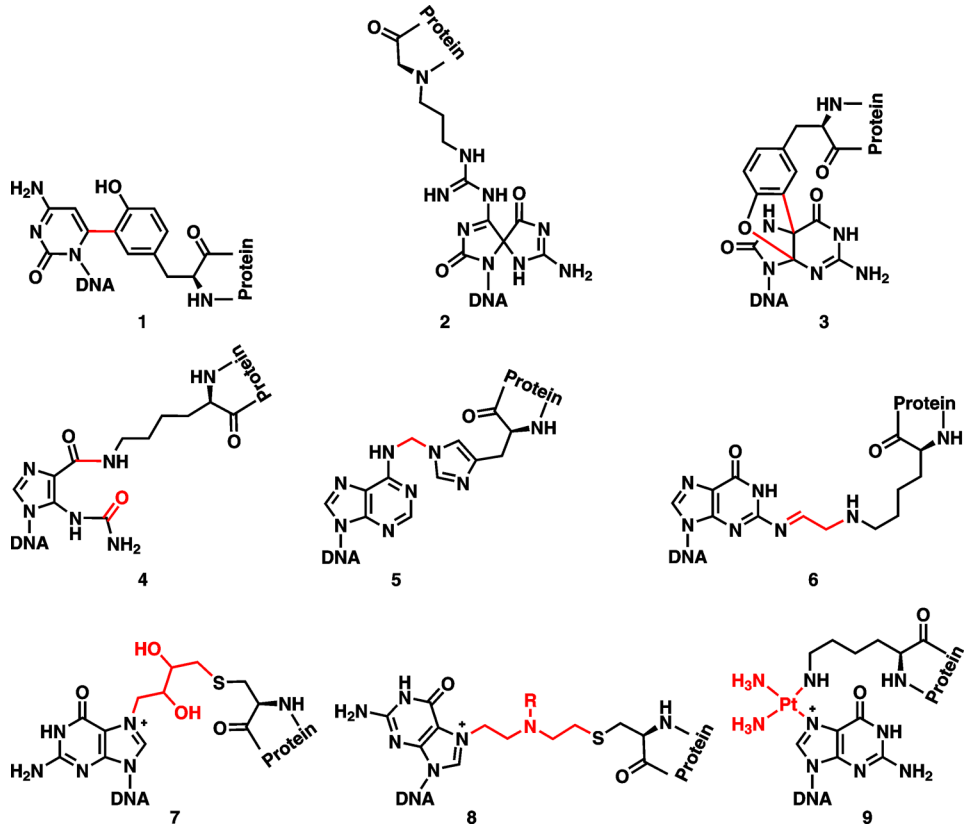

Figure 1. Chemical structures of DNA−protein cross-links induced by reactive oxygen species (**1**−**3**), reactive nitrogen species (**4**), formaldehyde (**5**), acrolein (**6**), 1,2,3,4-diepoxybutane (**7**), nitrogen mustards (**8**), and cisplatin (**9**).

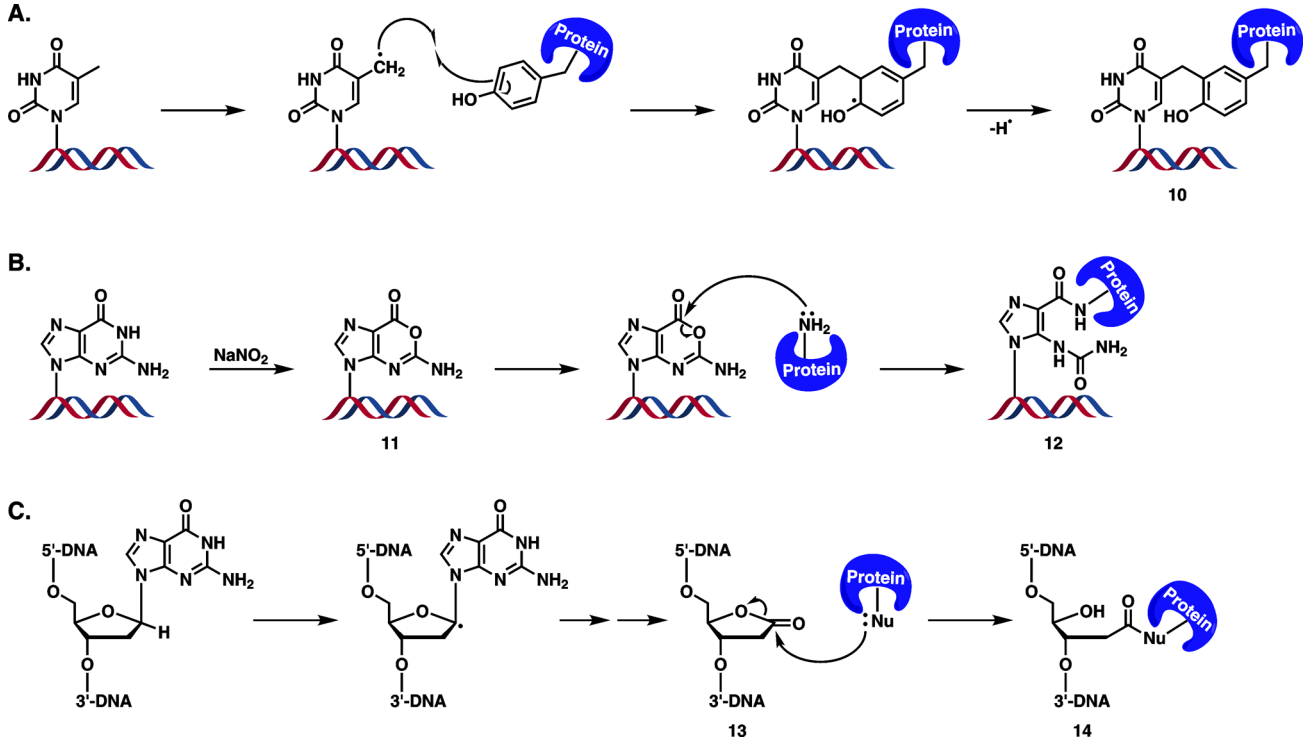

Figure 2. DPC induction by exposure to reactive oxygen and nitrogen species: direct cross-linking by a free radical mechanism (A), 2′-deoxyoxanosine-mediated reaction (B), and cross-linking via oxidized abasic site lesions (2-deoxyribonolactone) (C).

conjugates can arise as part of normal cellular metabolism, e.g. topoisomerase intermediates.[3] DPCs accumulate in heart and brain tissues with age[4] and may play a role in aging, cancer, and neurodegenerative diseases.

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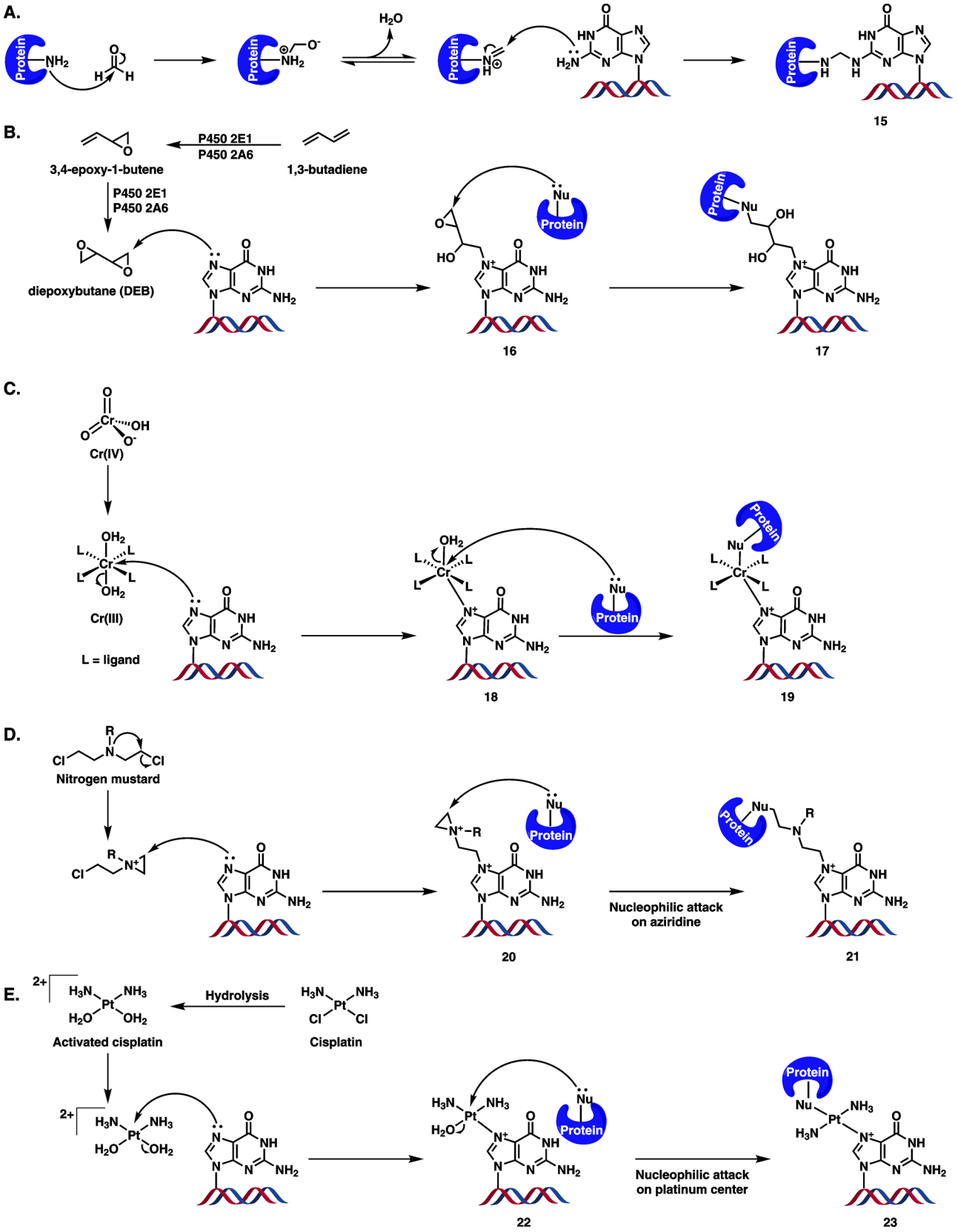

Figure 3. DPC induction by reactions with exogenous electrophiles: formaldehyde (A), 1,3-butadiene (B), chromate (C), antitumor nitrogen mustards (D), and cisplatin (E). In panel (C), "L" signifies ligands (cysteine, glutathione, histidine, or ascorbate).[24]

Reactive oxygen and nitrogen species (ROS and RNS) are physiologically produced during cellular respiration, immune response, and inflammation.[5] ROS reactions with guanine, cytosine, or thymine bases of DNA and lysine or tyrosine side chains of proteins create free radicals or electrophilic lesions that can subsequently react with another biomolecule to induce

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covalent DNA−protein cross-links (**1** and **2** in Figure 1; Figure 2A).[1−3,6−9]

DNA−protein cross-linking can be initiated by reactions of protein side chains with electrophilic DNA lesions.[1] For example, 5-amino-3-β-(2-deoxy-D-ribofuranosyl)-3*H*-imidazo[4,5-*d*]-[1,3]oxazin-7-one (2′-deoxyoxanosine) lesions (**11** in Figure 2B) are formed from deoxyguanosine in DNA upon exposure to RNS. The *O*-acylisourea functionality of oxanosine is susceptible to nucleophilic attack by protein side chains, forming an amide bond between the protein and the DNA (**4** in Figure 1, **12** in Figure 2B).[10] Similarly, oxidized abasic sites generated by exposure to ROS (2-deoxyribonolactone, **13** in Figure 2C) can form amide conjugates to proteins such as *Escherichia coli* endonuclease III (Endo III) and DNA polymerase β (Pol β) (**14** in Figure 2C),[11,12] while ROS-induced spirodiiminodihydantoin lesions form covalent DPCs to Arg and Lys side chains of proteins (**2** in Figure 1).[13]

Exposure to industrial, household, and environmental chemicals can produce DPCs in cells. For example, inhalation exposure of laboratory animals to the important industrial chemical formaldehyde (FA) induces large numbers of DPCs in nasal tissues, contributing to tissue-specific tumor development.[14,15] FA-mediated DPC formation involves Schiff base intermediates on proteins and DNA, which react with another biomolecule to generate methylene cross-links (**15** in Figure 3A).[15,16] DNA−protein cross-linking by FA is reversible, but is quite efficient and can involve a wide range of protein targets.[17] The ability of FA to reversibly trap proteins on DNA has been exploited in various biochemical applications such as ChIP-Seq, which combines chromatin immunoprecipitation (ChIP) with DNA sequencing to identify the binding sites of DNA-associated proteins.[18]

The important industrial and environmental chemical 1,3-butadiene (BD) is metabolically activated to 1,2,3,4-diepoxybutane (DEB), which is a *bis*-electrophile capable of cross-linking cellular biomolecules. DEB alkylates the N7 position of guanines in DNA, initially generating 2-hydroxy-3,4-epoxybut-1-yl monoadducts (**16** in Figure 3B), which can subsequently react with nucleophilic amino acid side chains of neighboring proteins to form butanediol DPCs (**7** in Figure 1, **17** in Figure 3B).[19]

Environmental and occupational exposure to transition metals such as chromium and nickel can cause DPC formation in cells and tissues.[20,21] Cr(VI) is transported into cells via a nonspecific anion carrier, where it can be reduced to Cr(III).[22] Cr(III) forms coordination complexes with phosphate groups and guanine bases of DNA, followed by binding to amino acid side chains of proteins to generate DPCs (**19** in Figure 3C).[23,24] In addition, Cr(VI)/Cr(III) redox cycling produces ROS, which can contribute to the formation of DPCs by the free radical mechanism shown in Figure 2A.[25]

Many common chemotherapeutic agents are capable of inducing both DNA−DNA and DNA−protein cross-links, thereby inhibiting DNA replication and initiating apoptosis. Nitrogen mustards are among the oldest and the most extensively utilized antitumor agents.[26,27] Drugs of this class, such as mechlorethamine, chlorambucil, and cyclophosphamide, contain two *N*-(2-chloroethyl) groups, which can sequentially alkylate two nucleophilic sites within proteins and DNA via aziridinium ion intermediates (Figure 3D). Recent mass spectrometry-based studies have shown that the majority of nitrogen mustard-induced DPCs are formed via alkylation of the N7 position of guanine in DNA and cysteine amino side chains in proteins (**8** in Figure 1).[28−30]

Platinum compounds such as *cis*-diamminedichloroplatinum(II) (cisplatin) are commonly used as anticancer drugs.[31] Although DNA−DNA cross-linking is considered the major mechanism of cisplatin-mediated antitumor activity,[32] platinum compounds also form large amounts of DPCs by binding to N7-guanine positions of DNA and cysteine, arginine, and lysine side chains of proteins (**9** in Figure 1; Figure 3E).[33,34] Other classes of DNA-modifying chemotherapeutic agents such as haloethylnitrosoureas also induce DPC lesions in cells.[35]

Over the past 10 years, research in our laboratory has focused on identifying the structures of DPCs induced by various cross-linking agents, including protein identities and the connectivity between proteins and DNA. We have also developed synthetic strategies for preparing DNA strands containing hydrolytically stable, structurally defined model DPCs and conducted polymerase bypass experiments to characterize the influence of DPC lesions on DNA replication.

IDENTITIES OF PROTEINS PARTICIPATING IN DPC FORMATION

Cellular DPCs are vastly complex and extremely heterogeneous DNA lesions due to the potential involvement of a wide range of proteins of varying size, physicochemical properties, cellular distribution, and cellular functions.[29,30,36,37] DNA−protein cross-linking may involve multiple sites on DNA and numerous amino acid side chains on proteins, further contributing to heterogeneity of these unusually bulky lesions (Figure 1).

Studies with Model Proteins

Our initial efforts to characterize DNA−protein cross-linking by *bis*-electrophiles have focused on model studies with recombinant AGT protein. AGT is an important DNA repair protein that recognizes promutagenic O^6-alkylguanine lesions. Following protein binding to damaged DNA, the O^6-alkylguanine nucleotide is flipped out of the base stack into the protein's active site, and the O^6-alkyl group is transferred to the activated side chain thiolate

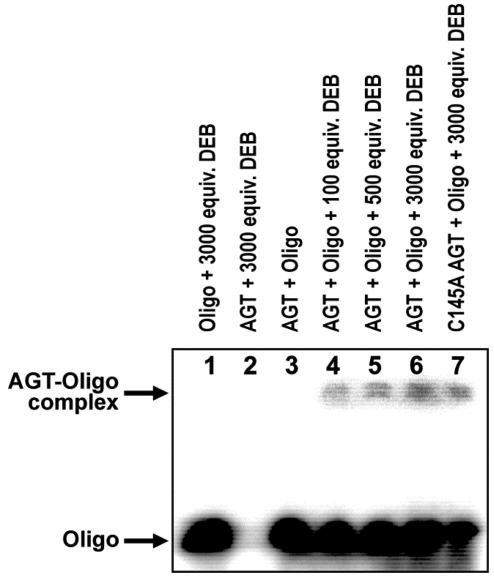

Figure 4. SDS-PAGE analysis of ^{32}P-endlabeled DNA duplexes (5′-GGA GCT GGT GGC GTA GGC-3′, (+) strand) following incubation with DEB and hAGT (lanes 4−6) or C145A hAGT (lane 7). AGT-DNA cross-links are observed as slowly moving bands on the gel. Lanes 1−3 serve as negative controls. Reprinted with permission from ref 42. Copyright 2006 American Chemical Society.

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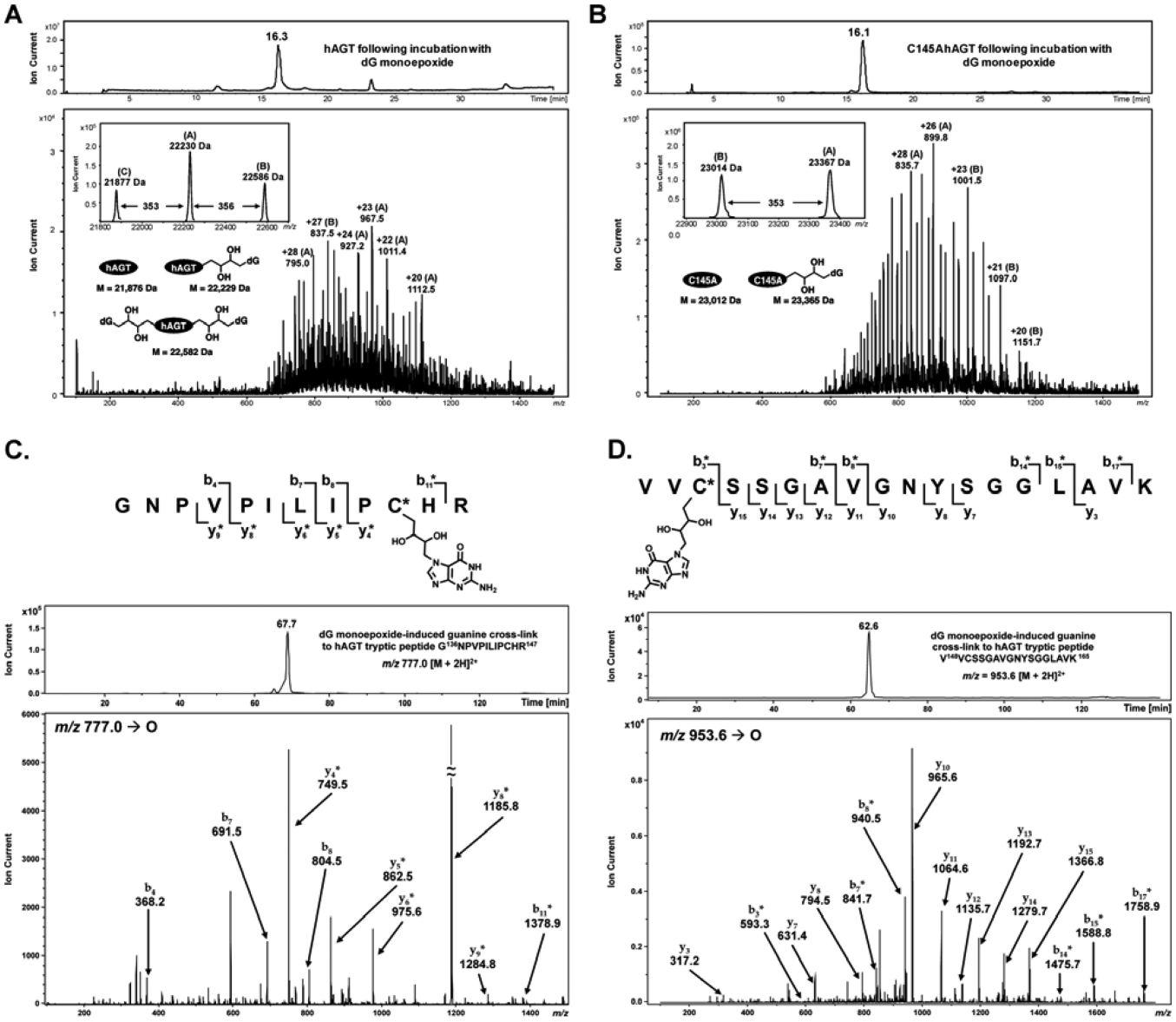

Figure 5. HPLC-ESI$^+$-MS analysis of hAGT protein (A) and dG monoepoxide-induced butanediol cross-links to hAGT (B). HPLC-ESI$^+$-MS/MS analysis of hAGT tryptic peptides G136NPVPILIPCHR147 (C) and V148VCSSGAVGNYSGGLAVK165 (D) containing a butanediol cross-link between Cys^{145} and guanine following treatment of the wild-type protein with dG monoepoxide. Reprinted with permission from ref 42. Copyright 2006 American Chemical Society

anion of Cys^{145}, restoring normal guanine.[38,39] AGT protects the human genome from the damaging effects of simple alkylating agents, but exacerbates the toxic and mutagenic effects of *bis*-electrophiles such as dibromoethane[40] and diepoxybutane,[41] supposedly a result of the formation of toxic AGT-DNA cross-links.

The molecular structures of hAGT-DNA conjugates were investigated in our laboratory using a mass spectrometry-based approach.[28,33,42] ^{32}P-labeled double-stranded oligonucleotides 5′-GGAGCTGGTGGCGTAGGC-3′ (+ strand, representing codons 10−15 of the *K-ras* protooncogene) were incubated with hAGT or C145A hAGT mutant in the presence of increasing amounts of DEB, mechlorethamine, or cisplatin. Denaturing SDS-PAGE analyses of the reaction mixtures have revealed distinct low mobility bands corresponding to covalent AGT-DNA conjugates (Figure 4).[28,33,42]

To identify the cross-linking sites within AGT upon reaction with DEB, hAGT or C145A AGT mutant was incubated with synthetic N^7-(2′-hydroxy-3′,4′-epoxybut-1′-yl)-guanine (dG monoepoxide) as a model for monoalkylated DNA.[42] The adducted proteins were analyzed by HPLC-ESI$^+$-MS. For wild type hAGT treated with dG monoepoxide, both monoalkylated hAGT containing a single butanediol-dG cross-link and hAGT containing two butanediol-dG cross-links were observed (Figure 5A). The corresponding reaction of C145A hAGT mutant with dG monoepoxide yielded monoalkylated protein containing a single butanediol-dG cross-link (Figure 5B).[42] These results indicate that cross-linking can take place via AGT Cys^{145}, but at least one other amino acid side chain can participate in DEB-mediated cross-linking. The identity of the second cross-linking site was established by HPLC-ESI$^+$-MS of tryptic peptides, which revealed doubly charged G^{136}NPVPILIP CHR^{147} and V^{148}VCSSGAVGNYSGGLAVK^{165} peptides containing butanediol cross-links to free guanine.[42] MS/MS spectra of these modified peptides were consistent with the alkylation

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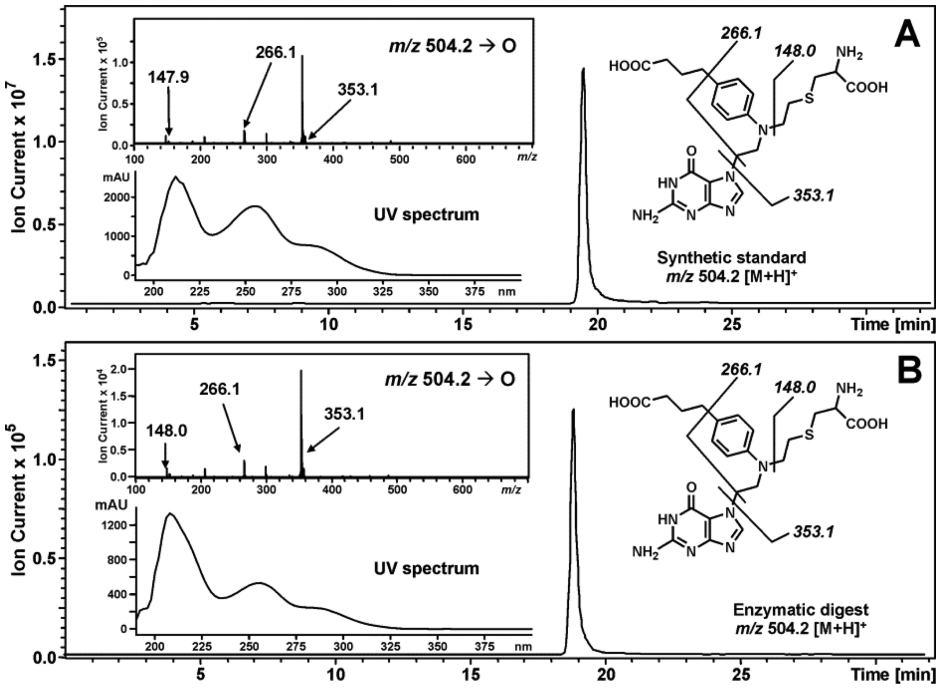

Figure 6. HPLC-ESI$^+$-MS/MS analysis of amino acid-guanine conjugates of chlorambucil. (A) Extracted ion chromatogram of synthetic Cys-N7G-PBA (m/z 504.2 [M + H]$^+$). Inset: MS/MS fragmentation and UV spectrum. (B) Extracted ion chromatogram of AGT-derived Cys-N7G-PBA (m/z 504.2 [M + H]+. Inset: MS/MS fragmentation and UV spectrum. Reprinted with permission from ref 28. Copyright 2008 American Chemical Society.

occurring at Cys^{145} and Cys^{150}, respectively (Figure 5C,D).[42] To establish the molecular structure of DEB-induced AGT-DNA conjugates, the cross-linked protein was digested to its constitutive amino acids using carboxypeptidase Y and proteinase K to yield 1-(*S*-cysteinyl)-4-(guan-7-yl)-2,3-butanediol (Cys-Gua-BD, m/z 359, [M + H]$^+$), which had the same HPLC retention time and tandem mass spectra as synthetic Cys-Gua-BD (**7** in Figure 1).[42]

Similar studies conducted with antitumor nitrogen mustards mechlorethamine and chlorambucil have identified the same two AGT residues (Cys^{145} and Cys^{150}) as targets for cross-linking to DNA.[28] HPLC-ESI$^+$-MS/MS analyses of total digests have identified *N*-(2-[*S*-cysteinyl]ethyl)-*N*-(2-[guan-7-yl]ethyl)-*p*-aminophenylbuyric acid and *N*-(2-[*S*-cysteinyl]ethyl)-*N*-(2-[guan-7-yl]ethyl)methylamine (**8** in Figure 1) in reaction mixtures treated with chlorambucil and mechlorethamine, respectively, which coeluted with the corresponding amino acid conjugates prepared synthetically (Figure 6). Analogous AGT-DNA reactions in the presence of cisplatin produced cross-links at multiple sites of the protein, including Glu^{110}, Lys^{125}, Cys^{145}, His^{146}, Arg^{147}, and Cys^{150}.[33] Unlike DEB and NM-mediated lesions, cisplatin-mediated DPCs were reversible, converting to the corresponding DNA−DNA cross-links upon heating.[33]

Studies with Cell Free Extracts

While studies with model proteins are useful for establishing the structural details of DNA−protein cross-linking, they cannot identify biologically relevant protein targets of *bis*-electrophiles. Such investigations are challenging because cellular DPCs can in theory encompass hundreds or even thousands of proteins. Additionally, many nuclear proteins have an intrinsic affinity for DNA, therefore one must distinguish between covalent DPCs and noncovalent DNA−protein complexes. Finally, the resulting DNA−protein conjugates can be difficult to detect because cross-linked proteins make up only a small fraction of total proteome, with a 100−1000-fold excess of unmodified protein present in the same sample.

In an attempt to overcome these challenges, we have developed an affinity based methodology for selective enrichment of proteins irreversibly trapped on DNA following exposure to *bis*-electrophiles (Figure 7). In this approach, biotinylated DNA duplexes were incubated with nuclear protein extracts from human cells in the presence of *bis*-electrophiles.[29,33,37] Following affinity capture of DNA and DNA-conjugated proteins on streptavidin beads and stringent washing steps to remove any noncovalently bound proteins, the cross-linked proteins were released from the DNA backbone by heating (Figure 7A).[29,33,37] This is possible because alkylation of the N7 position of dG in DNA destabilizes the glycosidic bond, making them susceptible to thermal hydrolysis.[43] The released protein-guanine conjugates were resolved by SDS-PAGE (Figure 7B) and subjected to in-gel tryptic digestion, followed by HPLC-ESI$^+$-MS/MS analysis of the resulting peptides to identify the proteins.[29,33,37] Gel electrophoresis analyses have revealed that treatment with 25 μM cisplatin, 500 μM mechlorethamine, and 25 mM DEB produced similar numbers of DPC lesions, revealing 100-fold differences in the efficiency of DPC formation by these *bis*-electrophiles.[29,33,37] This technique can be adapted to hydrolytically stable lesions by adding an enzymatic DNA digestion step (Groehler and Tretyakova, unpublished observations).

Our affinity capture-mass spectrometry experiments have identified a wide variety of proteins forming DNA−protein cross-links in the presence of nitrogen mustards, cisplatin, and DEB.[29,33,37] These gene products encompass a range of molecular functions including cellular homeostasis, DNA replication/repair, transcriptional regulation, cellular architecture, and translation/RNA splicing (Figure 7C).[29,33,37] While some proteins such as Msh2, Fen-1, PARP, actin, and GAPDH were targeted by all three electrophiles, significant differences between the protein targets of nitrogen mustards, DEB, and cisplatin were observed, probably

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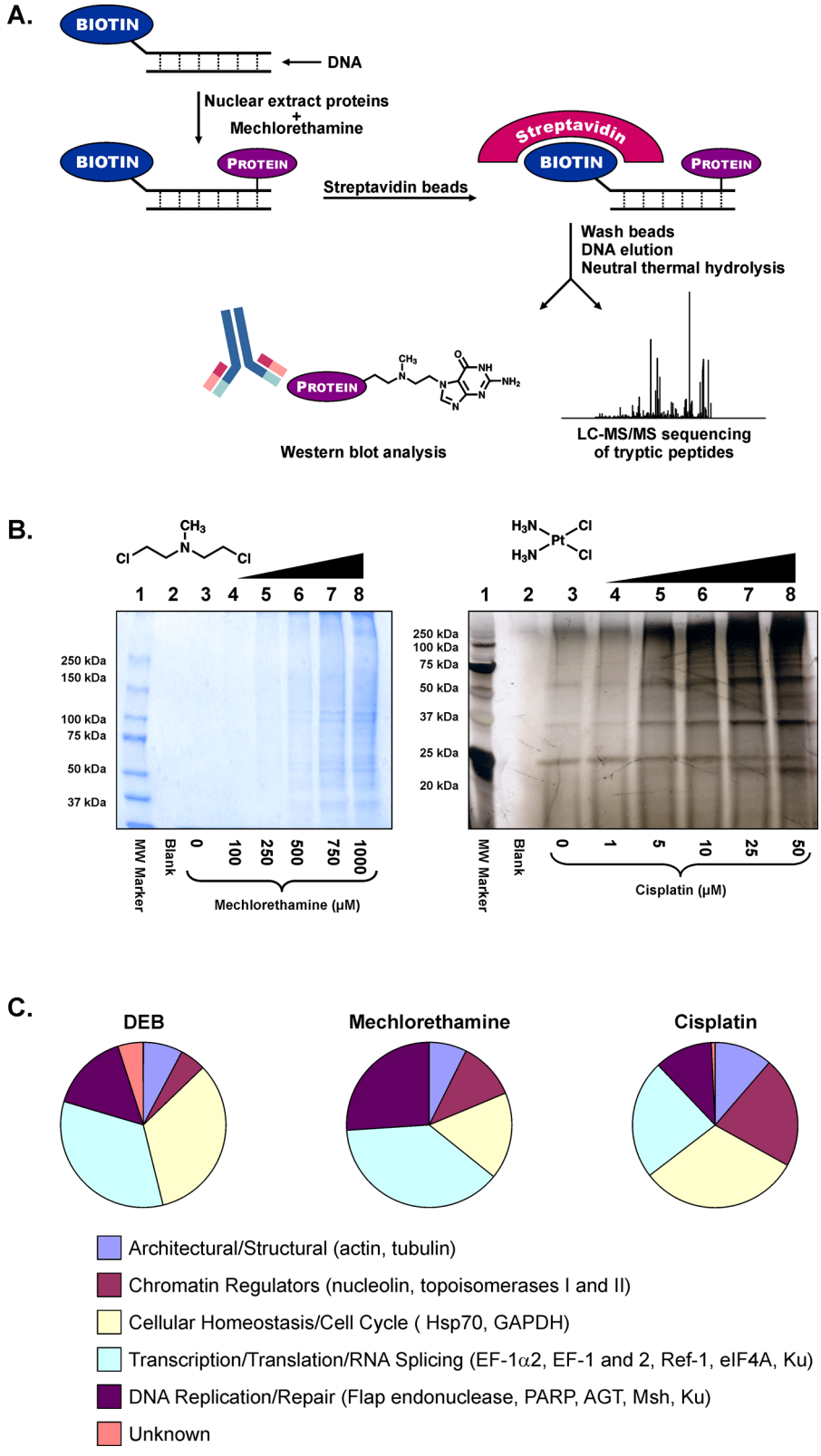

Figure 7. Experimental scheme for biotin capture methodology used to isolate DPCs from nuclear protein extracts incubated with mechlorethamine in the presence of double-stranded DNA (A), concentration-dependent formation of DPCs in nuclear protein extracts following exposure to mechlorethamine and cisplatin (B), and cellular functions of CHO and human proteins that form DPCs in the presence of DEB, mechlorethamine, and cisplatin (C).[19,29,33] Reprinted with permission from ref 29. Copyright 2009 American Chemical Society.

a result of different mechanisms of cross-link formation.[29,33,37] As discussed above, nitrogen mustards and diepoxides preferentially modify cysteine residues of proteins, while platinum compounds can target basic protein side chains such as arginine and lysine (Figure 1). Furthermore, the cross-linking order (with initial reaction happening on protein vs on DNA) may differ for different reagents, potentially leading to separate proteins being targeted.[44]

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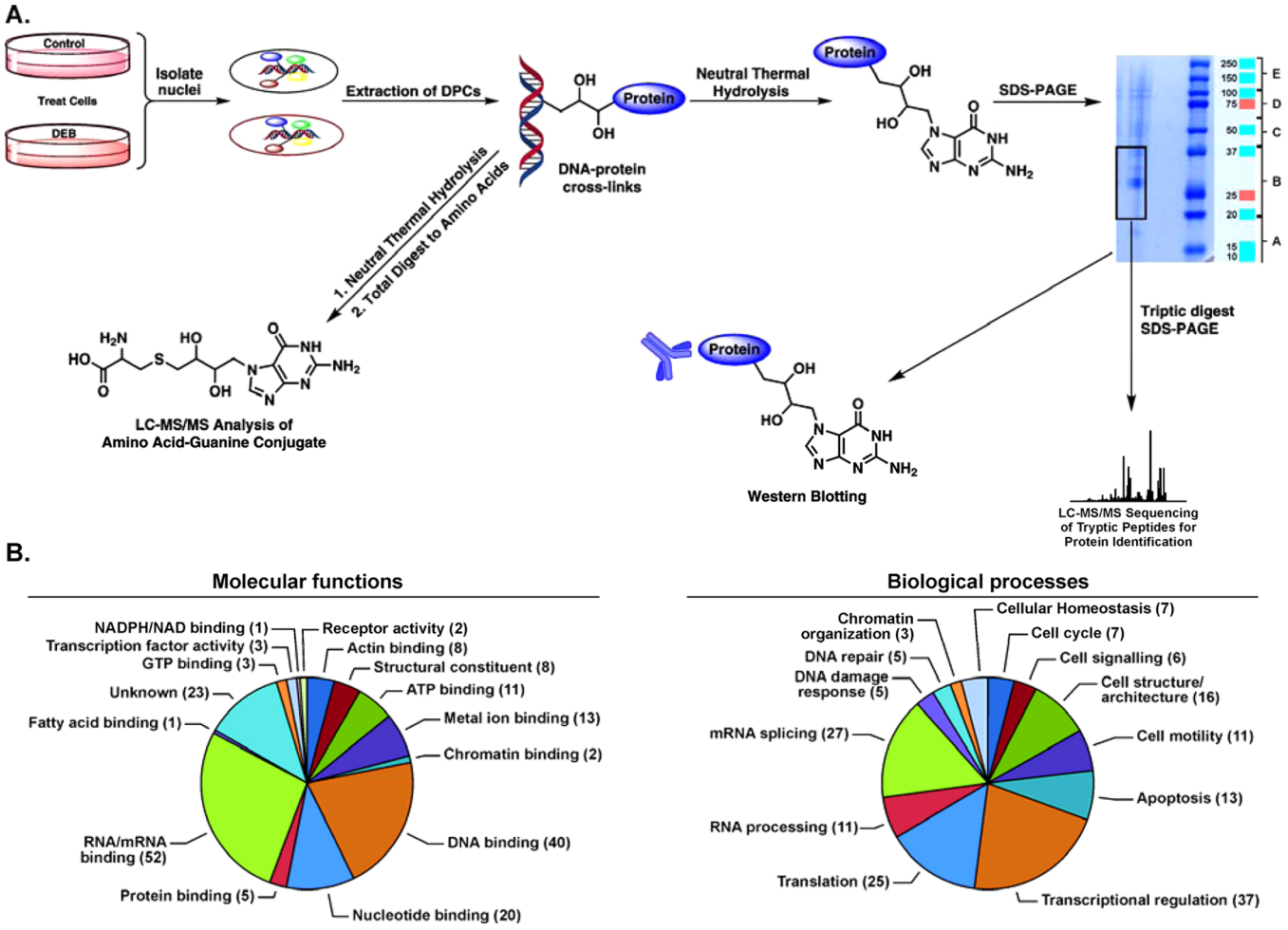

Figure 8. Strategy for the isolation and analysis of DPCs from diepoxybutane (DEB)-treated cells (A) and functional classification of proteins identified in proteomic analysis of DNA−protein cross-links in nuclear protein extracts from HeLa cells treated with DEB (B). Reprinted with permission from ref 19. Copyright 2013 American Chemical Society.

DPC Formation in Human Cell Culture

While the affinity capture approach employing biotinylated DNA duplexes allows for global analysis of DPCs following specific enrichment of cross-linked proteins, it employs synthetic DNA and cell-free protein extracts, which are devoid of normal DNA−protein interactions. A different methodology was needed to study DPC formation in cellular systems, where DNA is organized in chromatin and interacts with specific proteins in a sequence-dependent manner. We have developed a modified phenol-chloroform extraction procedure to isolate genomic DNA along with any covalently attached proteins (Figure 8A). DPC lesions are found on the interface between the aqueous and organic layers during extraction.[30] Following DNA precipitation, the cross-linked proteins are released from the DNA backbone by heating or enzymatic digestion, and the resulting guanine-protein conjugates are resolved by SDS-PAGE and identified using mass spectrometry of tryptic peptides as described above (Figure 8).[19,30,33]

Using this approach, DNA−protein cross-linking was investigated in human fibrosarcoma (HT1080) cells treated with mechlorethamine,[30] DEB,[19] and cisplatin.[33] We found that DNA−protein cross-linking in live cells is more efficient as compared to cell free extracts, requiring 10−100-fold lower drug concentrations.[19,30,33] This is probably a result of native protein−DNA interactions and sequence-specific DNA binding which cannot be modeled using short synthetic DNA duplexes. A large fraction of proteins that participated in DPC formation in HT1080 cells were nuclear proteins involved in transcriptional regulation as transcription factors, activators, or repressors (Figure 8B).[19,30,33] Other molecular functions of the identified proteins included protein binding, metal ion binding, and structural roles.[19,30,33] Several HMG box binding proteins, nucleophosmin, and matrin-3 were cross-linked to nuclear DNA by all three *bis*-electrophiles, but many others showed specificity for one of the three agents.[19,30,33]

SYNTHESIS OF DNA SUBSTRATES CONTAINING SITE-SPECIFIC DPCs

The availability of site-specific DNA substrates containing biologically relevant, hydrolytically stable DNA−protein conjugates is a requirement for any detailed studies aiming to understand the biological consequences of DPC formation and their repair mechanisms. This section of the Account discusses enzymatic and synthetic methodologies that have been developed for the preparation of site-specific, structurally defined DPCs that can be used in structural and biological investigations.

Enzymatic Methods for DPC Formation

Reductive trapping of enzyme−DNA complexes was among the first methods to create site-specific DNA−protein conjugates.[45] This approach traps DNA repair intermediates by reducing the

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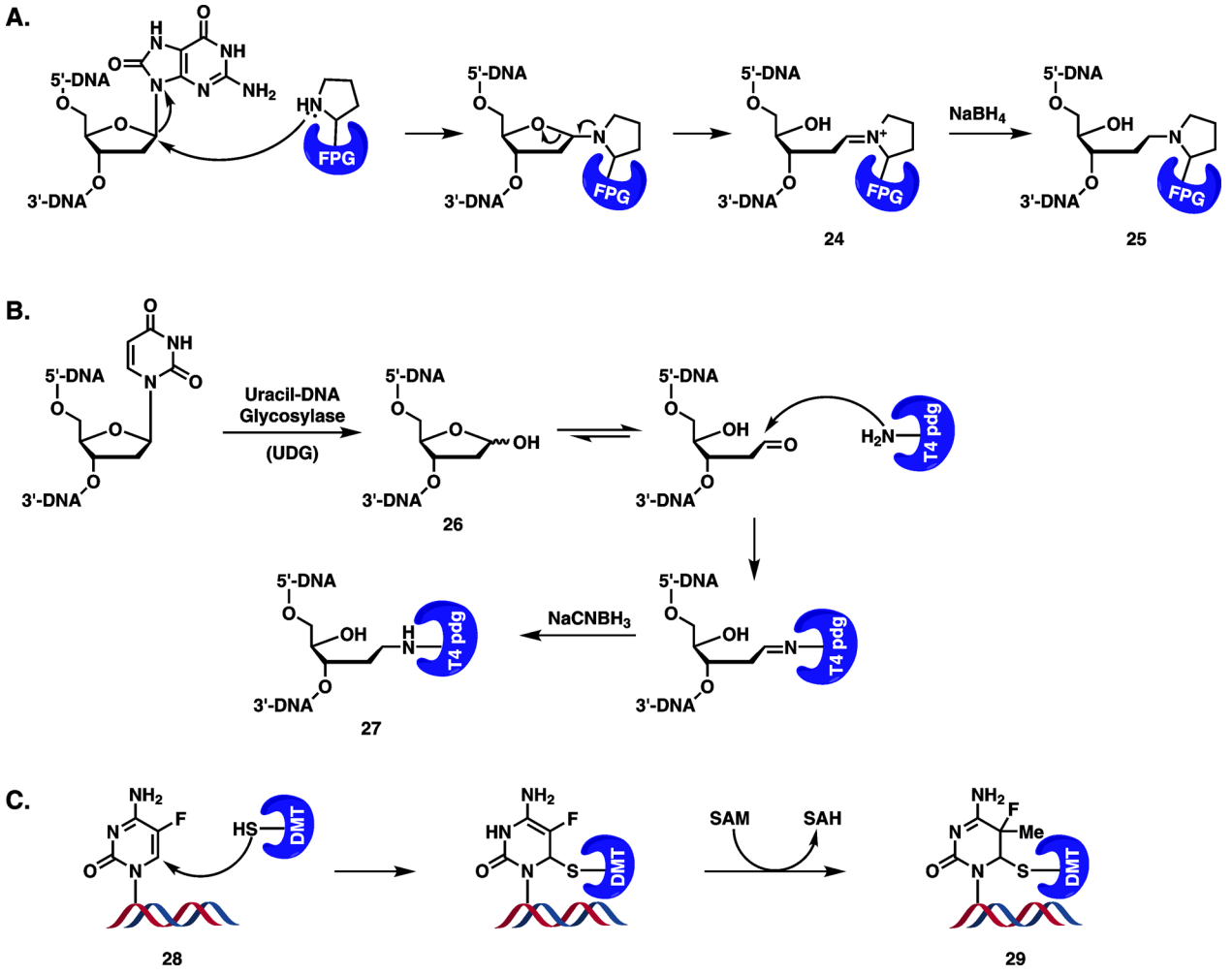

Figure 9. Enzymatic synthesis of site-specific DPC using abasic site-containing DNA and T4 pyrimidine dimer glycosylase (A), 8-oxo-dG-containing DNA and UDG protein (B), and 5-fluorocytosine containing DNA and DNA methyltransferase (DNMT) (C).

transient imino bond between base excision repair proteins and their DNA substrates. During base excision repair (BER), bifunctional DNA glycosylase/β-lyases use a basic amino acid side chain to displace the damaged base, forming a covalent DNA−protein intermediate. For example, *E. coli* formamidopyrimidine glycosylase (FPG) uses the N-terminal proline to displace 8-oxoguanosine (8-oxoG), resulting in a covalent linkage to the C-1′ position of the sugar (Figure 9A). The resulting aminal intermediate spontaneously isomerizes to the corresponding imine **24**, which can be reduced to stable amine **25** in the presence of sodium borohydride or cyanoborohydride.[46] While the original application of this approach was to understand the enzymatic mechanisms of BER enzymes, it can also be used to generate DNA strands containing site-specific DPC lesions (Figure 9A). For example, this methodology was employed to create DPCs to T4 pyrimidine dimer glycosylase,[47] endonuclease VIII (Nei),[48] formamidopyrimidine DNA glycosylase (Fpg),[46] and 8-oxo-guanine DNA glycosylase (Ogg).[49]

Monofunctional DNA glycosylases such as UDG, MUG, AlkA, Aag, and MutY do not form an aminal intermediate with their DNA substrate but rather catalyze depurination of the damaged bases. The resulting abasic sites **26** spontaneously convert between the closed ring structure and the open aldehyde form (Figure 9B). The latter can react with basic protein side chains within the DNA−protein complex to form transient Schiff bases, which can be subsequently reduced to form stable DPCs (**27** in Figure 9B).[45] Alternatively, masked abasic sites can be introduced into DNA strands synthetically and subsequently released by photolysis.[50]

In addition to DNA glycosylases, other DNA−enzyme intermediates can be trapped on DNA to generate site-specific DPCs. For example, DNA methyltransferases (DNMT) have been conjugated to DNA strands containing 5-aza-dC or 5-fluoro-dC.[45,51] The normal biological function of DNMT is to generate 5-methylcytosines by transferring a methyl group from *S*-adenosylmethionine (SAM) to cytosine bases within CG dinucleotides of DNA. The DNMT enzymatic mechanism involves the formation of a transient DNA−protein intermediate.[52] For DNA substrates containing 5-fluoro-dC or 5-aza-dC instead of native dC, the intermediate cannot dissociate, leading to irreversible trapping of methyltransferases on DNA (Figure 9C). This approach can be used to generate site-specific DNMT-DNA conjugates **29** by employing synthetic DNA strands with strategically placed 5-fluoro-dC or 5-aza-dC residues.[51,53]

Overall, enzymatic strategies for DPC generation have been extremely valuable in the field, providing structurally defined DPC substrates for the majority of previous studies of DPC replication and repair.[51,53] An advantage of this approach is that it efficiently forms chemical bonds between specific residues within proteins and DNA in a site-specific manner.[45] However, only a

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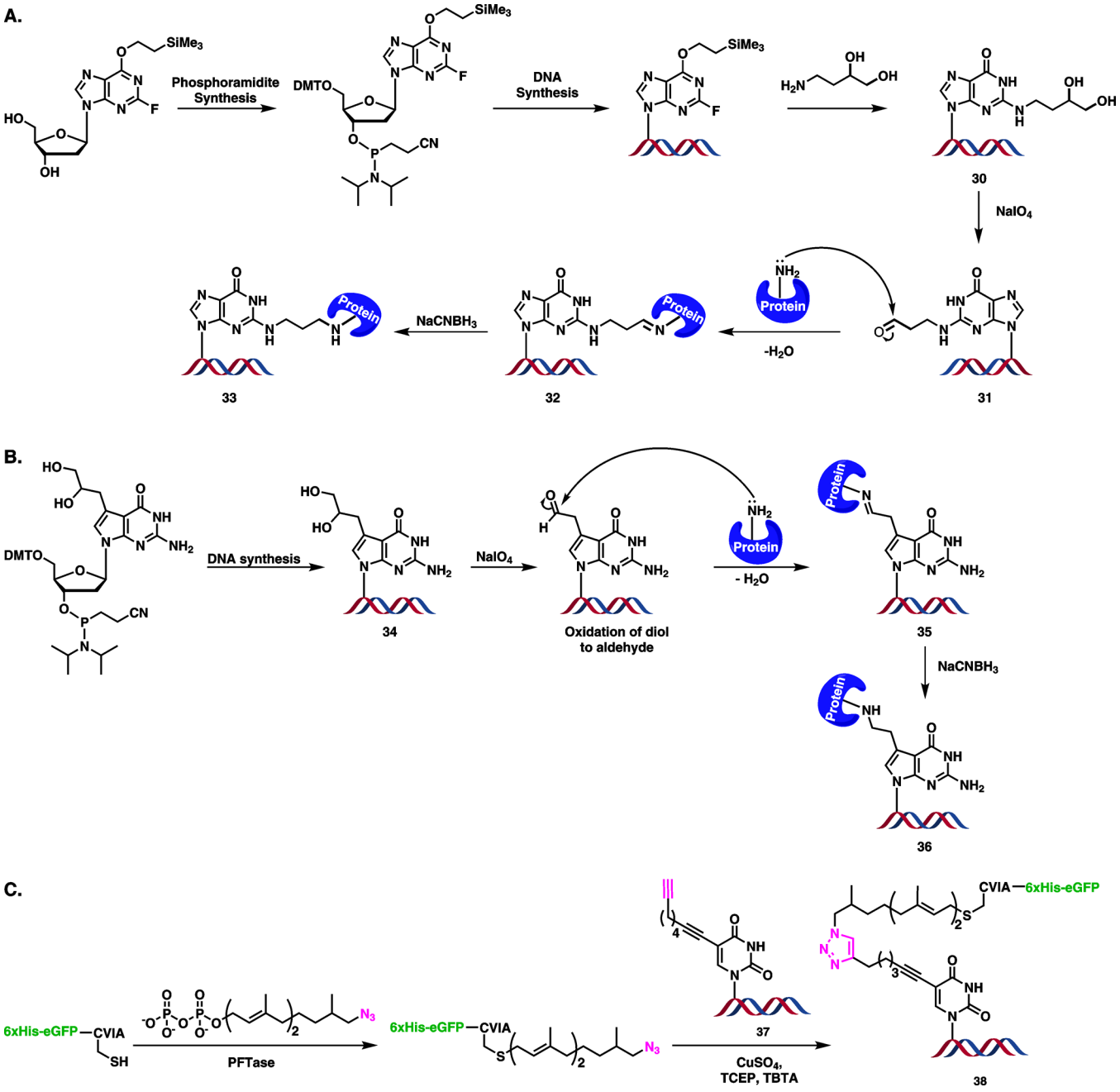

Figure 10. DPC synthesis using reductive amination of a masked aldehyde intermediate at the N^2-dG (A) and at the 7-deaza-dG position of DNA (B). Generation of site-specific DNA−protein conjugates by copper-catalyzed [3 + 2] Huisgen cycloaddition (click reaction) between an alkyne group from 5-(octa-1,7-diynyl)-uracil in DNA and an azide group within modified green fluorescent protein (6 × His-eGFP) (C).[60]

small group of proteins such as DNA glycosylases and DNA methyltransferases can be attached to DNA strands, not allowing for systematic studies of protein size, identity, and cross-linking site on DPC replication and repair. Furthermore, the chemical structures of DPCs generated by enzymatic approaches are not representative of the lesions produced upon exposure to free radicals, *bis*-electrophiles, and ionizing radiation (Figure 1).

Synthetic Methods to Form DPCs

Synthetic methodologies can be used to site-specifically conjugate a range of proteins to specified positions within DNA. In a typical experiment, DNA strands containing unnatural DNA nucleobases incorporating a protein-reactive moiety are prepared by solid phase synthesis, followed by incubation with protein of interest to form DPC. For example, Ide et al. have taken advantage of the propensity of dOxo lesions (**11** in Figure 2B) to form stable amide bonds with proteins.[10] dOxo was synthesized via nitrosative oxidation of 2′-deoxyguanosine, converted to the corresponding phosphoramidite, and site-specifically incorporated into synthetic DNA strands.[10] Upon incubation with an excess of a target protein, dOxo in DNA formed a stable amide bond to the protein (Figure 2B).

Reductive amination is commonly used to generate site-specific DPCs, using an approach analogous to mechanism-based strategy shown in Figures 9A,B. However, instead of relying on glycosylase-induced abasic sites, synthetic approaches incorporate aldehyde functionalities within DNA nucleobases (Figure 10A). Sanchez and Lloyd have prepared DNA strands

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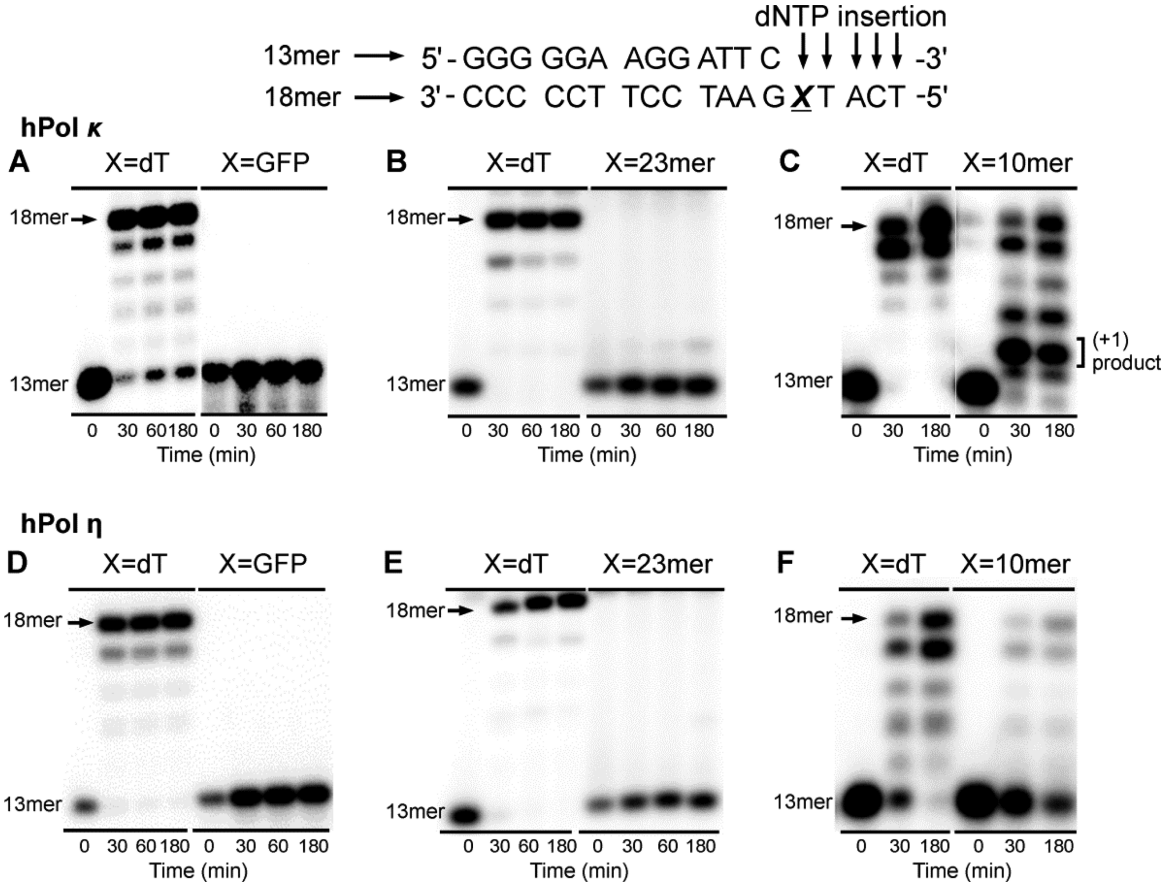

Figure 11. Extension of ^{32}P-labeled primers containing unmodified dT or DNA−protein and DNA−peptide conjugates of increasing size by human lesion bypass polymerases hPol κ (A−C) and hPol η (D−F) under standing start conditions. Reprinted with permission from ref 60. Copyright 2014 American Chemical Society.

incorporating site-specific oxopropyl-2′-deoxyguanosine (**31** in Figure 10A). When incubated with target peptides and proteins, **31** forms reversible Schiff base adducts **32**, which can be stabilized in the presence of $NaCNBH_3$ to generate stable conjugates **33**.[54] Marnett et al. and Lloyd et al. have utilized this methodology to prepare site specific DPCs connected to the N^2-G and the N^6-A positions of DNA.[55,56]

As discussed above, the most common site of DNA involved in DPC formation in the presence of *bis*-electrophiles is the N7 of guanine (structures **7**−**9** in Figure 1).[19,28−30,33,41,42,57] However, until recently, no methods had existed in the literature to generate DNA strands containing site-specific N7 guanine adducts. The main obstacle was that N7 guanine alkylation destabilizes the β-glycosidic bond of the modified nucleoside, leading to spontaneous depurination.[43] Schärer et al. have developed hydrolytically stable structural mimics of N7 guanine by replacing the N7 position of guanine with a carbon (7-deaza-G).[58] In collaboration with the Schärer group, we have adopted the same approach to create hydrolytically stable DPC conjugates.[59] To create a protein reactive group, the 2,3-dihydroxyprop-1-yl group was introduced at the 7-deaza-G position (**34** in Figure 10B). Treatment with sodium periodate converts the diol group to the corresponding aldehyde, which then reacts with Lys or Arg protein side chains to form a Schiff base **35**). The latter can be quantitatively reduced with $NaCNBH_3$ to produce a stable amine linkage **36**.[59] This approach has allowed us to covalently attach a range of DNA binding proteins (e.g., AlkB, NEIL1, RNase A, myoglobin, and Histone H4) and peptides (angiotensin 1, substance P) to the 7-Gua position in DNA in a high yield (75−90%).[59] HPLC-ESI$^+$-MS/MS of tryptic digests has revealed that the resulting model DPC substrates are site-specific within DNA, but involve multiple cross-linking sites within the protein.[59]

To enable the synthesis of model DPCs which are site specific in respect to both biomolecules (DNA and protein), we have adopted a bioorthogonal approach employing copper-catalyzed [3 + 2] Huisgen cycloaddition (click reaction) between azide-functionalized proteins and alkyne-containing DNA.[60] Azide-containing proteins were created by attaching an azide-containing farnesyl group to the C-terminal cysteine within the CVIA motif of the modified green fluorescent protein (GFP) using yeast farnesyl transferase.[61] Synthetic DNA oligomers containing C8-(octa-1,7-diynyl)-uracil (**37** in Figure 10C) were prepared by solid phase synthesis. The latter were conjugated to azide-containing GFP via cycloaddition in the presence of a copper catalyst (**38** in Figure 10C).[60] The same strategy was used to conjugate shorter azide-functionalized peptides of increasing size to the C-5 position of thymine within synthetic DNA strands.[60] In vitro polymerase bypass studies conducted with DPCs of an increasing size have revealed that large conjugates (23-mer peptides and proteins) completely blocked human DNA polymerases, while the 10-mer peptide lesions were bypassed (Figure 11).[60] Furthermore, primer extension experiments with human lesion bypass polymerases have revealed that ∼90% of extension products correspond to -1 and -2 deletions.[62] This low fidelity of bypass past the DNA−peptide conjugates by

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human polymerases is likely to contribute to the mutagenesis of endogenous and exogenous DPCs.

Future investigations in this field will be focused on the biological outcomes of DPC formation in cells, as well as their repair and processing pathways. In our recent studies, introduction of proteins containing DNA-reactive epoxide functionality into human fibrosarcoma cells via electroporation resulted in cell death and mutations at the *hprt* gene.[63] Jentsch and Walters groups have shown that, in yeast and *Xenopus* systems, DPC repair is facilitated by a protease-based DNA repair pathway specific for DPCs.[53,64,65] In the yeast, DPC-processing protease Wss1 degrades the protein component of DPCs, promoting replication of damaged DNA and maintaining genome stability.[64,65] A similar process operates in *Xenopus* egg extracts, where collision of a replication fork with a DPC located on the leading strand of DNA triggers proteolysis of the protein components of the DPC to small peptides.[53] The resulting DNA-peptide conjugates may be bypassed by translesion synthesis polymerases[60,62,66,67] or removed via the NER pathway.[68] However, the identity of the mammalian DPC-specific protease is yet to be established, and it remains to be determined how this process is regulated in mammalian cells to allow for selective degradation of DPCs while sparing productive DNA−protein complexes.

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