Chemical Research in Toxicology

Article

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O^6-2′-Deoxyguanosine-butylene-O^6-2′-deoxyguanosine DNA Interstrand Cross-Links Are Replication-Blocking and Mutagenic DNA Lesions

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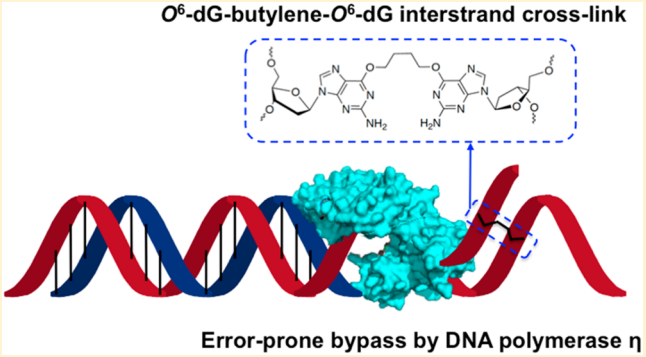
Supporting Information

ABSTRACT: DNA interstrand cross-links (ICLs) are cytotoxic DNA lesions derived from reactions of DNA with a number of anti-cancer reagents as well as endogenous bifunctional electrophiles. Deciphering the DNA repair mechanisms of ICLs is important for understanding the toxicity of DNA cross-linking agents and for developing effective chemotherapies. Previous research has focused on ICLs cross-linked with the N7 and N2 atoms of guanine as well as those formed at the N6 atom of adenine; however, little is known about the mutagenicity of O^6-dG-derived ICLs. Although less abundant, O^6-alkylated guanine DNA lesions are chemically stable and highly mutagenic. Here, O^6-2′-deoxyguanosine-butylene-O^6-2′-deoxyguanosine (O^6-dG-C4-O^6-dG) is designed as a chemically stable ICL, which can be induced by the action of bifunctional alkylating agents. We investigate the DNA replication-blocking and mutagenic properties of O^6-dG-C4-O^6-dG ICLs during an important step in ICL repair, translesion DNA synthesis (TLS). The model replicative DNA polymerase (pol) *Sulfolobus solfataricus* P2 DNA polymerase B1 (Dpo1) is able to incorporate a correct nucleotide opposite the cross-linked template guanine of ICLs with low efficiency and fidelity but cannot extend beyond the ICLs. Translesion synthesis by human pol κ is completely inhibited by O^6-dG-C4-O^6-dG ICLs. Moderate bypass activities are observed for human pol η and *S. solfataricus* P2 DNA polymerase IV (Dpo4). Among the pols tested, pol η exhibits the highest bypass activity; however, 70% of the bypass products are mutagenic containing substitutions or deletions. The increase in the size of unhooked repair intermediates elevates the frequency of deletion mutation. Lastly, the importance of pol η in O^6-dG-derived ICL bypass is demonstrated using whole cell extracts of Xeroderma pigmentosum variant patient cells and those complemented with pol η. Together, this study provides the first set of biochemical evidence for the mutagenicity of O^6-dG-derived ICLs.



INTRODUCTION

DNA is susceptible to numerous foreign and physiological reagents, forming 50,000−100,000 DNA adducts or DNA lesions per cell each day.[1] DNA lesions can affect the native double-helix structure, alter the genetic code, and disrupt genetic information flow, which altogether compromise human genome stability.[2] Genomic instability is a hallmark of cancer and many other human diseases.[3]

DNA interstrand cross-links (ICLs) are one of the most cytotoxic types of DNA lesions.[4,5] This is because the covalent link formed between two complementary strands prevents strand separation, which is a prerequisite for many DNA metabolisms including replication and transcription. Several important anti-tumor drugs, such as nitrogen mustards, cisplatin, and mitomycin C, exert cytotoxicity by forming ICLs.[6,7] Endogenous bis-electrophiles[8] and reactive abasic residues of DNA can also form ICLs,[9−14] which contribute to the toxicity of these compounds. To understand the chemical and biological consequences of ICLs, a number of synthetic ICL substrates have been developed, which include ICLs derived from platinum drugs, nitrogen mustard, endogenous malondialdehyde (reviewed by Schärer[15]), and different forms of abasic sites.[16,17]

Considerable progress has been made over the past decade in understanding how ICLs are processed during DNA replication

Received: August 18, 2016
Published: October 21, 2016

DOI: 10.1021/acs.chemrestox.6b00278
Chem. Res. Toxicol. 2016, 29, 1872−1882

and repair.[5,7,18,19] In mammalian cells, ICLs are repaired primarily in replication-dependent pathways (illustrated in Figure 1); other pathways outside the S and G2 phase also

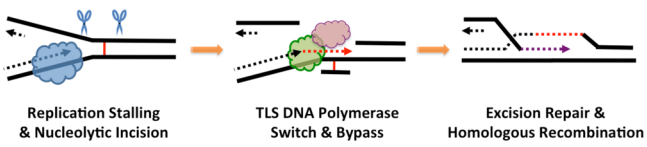


Figure 1. Replication-dependent repair of DNA interstrand cross-links.

contribute to ICL repair.[5,7,18,19] When the replication fork is blocked by ICLs, endonucleolytic enzymes cleave one strand on both sides of ICLs producing so-called unhooked repair intermediates. The unhooked intermediates can be further bypassed by one or several translesion synthesis (TLS) DNA polymerases (pols) during replication or during gap-filling DNA synthesis after replication is reinitiated downstream of ICLs. Eventually, the unhooked intermediates are removed by endonucleases and restored to the native duplex structure by homology-dependent recombination.[5,18]

The chemistry of DNA cross-links and the resulting structural impact on the DNA duplex influence the outcome of ICL repair.[5] Previously synthetic methods have been devoted to ICLs formed at the N7 and N2 atoms of guanine and N6 atom of adenine,[15−17] because these atoms are more susceptible to alkylation. Although O^6-guanine alkylation occurs to a lesser extent, the resulting DNA lesions are chemically stable and mutagenic.[20] To understand the mutagenicity of O^6-guanine-derived ICLs, we used our previously developed strategy to synthesize site-specifically modified oligomers containing O^6-2′-deoxyguanosine-butylene-O^6-2′-deoxyguanosine (O^6-dG-C4-O^6-dG) ICLs as model O^6-guanine ICLs.[21] The linker of the synthetic O^6-dG-C4-O^6-dG ICLs mimics the covalent linkage formed by bifunctional alkylating agents such as busulfan (1,4-butanediol dimethanesulfonate) with two guanines from opposite DNA strands.[22] Although busulfan confers its strong cytotoxicity via formation of intrastrand DNA cross-links and the formation of ICLs via busulfan has not been firmly established,[22] the O^6-dG-C4-O^6-dG ICLs can serve as a useful model to understand the mutagenicity of O^6-guanine-derived ICLs.

Here, we demonstrate that O^6-dG-C4-O^6-dG ICLs are replication-blocking and miscoding DNA lesions. O^6-dG-C4-O^6-dG ICLs block DNA synthesis by model replicative polymerase *Sulfolobus solfataricus* P2 DNA polymerase B1 (Dpo1) and human TLS pols η, κ, and *S. solfataricus* P2 DNA polymerase IV (Dpo4). Relative to the other three DNA pols tested, pol η exhibits the highest bypass activities. Nevertheless, pol η produces mutagenic products containing substitutions and deletions. The importance of pol η in bypassing O^6-dG-C4-O^6-dG ICLs is also demonstrated by primer-extension assays using pol η-deficient cell extracts of Xeroderma pigmentosum variant (XP-V) patient cells and those complemented with pol η. Collectively, our study provides the first insight into the mutagenic nature of O^6-dG-dervied ICLs.

MATERIALS AND METHODS

Materials. Unless otherwise noted, all chemicals were from Sigma-Aldrich (St. Louis, MO) or Alfa Aesar (Ward Hill, MA), and were of the highest quality available. Unlabeled deoxyribonucleotide triphosphates (dNTPs), T4 polynucleotide kinase, and uracil DNA glycosylase (UDG) were from New England BioLabs (Ipswich, MA). Unmodified oligonucleotides were synthesized and purified by Integrated DNA Technologies (Coralville, IA). [γ-^{32}P]ATP (specific activity of 3000 Ci mmol^{-1}) was from PerkinElmer (Waltham, MA). XP-V (XP30RO) fibroblasts (pol η-deficient, GM03617) were from Coriell Institute (Camden, NJ). A mutant of Dpo1 (D231A, E233A, D318A) deficient in 3′→5′ exonuclease activity was used to avoid complication in interpreting the kinetic data of nucleotide incorporation. Dpo1 and Dpo4 were expressed and purified as described.[23] The catalytic fragments of human pol η (1−432)[24] and pol κ (19−526)[25] were expressed and purified following previous procedures. Details of the synthesis, purification, and characterization of oligonucleotides containing site-specific O^6-dG-C4-O^6-dG ICLs are described in the Supporting Information.[21]

Primer-Extension Assays. A 13-, 19-, or 20-mer primer was 5′ [γ-^{32}P]ATP end-labeled and annealed to a 27-mer unmodified or ICL-bearing oligomer (sequences shown in Figure 2B, below). Assays were performed according to published procedures.[26] Briefly, full-length primer-extension experiments were conducted at 37 °C with 100 nM duplex DNA (with or without ICL), 80 nM DNA pol, four dNTPs at 100 μM each, 4% (v/v) glycerol, 5 mM DTT, 50 mM NaCl, 5 mM $MgCl_2$, and 100 μg mL^{-1} bovine serum albumin (BSA) in 50 mM Tris-HCl (pH 7.4 at 37 °C). Extension assays with cell extracts were performed with an ICL-S-containing duplex. Reaction conditions were the same as aforementioned except that 2 μg proteins (2 μL in volume) of cell extracts were used instead of purified enzymes.

Steady-state kinetic data were obtained with 80−100 nM duplex DNA, 0.5−20 nM DNA pol, and varying concentrations of a single dNTP. Reactions were quenched by addition of 50 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0) in 95% formamide (v/v). Reaction products were denatured at 90 °C and resolved using 18% acrylamide (w/v) gel electrophoresis containing 7 M urea. Products were quantified using a phosphorImaging system (GE Healthcare, Typhoon FLA7000) and ImageQuant software. Data were fit to the Michaelis−Menten equation to obtain k_{cat} and K_m using Prism software (GraphPad, San Diego, CA).

Preparation of the Whole-Cell Extract of XP-V Fibroblasts. XP-V fibroblasts were grown in Opti-MEM reduced serum medium plus 10% fetal bovine serum (Life Technologies, Grand Island, NY) in a humidified 5% CO_2 atmosphere. The pol η-complemented fibroblasts were prepared by transfecting XP-V fibroblasts with a pol η-expressing vector pJRM160 or a control vector without *POLH* gene (vectors were kind gifts from Dr. Roger Woodgate) using Lipofectamine 3000 (Life Technologies) according to the procedure recommended by the manufacturer. The fibroblasts with transiently over-expressed pol η were harvested 48 h after the transfection. Cell lysate was prepared following a previously described procedure with modifications.[27] Briefly, the cells were washed twice with ice-cold phosphate-buffered saline and resuspended in lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10% (w/v) sucrose, 10% (v/v) glycerol, 5 mM β-mercaptoethanol, and protease inhibitor cocktail (Thermo Fisher). The suspension was incubated at 4 °C for 1 h followed by sonication 3 × 5 s with an interruption of 15 s on ice. NaCl was added to a final concentration of 200 mM, and lysate was incubated at 4 °C for 10 min. After centrifugation (15000*g* for 20 min at 4 °C), supernatant was collected and flash frozen in liquid nitrogen. The amount of proteins determined by Bradford assay was 1 μg μL^{-1}. The expression of pol η was confirmed by Western blotting using a primary antibody against human pol η (Santa Cruz Biotechnology, Dallas, Texas) together with a horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology). The signals were developed using electrochemiluminescence (ECL) Western blotting substrate (Bio-Rad), and the image was taken using a Chemidoc Touch imaging system (Bio-Rad).

LC-MS Analysis of Full-Length Extension Products by Pol η. A 20-mer primer 5′- GTCGGTACCAGCGATGC(dU)AT-3′ was annealed to an unmodified or an ICL-bearing template oligomer at a 1:1.2 molar ratio. Reaction conditions were similar to those used in full-length primer-extension experiments except for the following concentrations: pol η, 1 μM; primer−template complex, 2 μM; glycerol, 2% (v/v); and physiological concentrations of dNTPs in

DOI: 10.1021/acs.chemrestox.6b00278
Chem. Res. Toxicol. 2016, 29, 1872−1882

nucleus (10 μM dGTP, 40 μM dATP, 40 μM dCTP, and 40 μM dTTP)[28] in a total volume of 50 μL. Polymerization reactions were allowed to proceed for 3 h at 37 °C to maximize the yield of extended products, and reactions were terminated with 10 mM EDTA. All concentrations were final concentrations in the reaction. The resulting products were incubated with 10 units of UDG overnight. Enzymes were extracted with phenol−chloroform extraction. Extracted oligomers were treated with 0.25 M piperidine at 90 °C for 1 h,[29] followed by speedvac treatment to remove the solvent and piperidine. Samples were resuspended in water and passed through C18 SampliQ solid-phase extraction cartridges (Agilent Technologies). The fractions containing DNA oligomers were pooled and dried using a speedvac concentrator.

LC-MS-based oligonucleotide sequencing was performed on a nanoAcquity ultraperformance liquid chromatography system (Waters Corp.)[30] connected to a Finnigan LTQ XL mass spectrometer (Thermo Scientific Corp.). Reaction products were separated using a PicoChip column (75 μm i.d., 105 mm bed length, and 15 μm tip, New Objective, Woburn, MA) packed with Reprosil-PUR C18 (3 μm, 120 Å) chromatography media. Chromatography mobile phase A was 400 mM 1,1,1,3,3,3-hexafluoro-2-propanol in water (pH adjusted to 7.0 with triethylamine), and mobile phase B was methanol. The flow rate was 250 nL min^{-1}, and the gradient program was 0−5 min, maintained at 95% A/5% B (v/v); 5−45 min, linear gradient to 25% B (v/v); 45−55 min, linear gradient to 50% B; 55−60 min, hold at 50% B; 60−105 min, linear gradient to 5% B; 105−125 min, hold at 5% B to re-equilibrate the column. A 1 μL aliquot was injected onto the column. Mass spectrometric data were collected under negative ionization mode. Ionization parameters were ionization voltage 3 kV, capillary temperature 300 °C, capillary voltage −45 V, tube lens voltage −110 V. MS/MS conditions were as follows: normalized collision energy 40%, activation Q 0.250, and activation time 30 ms. Product ion spectra were acquired over the range m/z 300−1800. The most abundant ions were selected for fragmentation. Theoretical fragmented ions were calculated using the Mongo Oligo mass calculator version 2.06 (hosted by the State University of New York at Albany).[31] The relative yields of extension products were estimated based on the integrated peak areas in extracted ion chromatograms, which were set to extract multiple species with different charged states. The peak area ratio of total products/residual primer with an unmodified duplex was set to 100%. The peak area ratios of product/residual primer with an ICL-containing duplex were normalized to the peak area ratios found with an unmodified duplex.

RESULTS

Design of O^6-dG-C4-O^6-dG ICLs To Mimic Unhooked Repair Intermediates. To understand the mutagenicity of O^6-guanine-cross-linked ICLs, we synthesized site-specifically modified oligonucleotides containing O^6-dG-C4-O^6-dG using our recently developed method (structure of O^6-dG-C4-O^6-dG shown in Figure 2A).[21] Several important factors affect the outcome the ICL repair, including the chemistry of linkage, the length of linker, distortion imposed by ICL, and the size of unhooked non-template strand (often referred to as unhooked repair intermediate).[4,5,7,18,19] The length of unhooked, non-template repair intermediate is thought to comprise 4−17 nucleotides.[32,33] We therefore synthesized two ICLs, one with a 4-nucleotide overhang (hereafter termed ICL-S) and the other with a 10-nucleotide overhang (hereafter termed ICL-L) to mimic the varying extent of resection around ICLs (the sequences of oligomers shown in Figure 2B). The 3′-nucleotide of the unhooked intermediate is a dideoxynucleotide to prevent DNA synthesis from the cross-linked oligomer. Therefore, our synthetic substrates serve as reasonable models for investigating the activity and outcome of TLS.

O^6-dG-C4-O^6-dG ICLs Are Replication-Blocking DNA Lesions. ICLs are absolute blocks for a variety of DNA

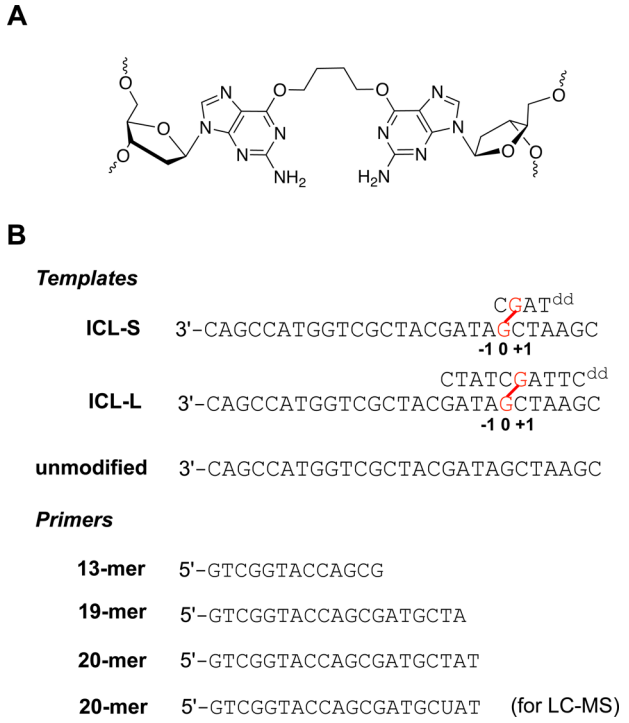


Figure 2. (A) Chemical structure of O^6-dG-C4-O^6-dG cross-link. (B) Primers and templates used in this study. T^{dd} and C^{dd} indicate dideoxynucleotides to prevent polymerization.

metabolisms and thus cannot be carried through a proliferative cycle without repair.[4] The collision of the replication fork with ICLs signals the recruitment of structure-specific endonucleases to cleave the non-template strand around ICLs, which subsequently creates unhooked intermediates amenable to TLS.[18] To examine the bypass capabilities of several representative TLS pols, ICL-containing substrates were annealed to a 13-mer oligomer primer for primer-extension experiments. As shown in Figure 3A, with an unmodified duplex, all four pols converted primer to full-length or nearly full-length products. On the contrary, DNA synthesis with ICL-bearing substrates stalled at different positions. Primer extension by the model replicative DNA polymerase Dpo1 was blocked by ICL-S and ICL-L, producing products terminated at the 0 position (80% for ICL-S and 70% for ICL-L, Figure 3B). Moderate bypass activity was observed for Dpo4, affording products extended to the −1 and 0 positions in a 10 min reaction; more bypass products were seen with longer reaction times. Pol κ is a relevant TLS pol capable of bypassing a number of ICL structures.[32,34] With the O^6-dG-C4-O^6-dG ICLs, more than 95% of the products from pol κ stalled at the −1 position (Figure 3A,B), indicating the strong inhibition of pol κ-catalyzed polymerization caused by O^6-dG-C4-O^6-dG ICLs. Extended reaction times (up to 30 min) did not increase the bypass yield of pol κ.

On the other hand, pol η, best known for its capability of accurately bypassing pyrimidine dimers,[35] only partially stalled at the −1 position and moderately bypassed both ICL-S and ICL-L substrates (Figure 3A). The percentages for total products past the lesion were 57% and 49% for ICL-S and ICL-L in a 10 min reaction catalyzed by pol η (Figure 3B). The partially extended products produced in pol η-catalyzed polymerization suggest the potential for deletion mutations (confirmed by LC-MS sequencing, *vide infra*). Reactions with

DOI: 10.1021/acs.chemrestox.6b00278
Chem. Res. Toxicol. 2016, 29, 1872−1882

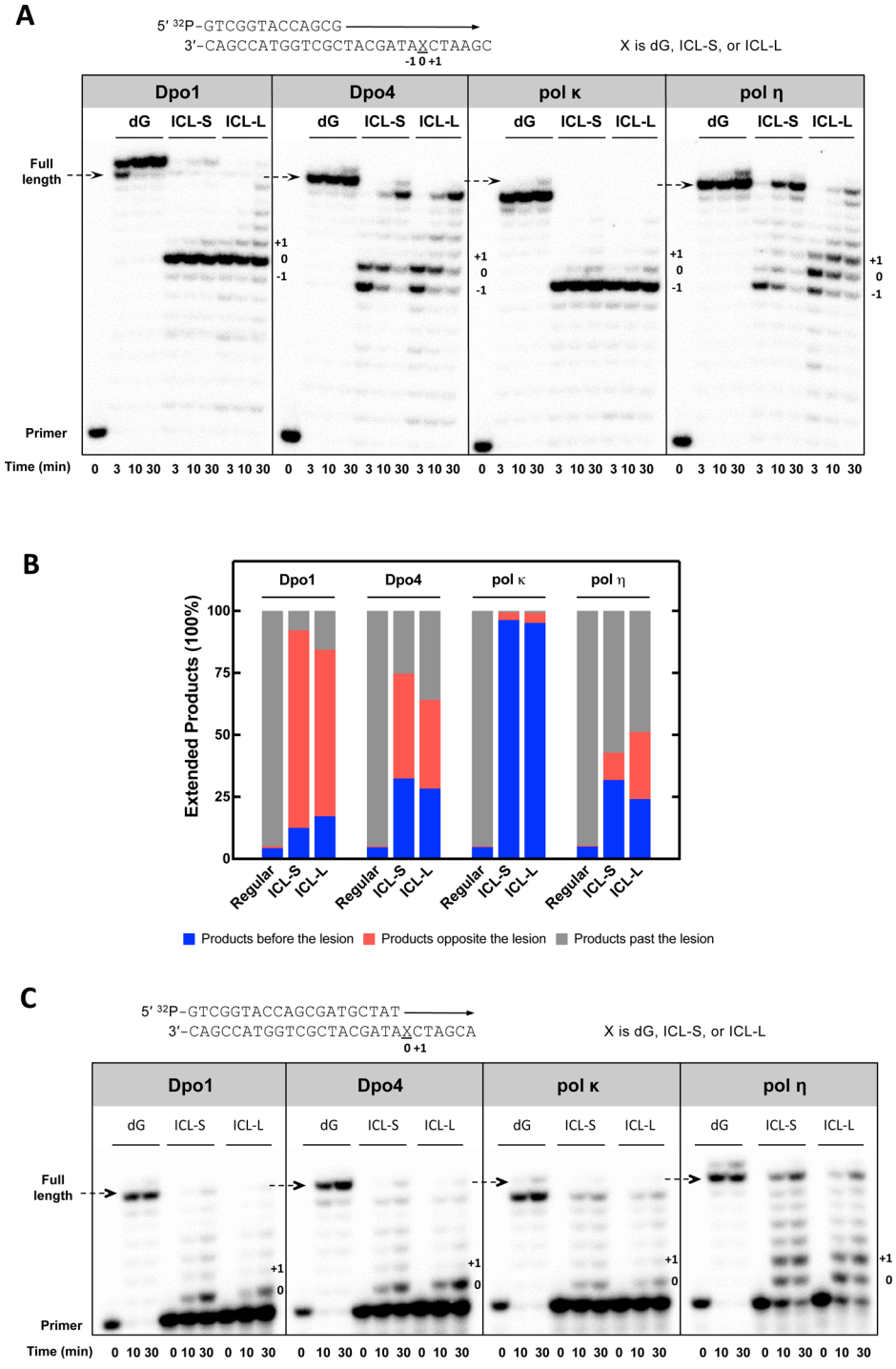


Figure 3. Primer-extension reactions catalyzed by Dpo1, Dpo4, pol *κ*, and pol *η*. (A) Extension reactions with a 13/27-mer duplex with or without ICL (running start assays). Arrows indicate full-length products. Major products terminated at the nucleotide prior to the lesion, at the lesion, or after the lesion are labeled as −1, 0, and +1. Assays were performed at 37 °C with 100 nM DNA duplex, dNTP mixtures at 100 μM each, 80 nM polymerase, 4% (v/v) glycerol, 5 mM DTT, 50 mM NaCl, 5 mM $MgCl_2$, and 100 μg mL^{-1} bovine serum albumin in 50 mM Tris-HCl pH 7.4. (B) Quantification of extended products from 10 min reactions shown in (A). (C) Extension reactions with a 20/27-mer duplex with or without ICL (standing start assays). Reaction conditions were the same as described in (A).

ICL-L resulted in a slightly higher yield of partially extended products relative to those with ICL-S (Figure 3B). Similar extension patterns were also observed when a 20-mer oligomer was used as a primer in primer-extension assays ("standing start" conditions, Figure 3C), except that DNA synthesis by Dpo1 seems to be able to proceed to the 0 position in Figure 3A whereas only weak extension capabilities were observed in Figure 3C. This difference in the extension pattern is likely due to the difference in the ability of Dpo1 to bind with the different ICL substrates (13/27-mer vs 20/27-mer duplexes) as well as different amounts of productive DNA pol/DNA/dNTP complexes formed. Together, O^6-dG-C4-O^6-dG ICLs block both replicative and bypass DNA pols tested; relative to the

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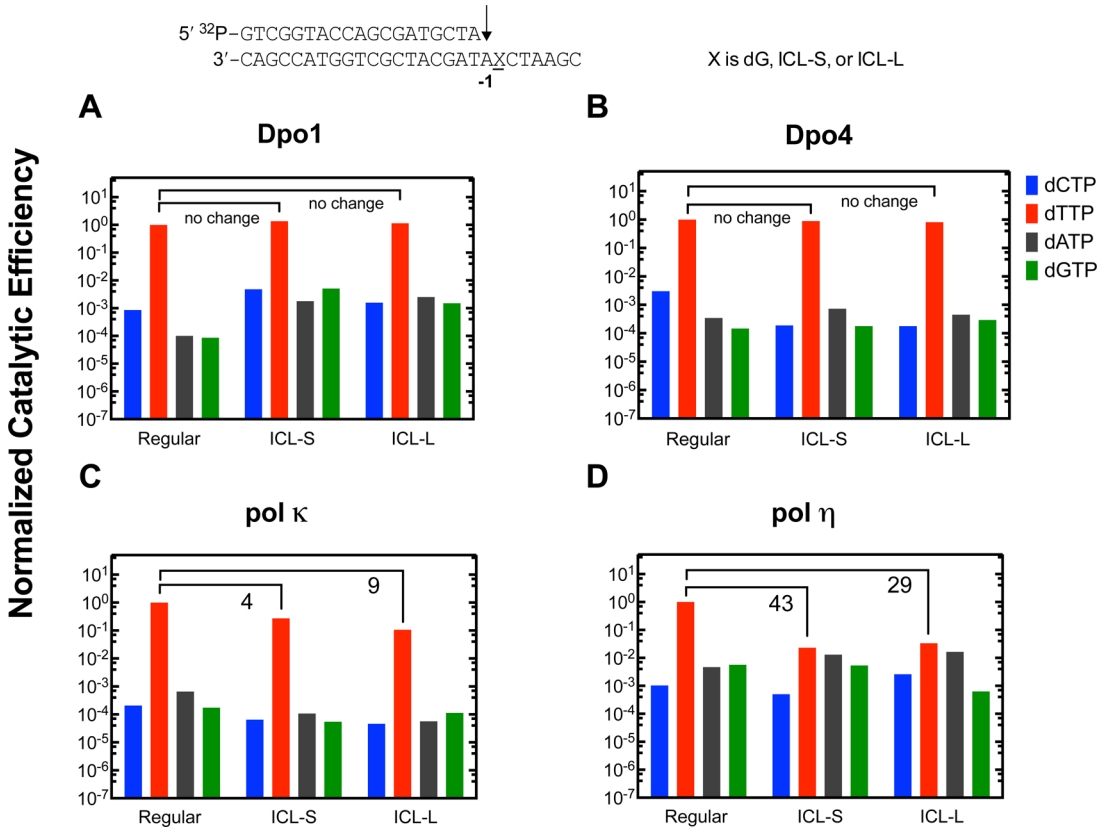

Figure 4. Normalized catalytic efficiencies of nucleotide incorporations at the −1 position with an unmodified template, ICL-S, or ICL-L for Dpo1 (A), Dpo4 (B), pol κ (C), and pol η (D). Results were derived from steady-state kinetic data shown in Table S1, Supporting Information. The catalytic efficiency of the native base pair with an unmodified duplex is considered as 1 for each enzyme. Normalized catalytic efficiencies were obtained using respective catalytic efficiency (k_{cat}/K_m) divided by the catalytic efficiency of the correct nucleotide with an unmodified duplex. Assays were performed at 37 °C with 80−100 nM duplex DNA (with or without ICL), 0.5−20 nM DNA pol, varying concentrations of a single dNTP, 4% (v/v) glycerol, 5 mM DTT, 50 mM NaCl, 5 mM $MgCl_2$, and 100 μg mL^{-1} BSA in 50 mM Tris-HCl (pH 7.4 at 37 °C). Changes in catalytic efficiency relative to a native base pair were calculated from $(k_{cat}/K_{m,dNTP})_{unmodified,\ dTTP}/(k_{cat}/K_{m,dNTP})_{ICL,\ dTTP}$, and are indicated as *x*-fold decrease. Changes less than 2-fold are considered as no change. Complete data are in Table S1.

other three pols, pol η showed the highest activity in replicating past O^6-dG-C4-O^6-dG ICLs.

***O*⁶-dG-C4-*O*⁶-dG ICLs Are Miscoding DNA Lesions.** On the basis of the mutagenicity of O^6-alkyl guanine and the size of an ICL relative to other alkylated DNA lesions, we hypothesized that O^6-dG-C4-O^6-dG ICLs are highly miscoding. To test this hypothesis, we performed steady-state kinetic analysis for nucleotide incorporations at the −1 and 0 positions. These positions are the sites of high mutation frequencies in replication-dependent ICL repair detected by deep sequencing.[36] Figure 4 shows the normalized catalytic efficiencies of different nucleotides obtained with unmodified or ICL-bearing substrates. Detailed steady-state kinetic parameters are shown in Table S1. The normalized catalytic efficiencies were obtained using the catalytic efficiencies of each nucleotide pairs divided by that of an unmodified, correct base pair for each DNA pol. As shown in Figure 4A,B, ICL-S and ICL-L had little impact on the efficiency and fidelity of nucleotide incorporation by Dpo1 or Dpo4 at the −1 position. For pol κ, the efficiency of correct nucleotide insertion at the −1 position decreased 4-fold for ICL-S and 9-fold for ICL-L relative to that obtained with an unmodified duplex, while the overall replication fidelity remained unchanged (Figure 4C). On the other hand, a greater decrease in the catalytic efficiency of the correct nucleotide incorporation was observed with the pol η-catalyzed ICL bypass (Figure 4D). Importantly, ICL-containing substrates compromised the nucleotide selectivity by pol η. Misincorporation occurred at 10^{-2} to 10^{-1} in frequency—much higher compared to the 10^{-3} error frequencies obtained with an unmodified duplex for pol η (Table S1). Taken together, at the −1 position ICLs did not significantly affect the replication efficiency and fidelity of Dpo1, Dpo4, and pol κ but compromised the efficiency and fidelity of nucleotide insertion by pol η.

At the 0 position (directly opposite the template cross-linked guanine) a dramatic decrease in efficiency of nucleotide incorporation was observed for all four polymerases, most likely due to the steric hindrance imposed by ICLs (Figures 3 and 5, Table S2). Dpo1 was highly inefficient in inserting a nucleotide at the 0 position with both ICL-S and ICL-L substrates and incorporated wrong nucleotides at higher or similar rates compared to the canonical base pair (Figure 5A). The drastic decrease in catalytic efficiencies of Dpo1 is consistent with the extension patterns shown in Figure 3C in which the same substrates were used. Dpo4 maintained preference for dCTP incorporation opposite the cross-linked guanine with ICL-S and ICL-L albeit with lower efficiency (Figure 5B). For pol κ, DNA replication was highly inefficient and error-prone with ICLs (Figure 5C), consistent with the weak extension activity at the 0 position observed in Figure 3A,C. For pol η, a moderate decrease in the efficiencies of nucleotide incorporations was observed. Notably, relative to

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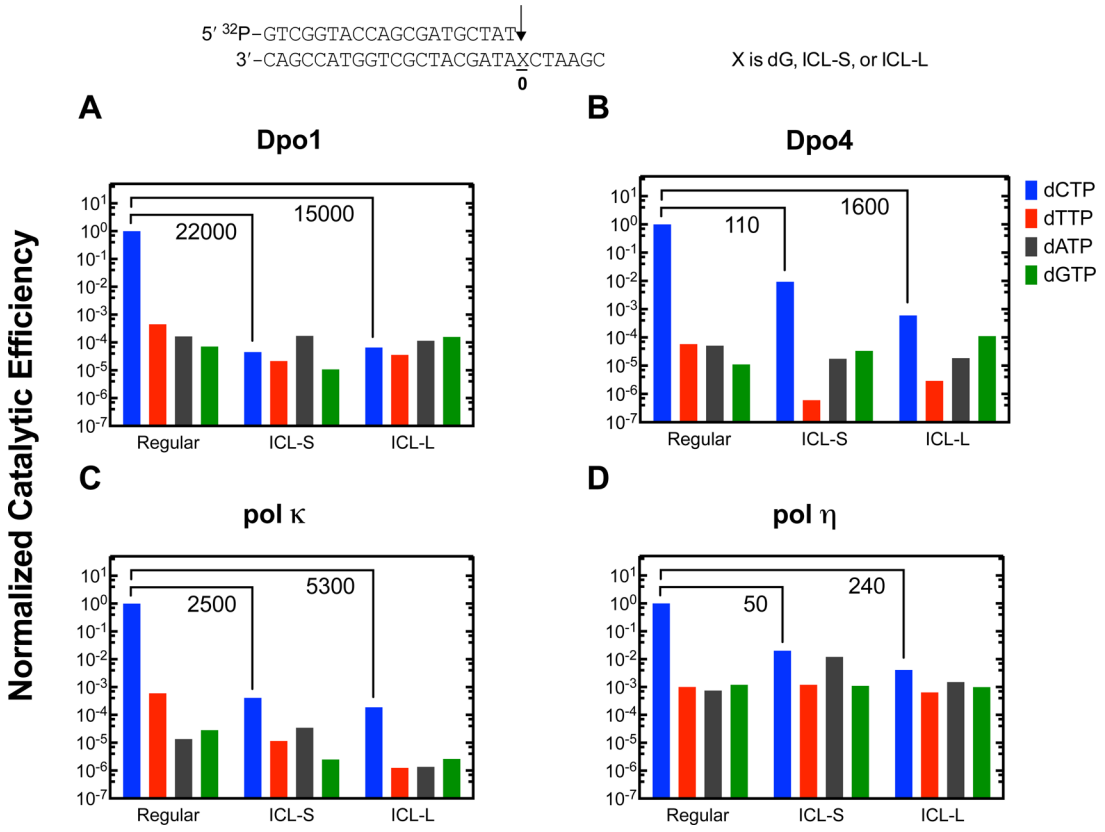

Figure 5. Normalized catalytic efficiencies of nucleotide incorporations at the 0 position with an unmodified template, ICL-S, or ICL-L for Dpo1 (A), Dpo4 (B), pol κ (C), and pol η (D). Results were derived from steady-state kinetic data shown in Table S2, Supporting Information. Experimental conditions were the same as those indicated in Figure 4. Normalized catalytic efficiencies and *x*-fold decrease were calculated in the same way as described in Figure 4. Changes in catalytic efficiency relative to a native base pair were indicated as *x*-fold decrease. Complete data are in Table S2.

Table 1. Summary of Polymerase η-Catalyzed Bypass Products from LC-MS/MS Analysis[a]

5′-GTCGGTACCAGCGATGCUAT → 3′-CAGCCATGGTCGCTACGATAG*C-TAAGC		normalized % of products	nucleotide opposite the 0 position	relative yield of error-prone products (%)[b]
unmodified	ATCGATTCG	100	error-free	
ICL-S	ATCGATTCG	19	error-free	
	ATTGATTCG	19		
	ATAGATTCG	13	substitutions	error-prone 71%
	ATGGATTCG	7		
	AT_GATTCG	7	deletion	
ICL-L	ATCGATTCG	15	error-free	
	ATTGATTCG	11		
	ATAGATTCG	5	substitutions	error-prone 69%
	ATGGATTCG	4		
	AT_GATTCG	13	deletion	

[a]The results were obtained using an undamaged, ICL-S, or ICL-L duplex. G* indicates an unmodified or cross-linked guanine, and U is 2′-deoxyuridine. Percents of products were calculated on the basis of the peak area in extracted ion chromatograms. Total products/cleaved primer ratio obtained with an undamaged duplex was considered as 100%. Products of pol η-catalyzed bypass were normalized to the product ratio of an undamaged duplex. Nucleotides incorporated opposite the cross-linked guanine (0 position) are underlined in the extended products. [b]Relative percent yields of error-prone products were calculated using the sum of normalized percent yields of error-prone products divided by the sum of the normalized yields of all extended products for each duplex.

other three polymerases, pol η showed a minimal decrease in the catalytic efficiency of the correct dCTP with ICL-harboring substrates (Figure 5D), in keeping with the relatively high yield of bypass products in pol η-catalyzed primer extensions (Figure 3A,C). Nevertheless, pol η-mediated TLS was highly error prone—incorrect nucleotides were incorporated at similar or slightly lower efficiency relative to the correct nucleotide (dCTP) for both ICL-containing substrates. Overall, these steady-state kinetic data are consistent with the limited length of bypass products observed for Dpo1 and pol κ in primer-

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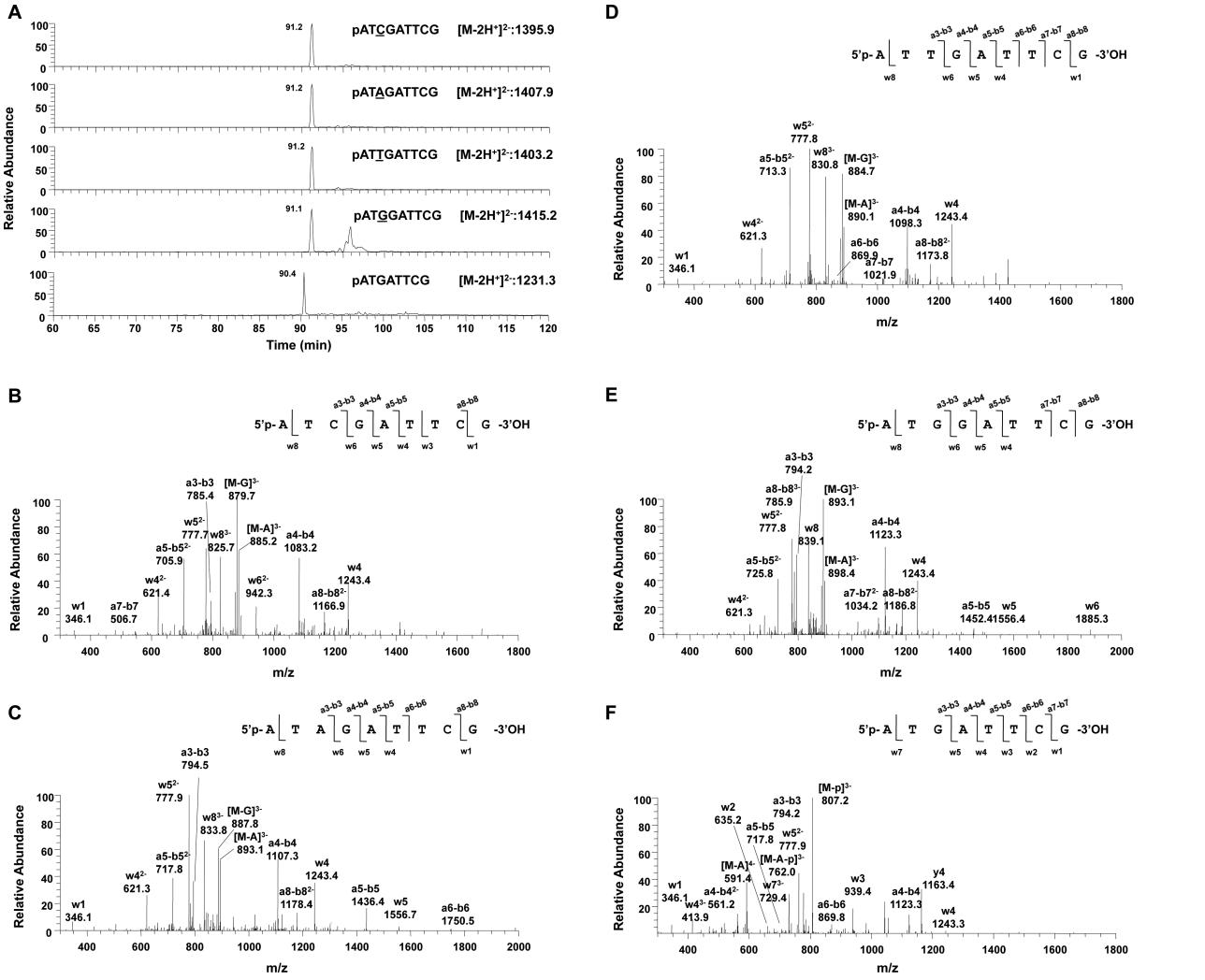

Figure 6. LC-MS sequencing of pol η-catalyzed ICL bypass products. (A) Extracted ion chromatograms of products with different nucleotides incorporated opposite the lesion (underlined), i.e., m/z 1395.9 with C, m/z 1407.9 with A, m/z 1403.2 with T, and m/z 1415.2 with G. Product m/z 1231.3 is with a one-nucleotide deletion. (B) Fragmentation mass spectra of the different species in (A). The observed product ions that match theoretical fragmented ions are labeled. Reactions contained 1 μM pol η, 2 μM primer−template complex; 2% (v/v) glycerol, physiological concentrations of dNTPs in the nucleus (10 μM dGTP, 40 μM dATP, 40 μM dCTP, and 40 μM dTTP), 5 mM DTT, 50 mM NaCl, 5 mM $MgCl_2$, and 50 μg mL^{-1} BSA in a total volume of 50 μL.

extension experiments and demonstrate the miscoding nature of O^6-dG-C4-O^6-dG ICLs in TLS.

ICL Bypass by Pol η Results in Substitution and Deletion Mutations. According to results obtained with primer-extension assays and steady-state kinetic analysis, pol η is the most robust DNA pol in replicating past the cross-linked template guanine. The observed partially extended products in gel electrophoretic analysis suggest the potential for truncation or deletion mutations (likely due to looping-out the lesion during catalysis, see refs 26 and 37 for examples); however, the sequence of these products remains obscure. To address this question, we used LC-MS-based oligonucleotide sequencing to identify the bypass products as well as to determine their relative yields. This powerful technique is complementary to the kinetic analysis and is particularly suitable for detecting insertions/deletions (indels).[30,38]

As summarized in Table 1, the normalized product yield with an unmodified duplex was considered as 100% in order to reflect the decrease in the total product yield with ICL-bearing substrates. The normalized yields of extended primers by pol η with ICL-S and ICL-L were 65% and 48%, respectively, consistent with the replication-blocking nature of ICLs observed in Figure 3. Remarkably, approximately 70% of the total bypass products contained substitution and deletion mutations with both ICL-S and ICL-L (Table 1). For pol η-catalyzed ICL bypass, the substitutions occurred at comparable yields relative to the error-free bypass, consistent with the comparable efficiencies of four nucleotides at the 0 position in steady-state kinetic analysis (Figure 5D). Reactions with the bulkier repair intermediate (ICL-L) afforded twice as much products with deletion (13% for ICL-L vs 7% for ICL-S), similar to the higher yield of partially extended products with ICL-L relative to ICL-S observed in Figure 3A. The sequences and fragmented ions of product oligomers are shown in Figure 6. Overall, using LC-MS-based sequencing, we identified products containing substitution and deletion mutations in pol η-catalyzed TLS. Although pol η is relatively efficient in replicating past ICLs, the process is highly error-prone. Together with the kinetic data, these results suggest that O^6-dG-C4-O^6-dG ICLs could be highly mutagenic *in vivo*.

DOI: 10.1021/acs.chemrestox.6b00278
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Pol η Is an Important DNA Polymerase in Bypassing the Repair Intermediate of O^6-dG-C4-O^6-dG ICLs. Multiple TLS pols can act redundantly in TLS.[39] To verify whether pol η plays an important role in bypassing O^6-dG-C4-O^6-dG ICL unhooked intermediate in the presence of multiple DNA pols, we tested the bypass capability of human cell extracts from XP-V patient cells and those complemented with pol η. XP-V fibroblasts harbor a 13-bp deletion from nucleotide 104 to 116 in exon 2 of the *POLH* gene, which results in the expression of a truncated and non-functional pol η.[35] We transfected XP-V cells with a vector that over-expresses wild-type pol η and confirmed the transient expression of pol η by Western blot (Figure 7A). Control cells were prepared by transfecting XP-V cells with a vector lacking the *POLH* gene. We then performed primer-extension assays with ICL-S-containing duplex and whole-cell extracts from both types of cells. As shown in Figure 7B, minimal bypass activity was observed for XP-V cell extract. A small amount of primer was extended to the 0 position, and nearly no products extended beyond the cross-linked guanine. On the other hand, bypass activities were observed with the cell extract complemented with pol η. The relatively low yield of the extended primers could be potentially due to the suboptimal template length for binding of pol η and other accessory proteins. Nevertheless, the clear difference seen in the bypass activities of both types of cell extracts suggests an important role for pol η in bypassing O^6-dG-C4-O^6-dG ICLs.

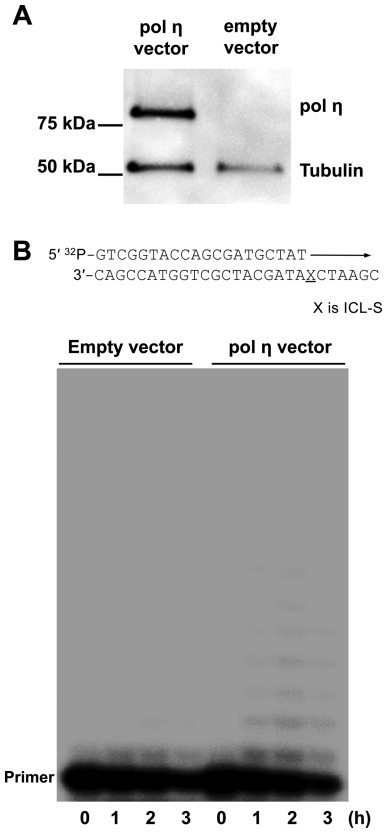

Figure 7. (A) Western blot of whole cell extracts of XP-V cells complemented with empty vector or pol η-expressing vector. (B) Primer-extension reactions with whole cell extracts of XP-V cells complemented with empty vector or pol η-expressing vector. The substrate is an ICL-S-containing duplex. Reactions were performed with 40 nM DNA duplex, dNTPs at 100 μM each, 2 μg proteins from whole cell extract (2 μL), 5 mM DTT, 50 mM NaCl, 5 mM $MgCl_2$, and 100 μg mL^{-1} BSA in 50 mM Tris-HCl pH 7.4.

DISCUSSION

O^6-Alkyl guanine DNA lesions are chemically stable and highly mutagenic.[20] The highly conserved DNA repair gene *MGMT* safeguards the genome by removing O^6-guanine DNA lesions.[40] On the basis of the mutagenicity of O^6-guanine DNA lesions and the bulkiness of ICLs, we hypothesized that O^6-guanine ICLs are highly mutagenic. To test this, we constructed oligonucleotides with a butylene bridge cross-linking two O6 atoms of guanines from complementary strands to mimic the repair intermediate after nucleotidyl incision. We examined the outcome of DNA replication during an essential step of ICL repair, i.e., TLS.

Our results demonstrate that O^6-dG-C4-O^6-dG ICLs are replication-blocking and mutagenic DNA lesions. O^6-dG-C4-O^6-dG ICLs blocked DNA synthesis by pol κ and processive DNA polymerase Dpo1, and weak bypass and extension activities were observed for Dpo4 (Figure 3). Previously, Lloyd and colleagues showed that DNA synthesis by *E. coli* pol II pauses at the −1 position when encountering N^2-dG-N^2-dG ICLs (a mimic of acrolein-derived ICLs), and that *E. coli* pol IV is able to bypass the ICLs, depending on the size of the unhooked intermediate.[41] Walter et al. demonstrated using *Xenopus* egg extracts that during replication-dependent ICL repair, the replicase first pauses 20−40 nucleotides from the ICLs owing to the steric hindrance imposed by the CMG helicase, and then replicative DNA pol proceeds to the −1 position before switching to TLS pols.[42] Since ICLs are blocks to replicative DNA pols, our observation with model replicative DNA polymerase Dpo1 and TLS polymerase Dpo4 is somewhat unexpected. Dpo1 readily approached to the O^6-dG-C4-O^6-dG ICLs and inserted a nucleotide opposite the cross-linked guanine, whereas Dpo4 partially paused at the −1 position. This may be due to the fact that an exonuclease-deficient mutant of Dpo1 was used. In addition, Dpo1 is known to be able to replicate past a number of DNA lesions, including N^2-methylguanine,[43] O^6-methylguanine,[43] and highly distorting (5′*S*)-8,5′-cyclo-2′-deoxyguanosine.[26] Thus, Dpo1 and Dpo4 are likely to coordinate the bypass of O^6-dG-dervied ICLs *in vivo*.

In mammalian cells, a number of the TLS pols play important roles in ICL repair pathways, including pol ζ, pol η, pol κ, and Rev1. Using proteomic analysis and *Xenopus* egg extracts, Räschle et al. demonstrated that upon encountering psoralen-induced ICLs, pol η, pol κ, Rev1, Rev3−Rev7 (two subunits of pol ζ), together with the Fanconi core complex are recruited concomitantly with the unloading of the replicative DNA pols.[44] Pol κ is known for its role in bypassing bulky minor groove DNA lesions, such as (−)-*trans*-*anti*-benzo[*a*]pyrene-N^2-dG adduct.[45] In addition, pol κ is capable of replicating past acrolein- and mitomycin C-derived ICLs (both are N^2-dG minor groove lesions) in vitro either in the form of recombinant enzyme[32,34] or in *Xenopus* egg extracts.[46] Pol κ-deficient cells or mice are sensitive to mitomycin C exposure.[32,46,47] By contrast, pol κ has minimal bypass activities toward major groove ICLs (*N*7-dG-*N*7-dG) derived from cisplatin, nitrogen mustard,[48] and oxidized abasic lesion.[30] Consistent with these properties of pol κ, our results demonstrate that major groove adducts O^6-dG-C4-O^6-dG ICLs are poor substrates for the TLS activity of pol κ (Figures 3−5, Tables S1 and S2). Together, these results suggest that the

DOI: 10.1021/acs.chemrestox.6b00278
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catalytic activity of pol κ is unlikely to contribute to replication-dependent repair of O^6-dG ICLs *in vivo*.

Pol η is probably the most versatile TLS DNA pol known to bypass a spectrum of bulky DNA lesions.[37] A genetic deficiency in the *POLH* gene is associated with a variant type of Xeroderma pigmentosum, a cancer predisposition syndrome.[49,50] In addition, pol η is able to carry out insertion as well as extension with a number of structurally distinct ICLs, including those induced by acrolein,[51] cisplatin,[48] nitrogen mustards,[48,52] and oxidized abasic sites.[30] In the present study, pol η showed the highest bypass activity toward O^6-dG-C4-O^6-dG ICL substrates compared to the other three DNA pols. The clear difference observed in bypass activities using cell extracts from human XP-V fibroblasts and those complemented with pol η suggests that pol η is important for rescuing a stalled replication fork in the replication-dependent ICL repair pathway (Figure 7). We cannot, however, exclude the possibility of other pols involved in TLS directly at the stalled fork or post-replication gap-filling. Additional studies are required to verify the extent of involvement of pol η in ICL repair *in vivo*.

TLS pols are known for promiscuous DNA synthesis and thus play a major role in DNA damage-induced mutagenesis.[2] Our results from both steady-state kinetic analysis and LC-MS sequencing have shown that pol η-assisted bypass of O^6-dG-C4-O^6-dG ICLs is highly error-prone. Products with substitution and deletion mutations account for approximately 70% of the total extended primers (Table 1). These results demonstrate that O^6-dG-C4-O^6-dG ICLs are highly miscoding in vitro and suggest their strong mutagenic potential in vivo. Using deep sequencing, Budzowska et al. showed that the mutation occurs primarily at the 0 position (opposite the cross-linked nucleotide) during replication-dependent ICL repair.[36] Consistent with these results, we found that replication errors with pol η during O^6-dG-C4-O^6-dG ICL bypass occurred primarily at the 0 position (summarized in Table 1). The competency of pol η in bypassing a variety of DNA adducts and the error-prone nature of the bypass are thought to contribute to the chemotherapy resistance and an elevated chemotherapy-induced mutation load.[19,53,54] Therefore, targeting TLS holds great promise in improving the effectiveness of conventional chemotherapy.

In conclusion, we have provided the first set of biochemical evidence to show that O^6-dG-C4-O^6-dG ICLs are replication-blocking and miscoding DNA lesions and that pol η is an important TLS polymerase in the replication-dependent ICL repair pathway. Our study broadens the understanding of the versatility of pol η in bypassing structurally diverse ICLs and underscores the importance of further evaluation of the extent of participation of pol η in ICL repair pathways in biological systems.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.6b00278.

Synthesis, purification, and characterization of ICL-containing oligonucleotides; Tables S1 and S2, listing steady-state kinetic parameters for single-base incorporation at the −1 and 0 positions (PDF)

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Funding

This work was supported by in part by Central Michigan University start-up funds (to L.Z.), Central Michigan University Faculty Research and Creative Endeavors Award (to L.Z.), U.S. Army Research Office (grant no. W911NF-15-1-0140 to L.Z.), U.S. NIH P01 CA160032 (to M.E.), the Natural Sciences and Engineering Research Council of Canada (NSERC, grant No. 299384-2011 to C.J.W.), and the Canada Research Chair Program (grant no. 950-213807 to C.J.W.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Dr. F. Peter Guengerich for providing expression plasmids of human pol η and pol κ, and to Dr. Roger Woodgate for pJRM160 and control plasmids for transfection.

■ ABBREVIATIONS

BSA, bovine serum albumin; dG, 2′-deoxyguanosine; dNTP, deoxyribonucleotide triphosphate; ICL, DNA interstrand cross-link; pol, DNA polymerase; O^6-dG-C4-O^6-dG, O^6-2′-deoxyguanosine-butylene-O^6-2′-deoxyguanosine; TLS, translesion synthesis; UDG, uracil DNA glycosylase; XP-V, Xeroderma pigmentosum variant

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