Article

Mutagenicity and Genotoxicity of (5′S)-8,5′-Cyclo-2′-deoxyadenosine in *Escherichia coli* and Replication of (5′S)-8,5′-Cyclopurine-2′-deoxynucleosides in Vitro by DNA Polymerase IV, Exo-Free Klenow Fragment, and Dpo4

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S *Supporting Information*

ABSTRACT: Reactive oxygen species generate many lesions in DNA, including R and S diastereomers of 8,5′-cyclo-2′-deoxyadenosine (cdA) and 8,5′-cyclo-2′-deoxyguanosine (cdG). Herein, the result of replication of a plasmid containing S-cdA in *Escherichia coli* is reported. S-cdA was found mutagenic and highly genotoxic. Viability and mutagenicity of the S-cdA construct were dependent on functional pol V, but mutational frequencies (MFs) and types varied in pol II- and pol IV-deficient strains relative to the wild-type strain.

Both S-cdA → T and S-cdA → G substitutions occurred in equal frequency in wild-type *E. coli*, but the frequency of S-cdA → G dropped in pol IV-deficient strain, especially when being SOS induced. This suggests that pol IV plays a role in S-cdA → G mutations. MF increased significantly in pol II-deficient strain, suggesting pol II's likely role in error-free translesion synthesis. Primer extension and steady-state kinetic studies using pol IV, exo-free Klenow fragment (KF (exo⁻)), and Dpo4 were performed to further assess the replication efficiency and fidelity of S-cdA and S-cdG. Primer extension by pol IV mostly stopped before the lesion, although a small fraction was extended opposite the lesion. Kinetic studies showed that pol IV incorporated dCMP almost as efficiently as dTMP opposite S-cdA, whereas it incorporated the correct nucleotide dCMP opposite S-cdG 10-fold more efficiently than any other dNMP. Further extension of each lesion containing pair, however, was very inefficient. These results are consistent with the role of pol IV in S-cdA → G mutations in *E. coli*. KF (exo⁻) was also strongly blocked by both lesions, but it could slowly incorporate the correct nucleotide opposite them. In contrast, Dpo4 could extend a small fraction of the primer to a full-length product on both S-cdG and S-cdA templates. Dpo4 incorporated dTMP preferentially opposite S-cdA over the other dNMPs, but the discrimination was only 2- to 8-fold more proficient. Further extension of the S-cdA:T and S-cdA:C pair was not much different. For S-cdG, conversely, the wrong nucleotide, dTMP, was incorporated more efficiently than dCMP, although one-base extension of the S-cdG:T pair was less efficient than the S-cdG:C pair. S-cdG, therefore, has the propensity to cause G → A transition, as was reported to occur in *E. coli*. The results of this study are consistent with the strong replication blocking nature of S-cdA and S-cdG, and their ability to initiate error-prone synthesis by Y-family DNA polymerases.
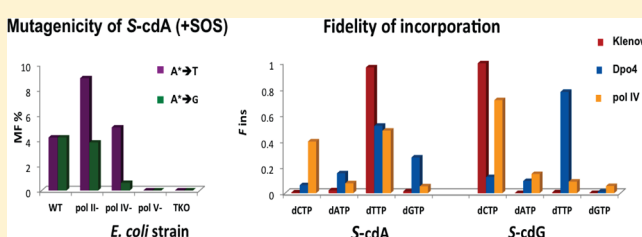
■ INTRODUCTION

The oxidative DNA damages, 8,5′-cyclo-2′-deoxyadenosine (cdA) and 8,5′-cyclo-2′-deoxyguanosine (cdG) diastereomers (Figure 1), have been detected in DNA derived from various
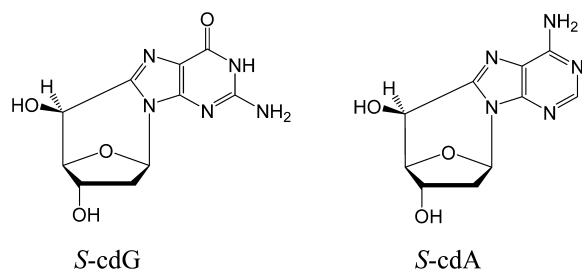

Figure 1. Structure (5′S)-8,5′-cyclo-2′-deoxyguanosine (S-cdG) and (5′S)-8,5′-cyclo-2′-deoxyadenosine (S-cdA).

cells and organisms.[1] A characteristic of these lesions is that both the 2′-deoxyribose and the purine base are damaged, and the C5′−C8 intramolecular cyclization induces an unusual O4′-*exo* (west) pseudorotation in DNA.[2] The O4′-*exo* (west) pseudorotation causes a perturbation in the helical twist and base pair stacking of DNA, resulting in thermodynamic destabilization of the DNA duplex. However, Watson−Crick base-pairing can be maintained. Owing to the presence of the C5′-C8 covalent bond between the base and the sugar, base excision repair cannot excise cdA and cdG, and indeed, no glycosylase has been found to repair these oxidative lesions.[3,4] However, nucleotide excision repair (NER) system can repair these DNA damages,[3,4] and the efficiency of repair varies with the base located opposite them in the complementary strand.[5]

These lesions are suspected to play a role in neurologic diseases in Xeroderma Pigmentosum patients with defects in NER.[6] S-cdA accumulates in genomic DNA of Cockayne syndrome B-deficient mice, signifying that the cyclopurine lesions may accumulate in Cockayne syndrome patients.[7] S-cdA is also a strong block of gene expression in human cells.[4]

In a previous investigation, we found that S-cdG is highly toxic and mutagenic in *Escherichia coli*.[8] High toxicity of this lesion was unexpected since the C5′—C8 bond locks the base in the anti-orientation but does not adversely affect Watson—Crick base-paring.[2] We also determined that error-prone bypass by DNA polymerase V (pol V) is essential for its viability and mutagenesis. Genotoxic and mutagenic properties of S-cdG and S-cdA were reported also by others.[9] In *E. coli*, pol V is one of the three SOS polymerases, which also include pol II and pol IV, for executing translesion synthesis (TLS) of replication-blocking lesions.[10] Many TLS polymerases, including pol IV and pol V in *E. coli*, belong to the Y-family of specialized DNA polymerases,[10,11] whereas pol II belongs to the B-family.[12] The TLS polymerases have more spacious active sites allowing them to accommodate many DNA lesions. In addition, unlike the replicative polymerases, the lack of proofreading exonuclease function of the TLS polymerases permits them to continue DNA synthesis past many lesions, albeit with a lower fidelity. For S-cdG and S-cdA, in vitro primer extension studies were performed with *Saccharomyces cerevisiae* and human polymerase η, and both bypass these lesions accurately and efficiently.[13]

In the current work, we have evaluated error-free and error-prone TLS of S-cdA in *E. coli*. We show that, like S-cdG, S-cdA is a strong block of replication and its bypass is entirely dependent on pol V. Even so, pol II and pol IV play intriguing roles in bypass and mutagenicity of S-cdA. In order to better understand the intricacies of TLS past S-cdG and S-cdA, we have investigated the in vitro nucleotide incorporation and extension kinetics of S-cdA and S-cdG by pol IV, one of the polymerases involved in S-cdA mutagenesis in *E. coli*. In addition, we have investigated in vitro TLS by exo-free Klenow fragment (KF (exo⁻)) and Dpo4, two model DNA polymerases that, like pol IV and pol V, lack the proofreading 3′ → 5′ exonuclease editing function. The Klenow fragment retains the polymerase activity of *E. coli* pol I, an A-family DNA polymerase, which plays roles in processing Okazaki fragments and in gap-filling during excision-repair processes, whereas Dpo4, an archebacterial DNA polymerase, is a homologue of *E. coli* pol IV and is regarded as a prototypical Y-family DNA polymerase.

■ MATERIALS AND METHODS

Materials. [γ-³²P] ATP was from Du Pont New England Nuclear (Boston, MA). KF (exo⁻), *Eco*RV restriction endonuclease, T4 DNA ligase, T4 polynucleotide kinase, uracil DNA glycosylase, exonuclease III, Dnase I, and Rnase TI were obtained from New England Bioloabs (Beverly, MA). S-cdA-cyanoethyl phosphoramidite was purchased from Berry and Associates (Dexter, MI). pMS2 plasmid and Dpo4 were gifts from M. Moriya (SUNY, Stony Brook, NY) and Z. Suo (Ohio State University, Columbus, OH), respectively.

The *E. coli* strains used were AB1157 [F⁻ *thr-1 araC14 leuB6*(Am) Δ(*gpt-proA*)62*lacY1 tsx-33 supE44*(AS) *galK2*(Oc) *hisG4*(Oc) *rfbD1 mgl51 rpoS396*(Am) *rpsL31*(Str^r) *kdgK51 xylA5 mtl-1 argE3*(Oc) *thi-1*], pol II⁻ (AB1157 but *polBΔ1*::Ω Sm-Sp), pol IV⁻ (AB1157 but Δ*dinBW2*::cat), GW8017 (AB1157 but *umuDC595*::cat), and pol II⁻/pol IV⁻/pol V⁻ (AB1157 but *polBΔ1*::Ω Sm-Sp *dinB umuDC595*::cat). All *E. coli* strains were provided by G. Walker (MIT, Cambridge, MA).

Methods. *Overexpression and Purification of HT- DinB.* The gene sequence of *dinB* was cloned into pYG648 ampicillin resistant plasmid, and it was incorporated in to the commercially available pET-16b vector (a gift of Drs. M. Yamada and T. Nohmi, NIHS, Tokyo, Japan).[14] Fifty nanograms of the modified vector was transformed in BL21 (DE3) cells and plated on LB agar plates containing 70 μg/mL ampicillin and 50 μg/mL chloramphenicol. Individual colonies were picked and grown in a 10 mL of LB broth overnight at 37 °C. Starting with an aliquot of the overnight culture, a log phase culture with ampicillin and chloramphenicol was grown, and the expression of the proteins were induced with 1 mM IPTG at 0.8 OD_{260}. After 30 min incubation, rifampicin (100 μg/mL) was added. Cells were then harvested, washed with buffer A (50 mM NaH_2PO_4, 5 mM Tris-Cl, 300 mM NaCl, and 20 mM imidazole (pH 8)), and resuspended in the same buffer (10 mL of total volume). Cells were lysed by sonication and the lysates were treated with Dnase I (40 mg/mL) and Rnase TI (130 U/mL) (New England Biolabs). The final volume was brought to 14 mL with 20 mM 2-mercaptoethanol (buffer B). The lysate was then spun down at 13,000 × g. The supernatant was applied to a Ni-NTA resin 5 mL column (Qiagen). The column was washed with 15 mL of wash buffer 1 (10 mM imidazole, 20 mM Na_2HPO_4, and 300 mM NaCl) and subsequently with 10 mL of wash buffer 2 (25 mM imidazole, 20 mM Na_2HPO_4, and 300 mM NaCl) after application of lysate to the column. The column was eluted with the elution buffer (250 mM imidazole, 20 mM NaH_2PO_4, and 300 mM NaCl at pH 8.0). Fractions containing the pure protein at 42 kDa were combined and analyzed by SDS PAGE. The purified protein fractions were frozen in liquid nitrogen and stored at −80 °C.

Synthesis and Characterization of Oligonucleotides. The S-cdA-modified oligonucleotide 5′-GTGCA*TGTTTGT-3′ was synthesized and characterized using an approach similar to the synthesis of S-cdG-containing oligonucleotides.[2] Likewise, the 26-mers 5′-GTGC-XTGTTTGTATCGCTTGCAGGGG-3′, where X = S-cdG or S-cdA, were synthesized and characterized.[2] The DNA sequence of the 12-mer and the first 12-nucleotides of the 26-mer template was the same, and this sequence was taken from codon 272−275 of the *p53* gene, in which the lesion was incorporated at the second nucleotide of codon 273, a well-known mutational hotspot for human cancer.[15] The unmodified oligonucleotides were analyzed by MALDI-TOF MS analysis, which gave a molecular ion with a mass within 0.005% of theoretical, whereas the adducted oligonucleotides were analyzed by ESI-MS in addition to digestion followed by HPLC analysis. The m/z of the S-cdG and S-cdA 26-mers gave 8078 and 8062 Da, respectively, whereas the same for the S-cdA 12-mer was 3680 Da, consistent with the presence of the corresponding cyclopurine lesions. Digestion of the oligonucleotides followed by HPLC analyses further confirmed the presence of the lesion (data not shown).

Construction and Characterization of pMS2 Vectors Containing a Single S-cdA. The single stranded pMS2 shuttle vector, which contains its only *Eco*RV site in a hairpin region, was prepared as described.[16] The pMS2 DNA (58 pmols, 100 μg) was digested with a large excess of *Eco*RV (300 pmol, 4.84 μg) for 1 h at 37 °C followed by room temperature overnight. A 58-mer scaffold oligonucleotide was annealed overnight at 9 °C to form the gapped DNA. The control and lesion-containing dodecamers, after phosphorylation with polynucleotide kinase, were combined with gapped pMS2 DNA and ligated overnight at 16 °C. Unligated oligonucleotides were removed by passing through Centricon-100, and the DNA was precipitated with ethanol. The scaffold oligonucleotide was digested by treatment with uracil DNA glycosylase and exonuclease III, the proteins were extracted with phenol/chloroform, and the DNA was precipitated with ethanol. The final construct was dissolved in 1 mM Tris-HCl-0.1 mM EDTA, pH 8, and a portion was subjected to electrophoresis on 1% agarose gel in order to assess the amount of circular DNA.

Transformation in E. coli and Analyses of Progeny. The control and S-cdA construct were used to transform *E. coli* cells, and transformants were analyzed by oligonucleotide hybridization.[8,17] Oligonucleotide probes containing the complementary 15-mer sequence were used for analysis. Two 14-mer left and right probes were used to select phagemids containing the correct insert, and

transformants that did not hybridize with both the left and right probes were omitted. Any transformant that hybridized with the left and right probes but failed to hybridize with the 15-mer wild-type probe were subjected to DNA sequence analysis. Lesion bypass efficiency was calculated by comparing the transformation efficiency of the S-cdA construct with that of the control, whereas mutation frequency (MF) was calculated on the basis of hybridization and sequence analysis.

In Vitro Nucleotide Incorporation and Chain Extension. A 26-mer template, 5′-GTGCXTGTTTGTATCGCTTGCAGGGG-3′, where X = S-cdG or S-cdA, was used for the in vitro studies. We used both running-start and standing-start conditions to evaluate bypass of the lesion. Template-primer complex (50 nM) was incubated with increasing concentration of pol IV, KF (exo⁻), or Dpo4 at 37 °C for 30 min in the presence of all four dNTPs (100 μM). For the running-start experiments, a 5′-³²P-radiolabeled 14-mer primer, 5′-CTGCAAGCGATACA-3′, was annealed to the template so that it was five bases 3′ to the lesion. To determine the nucleotide preferentially incorporated opposite S-cdG or S-cdA, the steady-state kinetic analyses were performed by the method of Goodman and co-workers.[18,19] The primed template was obtained by annealing 2-fold molar excess of the modified or control 26-mer template (~2.5 pmol) to a complementary 5′-³²P-labeled primer. Primer extension in standing-start conditions was carried out with pol IV (5–50 nM), KF (exo⁻) (5.5–40.5 nM), or Dpo4 (9.4–38 nM) with varying concentration of individual dNTPs (from 1 nM to 1 mM) in 25 mM Tris-HCl buffer (pH 7.5), 5 mM MgCl₂, and 5 mM dithiothreitol at 37 °C for 2 min. For certain experiments, the primer extension in running-start conditions was carried out with a mixture of all four dNTPs for various times. The reactions were terminated by adding an equal volume of 95% (v/v) formamide, 20 mM EDTA, 0.02% (w/v) xylene cyanol, and 0.02% (w/v) bromophenol blue and heating at 90 °C for 2 min, and the products were resolved on a 20% polyacrylamide gel containing 8 M urea. The DNA bands were visualized and quantitated using a Phosphorimager. The dNTP concentration and the time of incubation were optimized to ensure that primer extension was less than 20%. The K_m and k_{cat} were extrapolated from the Michaelis–Menten plot of the kinetic data. Fidelity (F) of incorporation or extension was determined by the following equation: $(k_{cat}/K_m)_{incorrect}/(k_{cat}/K_m)_{correct}$.

RESULTS

Viability and Mutagenicity of S-cdA in E. coli. The lesion bypass efficiency or viability can be determined by comparing the transformation efficiencies of the lesion-containing and control construct. Compared to the control, progeny derived from the S-cdA construct upon transformation was 0.5% in E. coli with normal repair and replication functions, which increased more than 17-fold with SOS (Table 1). Viability was also low in pol II- and pol IV-deficient strains (Table 1). Upon induction of SOS, in pol II-deficient strain viability increased only 3-fold, whereas in pol IV-deficient strain viability increased more than 25-fold (Table 1). Remarkably, however, no progeny was recovered from pol V-deficient strain

Table 1. Viability (%) of S-cdA in E. coli[a]

polymerase knocked out	−SOS	+SOS[b]
none	0.5 ± 0.2	8.7 ± 2.5
pol II	0.6 ± 0.1	1.7 ± 0.3
pol IV	0.4 ± 0.2	10.6 ± 1.4
pol V	<0.001	<0.005
pol II/pol IV/pol V	<0.001	<0.001

[a]Viability was determined in percentage by comparing transformation efficiency of the S-cdA plasmid with that of the control construct (considered to be 100%). The data represent 3−6 independent experiments. [b]SOS was induced with 20 J/m² UV irradiation.

or the strain deficient in all three SOS polymerases. These results are similar to what was observed on replication of a S-cdG construct reported earlier,[8] and we conclude that pol V (UmuD′₂C) is required for replication of both S-cdA and S-cdG. On the basis of the noteworthy increase in viability of both S-cdA and S-cdG construct with SOS in the pol IV-deficient strain, we postulate that when both polymerases are present, pol V and pol IV compete to conduct TLS, but pol IV is unable to bypass the lesion, generating many unproductive polymerase-DNA complexes, although some of them might be extended by pol V. However, in pol IV-deficient strain, pol V is able to carry out TLS of many more templates. The role of pol II may be more complex in that it plays a secondary role in bypassing these lesions, but it also is unable to bypass them independently.

The progeny from each transformation was analyzed by oligonucleotide hybridization followed by DNA sequencing. As shown in Table 2, mutational frequency (MF) in repair and replication proficient strain was 6.2% and 8.4% without and with SOS, respectively, and equal fraction of mutants carried A → T and A → G in each case. In pol II-deficient strain, the MF enhanced 2.5-fold to 15% in the absence of SOS, which did not increase with SOS and, in fact, dropped slightly to 13% (Table 2). However, the enhancement in MF in the pol II-deficient strain was mainly due to the increase in the A → T events. In contrast, MF was lower in pol IV-deficient strain with 4.2% and 5.6% without and with SOS, respectively, and the major type of mutation with SOS was A → T (Table 2). Although a clear picture of the type of errors by individual polymerases did not emerge, it seems likely that pol IV plays a role in most A → G mutations, whereas pol V may be involved in A → T mutations. The role of pol II is less certain, but it seems to play a role in error-free bypass. Increased MF in pol II-deficient strain in the absence of SOS was also observed with S-cdG.[8]

In Vitro Replication of S-cdG and S-cdA by Pol IV. As evidenced in the previous section and in our earlier published work,[8] an intriguing aspect of the cyclopurine lesions is that they are highly toxic in E. coli, even though these lesions only contain an extra covalent bond. High toxicity of DNA lesions is a consequence when they are strong blocks to replication. Yet, published reports indicate that both S-cdG and S-cdA are readily bypassed by the Y-family DNA polymerase pol η from yeast and human.[13] In order to explore this issue critically, we have investigated in vitro TLS by pol IV, one of the Y-family SOS polymerases in E. coli. This polymerase is also of interest because of its potential role in A → G mutations in E. coli.

Although 50 nM or higher concentration of pol IV readily extended the 14-mer primer to a full-length 23-mer product on the control template, it was strongly blocked by the cyclopurine lesions (Figure 2A). Even with 300 nM pol IV, a full-length product was undetectable (Figure 2A). The major block was before the lesion, as evidenced by a strong 18-mer band, but a weak 19-mer band was formed when high concentration of the enzyme was used (Figure 2A). Of the two cyclopurine lesions, S-cdG was a stronger block to pol IV compared to S-cdA. The time course of primer extension on control template with 100 nM enzyme showed that full-length 23-mer product was formed within 5 min, whereas only a small fraction of the 19-mer band was formed in 30 min on lesion-containing templates (Figure 2B).

Steady-State Kinetic Analyses of Nucleotide Incorporation Opposite S-cdA and S-cdG and Further Extension by Pol IV. Since pol IV can incorporate a nucleotide opposite

Table 2. Mutations Induced by S-cdA in E. coli

polymerase knocked out	SOS[a]	exptl no.	no. of colonies screened	no. of mutations[b] (%)	no. of A* → T (%)	no. of A* → G (%)	other mutations (%)
none	−	1	56	5	2	3	0
		2	57	3	3	0	0
		3	49	2	0	2	0
		total	162	10 (6.2)	5 (3.1)	5 (3.1)	0
	+	1	80	6	3	3	0
		2	86	8	4	4	0
		total	166	14 (8.4)	7 (4.2)	7 (4.2)	0
pol II	−	1	47	8	6	2	0
		2	53	6	4	2	0
		3	50	9	5	3	1[c]
		total	150	23 (15.3)	15 (10.0)	7 (4.7)	1[c](0.6)
	+	1	79	10	6	4	0
		2	78	10	8	2	0
		total	157	20 (12.7)	14 (8.9)	6 (3.8)	0
pol IV	−	1	72	4	1	1	2[d]
		2	73	2	1	1	0
		total	145	6 (4.2)	2 (1.4)	2 (1.4)	2[d](1.4)
	+	1	85	4	3	1	0
		2	76	5	5	0	0
		total	161	9 (5.6)	8 (5.0)	1 (0.6)	0

[a]SOS was induced with 20 J/m² UV irradiation. [b]For each strain, MF of control construct was <1% (data not shown). [c]A*T → TG. [d]A* → C.

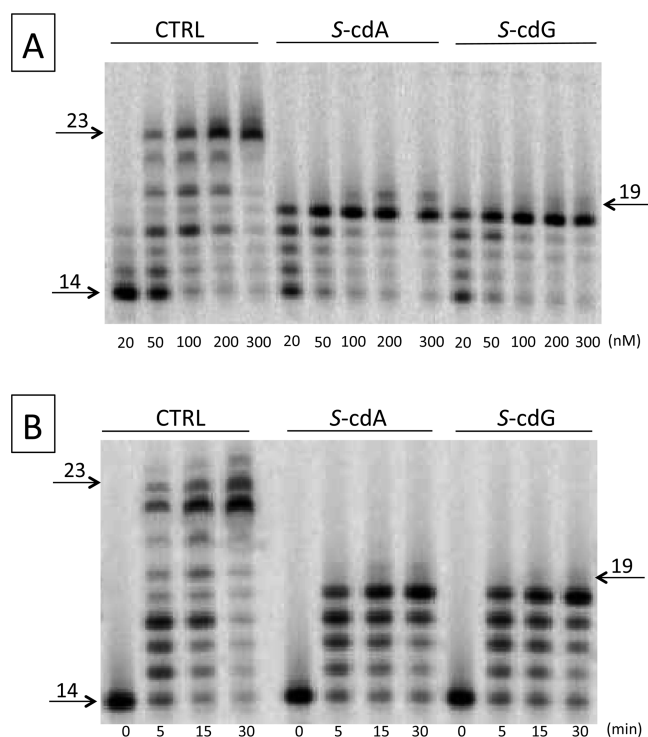5'-GTGCXTGTTTGTAT CGCTTGCAGGGG-3'
ACATAGCGAACGTCp32

Figure 2. Extension of a 14-mer primer by pol IV DinB by varying concentration (20, 50, 100, 200, and 300 nM) of the enzyme incubated for 30 min (panel A) and with increasing time (0, 5, 15, and 30 min) with 100 nM enzyme (panel B) on control, S-cdA, and S-cdG 23-mer templates. Template-primer complex (50 nM) was incubated at 37 °C in the presence of all four dNTPs (100 μM).

S-cdA and S-cdG, we used steady-state kinetic assays to determine the kinetic parameters of nucleotide insertion opposite these lesions relative to the unmodified bases. Albeit 10,000-fold less efficiently than the control, pol IV preferen-

tially incorporated the correct nucleotide opposite both lesions (Table 3). For S-cdG, incorporation of dCMP opposite the lesion was approximately 10-fold more efficient than incorporation of the other dNMPs (Table 3). In contrast, for S-cdA, incorporation of dCMP was 80% as efficient as incorporation of dTMP (Table 3). Next, we evaluated extension past these lesions after a nucleotide has been incorporated opposite the lesion. It is evident from Table 4 that extension past either S-cdA or S-cdG by pol IV was extremely sluggish, with nearly 10⁴- to 10⁵-fold less efficient extension, even when a correct base (C opposite S-cdG or T opposite S-cdA) was placed opposite the lesion (Table 4). Extension of a wrong base (T opposite S-cdG or C opposite S-cdA) was 10-fold less efficient than the correct base opposite these lesions.

In Vitro Replication of S-cdG and S-cdA by Exo-Free Klenow Fragment and Dpo4. In addition to pol IV, we investigated in vitro TLS by KF (exo-) and Dpo4, two other DNA polymerases that lack the 3′ → 5′ proofreading exonuclease activity. As shown in Figures 3 and 4, both S-cdG and S-cdA were strong blocks to these DNA polymerases. The most intense band was a 19-mer, indicating replication block after incorporation of a nucleotide opposite the lesion. For KF (exo⁻), with 20 nM enzyme, two additional bands of 20-mer and 21-mer were observed for both S-cdA and S-cdG (Figure 3A), but even with significantly increased concentration of the enzyme, full-length 23-mer products were nearly undetectable. In contrast, for Dpo4, though 19-mer was the longest band observed with 20 nM enzyme, with 100 and 200 nM enzyme concentration, a weak band of full length 23-mer was noticeable for both S-cdA and S-cdG (Figure 4A). Despite partial extension, however, in each case 19-mer was the strongest band for these lesions (Figures 3A and 4A). The time course of primer extension also was similar in that with 100 nM concentration of KF (exo-), the extension did not progress to appreciable extent beyond a 21-mer product with increasing time up to 30 min (Figure 3B). For 316 nM Dpo4, in contrast, full length 23-mer product was formed within 5 min, which

Table 3. Kinetic Parameters for dNTP Incorporation by Pol IV on an Undamaged and *S*-cdG or *S*-cdA Containing Substrate

dNTP	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})	F_{inc}[a]
5'-GTGC**G***TGTT TGTATC GCTTGCAGGGG-3' ACAAACATAGCGAACp32				
Undamaged substrate				
dCTP	15 ± 2	15 ± 1	1	1
dATP	0.10 ± 0.03	354 ± 87	2.8×10^{-4}	2.8×10^{-4}
dTTP	0.056 ± 0.007	27 ± 3	2.1×10^{-3}	2.1×10^{-3}
dGTP	0.070 ± 0.008	305 ± 14	2.3×10^{-4}	2.3×10^{-4}
S-cdG–containing substrate				
dCTP	0.07 ± 0.02	403 ± 188	1.7×10^{-4}	1.7×10^{-4}
dATP	0.011 ± 0.001	317 ± 96	3.5×10^{-5}	3.5×10^{-5}
dTTP	0.020 ± 0.005	965 ± 202	2.1×10^{-5}	2.1×10^{-5}
dGTP	0.0056 ± 0.0004	442 ± 128	1.3×10^{-5}	1.3×10^{-5}
5'-GTGC**A***TGTT TGTATC GCTTGCAGGGG-3' ACAAACATAGCGAACp32				
Undamaged substrate				
dCTP	0.055 ± 0.005	605 ± 129	9.1×10^{-5}	3.4×10^{-5}
dATP	0.0153 ± 0.0009	93 ± 29	1.7×10^{-4}	6.3×10^{-5}
dTTP	24.6 ± 0.5	9 ± 3	2.7	1
dGTP	0.008 ± 0.001	295 ± 36	2.7×10^{-5}	1.0×10^{-5}
S-cdA–containing substrate				
dCTP	0.131 ± 0.002	260 ± 23	5.0×10^{-4}	1.9×10^{-4}
dATP	0.048 ± 0.009	501 ± 76	9.6×10^{-5}	3.6×10^{-5}
dTTP	0.0555 ± 0.0009	88 ± 25	6.3×10^{-4}	2.3×10^{-4}
dGTP	0.031 ± 004	455 ± 164	6.8×10^{-5}	2.5×10^{-5}

[a]Fidelity (*F*) of incorporation or extension was determined by the following equation: $(k_{cat}/K_m)_{incorrect}/(k_{cat}/K_m)_{correct}$.

Table 4. Kinetic Parameters for One-Base Extension Past the Lesion (i.e., dGTP Incorporation Opposite the 5′C to the Lesion or Control) with a Correct or Mismatched Base Opposite It by Pol IV

Template (X)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})
5'-GTGCXTGT TTG TATCGCT TGCAGGGG-3' CACAAACATAGCGAACp32			
G	3.1 ± 1.3	6.4 ± 2.4	0.5
S-cdG	0.039 ± 0.016	223 ± 89	1.8×10^{-4}
5'-GTGCXTGT TTGTATCGCT TGCAGGGG-3' TACAAACATAGCGAACp32			
G	0.011 ± 0.003	137 ± 53	8.0×10^{-5}
S-cdG	0.011 ± 0.002	341 ± 122	3.2×10^{-5}
5'-GTGCXTGT TTGTATCGCT TGCAGGGG-3' TACAAACATAGCGAACp32			
A	3.2 ± 0.5	8.6 ± 2.1	0.4
S-cdA	0.02 ± 0.01	196 ± 81	1.0×10^{-4}
5'-GTGCXTGT TTG TATCGCT TGCAGGGG-3' CACAAACATAGCGAACp32			
A	0.21 ± 0.06	103 ± 35	2.0×10^{-3}
S-cdA	0.0039 ± 0.0005	233 ± 25	1.7×10^{-5}

5'-GTGCXTGTTTGTAT CGCTTGCAGGGG-3'
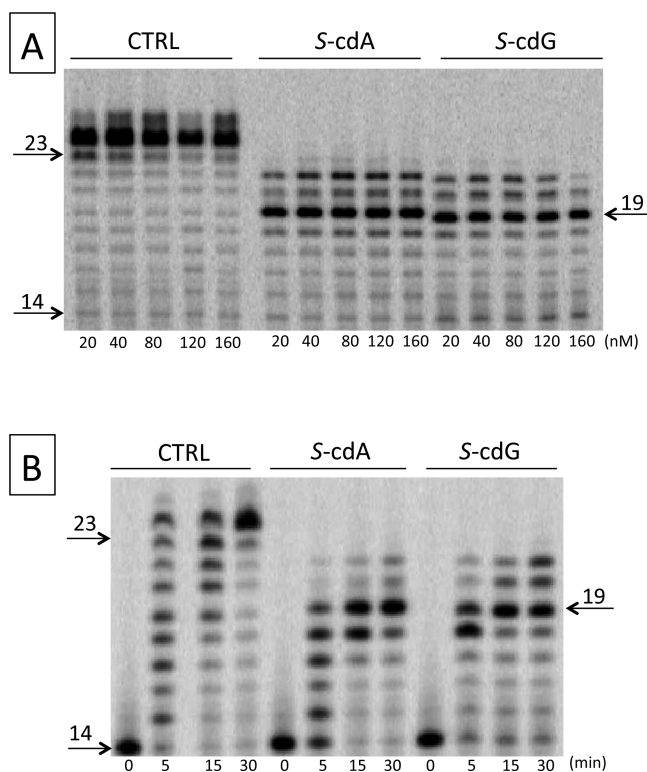ACATAGCGAACGTCp32

Figure 3. Extension of a 14-mer primer by KF (exo⁻) by varying concentration (20, 40, 80, 120, and 160 nM) of the enzyme incubated for 30 min (panel A) and with increasing time (0, 5, 15, and 30 min) with 100 nM enzyme (panel B) on control, S-cdA, and S-cdG 23-mer templates. Template−primer complex (50 nM) was incubated at 37 °C in the presence of all four dNTPs (100 μM).

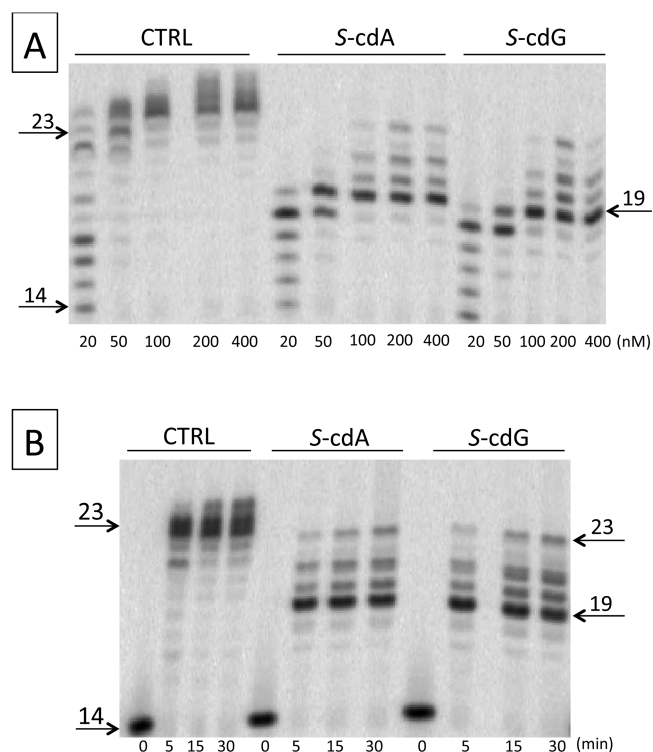5'-GTGCXTGTTTGTAT CGCTTGCAGGGG-3'
ACATAGCGAACGTCp32

Figure 4. Extension of a 14-mer primer by Dpo4 by varying concentration (20, 50, 100, 200, and 400 nM) of the enzyme incubated for 30 min (panel A) and with increasing time (0, 5, 15, and 30 min) with 316 nM enzyme (panel B) on control, S-cdA, and S-cdG 23-mer templates. Template−primer complex (50 nM) was incubated at 37 °C in the presence of all four dNTPs (100 μM).

increased with time, but for both S-cdA and S-cdG, the 19-mer was the strongest band (Figure 4B).

Steady-State Kinetic Analyses of Nucleotide Incorporation Opposite S-cdA and S-cdG by KF (exo-) and Dpo4. Next, we determined the kinetic parameters of nucleotide incorporation opposite these lesions and the control by KF (exo⁻) and Dpo4. KF (exo⁻) incorporated the correct nucleotide opposite both S-cdA and S-cdG, but dTMP incorporation opposite S-cdA and dCMP incorporation opposite S-cdG were more than 100-fold less efficient compared to the unmodified base A and G, respectively (Table 5). Nevertheless, correct nucleotide incorporation was preferred, and misincorporation frequency opposite S-cdA and S-cdG was 10- to 100-fold less efficient than correct nucleotide incorporation (Table 5). Similarly, for Dpo4, the efficiency of dCMP incorporation, as determined by the k_{cat}/K_m values, opposite S-cdG was only 0.06% relative to G (Table 6), but the nucleotide preferentially incorporated opposite S-cdG by Dpo4 was the wrong nucleotide dTMP, which was incorporated nearly 10-fold more efficiently than dCMP. The order of nucleotide incorporation opposite S-cdG was dTMP > dCMP > dAMP > dGMP. For S-cdA, although nearly 1000-fold less efficient relative to a template A, the correct nucleotide dTMP incorporation was preferred by Dpo4 (Table 6), but the efficiency of misincorporation was not significantly different from the correct nucleotide incorporation in that dGMP,

dAMP, and dCMP incorporations were 53%, 29%, and 12%, respectively, as efficient as incorporation of dTMP.

Steady-State Kinetic Analyses of Extension Past S-cdA and S-cdG by KF (exo⁻) and Dpo4. We also evaluated extension past these lesions after a nucleotide has been inserted opposite these lesions. It is evident from Table 7 that extension past these lesions by KF (exo⁻) was extremely sluggish, with nearly 10^5- to 10^6-fold less efficient extension, even when a correct base (C opposite S-cdG or T opposite S-cdA) was located opposite the lesion (Table 7). Indeed, extension of a G:A or A:C mismatch occurred more efficiently by KF (exo⁻) than either the correct or wrong base-pair with S-cdG or S-cdA (Table 7). Also, the extension of S-cdG:T mismatch was nearly as efficient as S-cdG:C pair, whereas S-cdA:C pair was extended slightly more efficiently than S-cdA:T pair (Table 7). For Dpo4, the S-cdG:T mismatched pair was extended 10-fold more slowly relative to the S-cdG:C pair, which itself extended quite inefficiently (i.e., >10^4-fold less efficiently than a G:C pair) (Table 8). Conversely, S-cdA:T pair was extended by Dpo4 almost as efficiently as the S-cdA:C pair, although it was more than 10-fold less efficient than an A:C mismatched pair (Table 8).

■ DISCUSSION

Both S-cdG and S-cdA contain an extra covalent bond between the base and the sugar. Therefore, it is not surprising that some of their chemical and biological characteristics are similar. Even

Table 5. Kinetic Parameters for dNTP Incorporation by KF (exo⁻) on an Undamaged and S-cdG or S-cdA Containing Substrate

dNTP	k_{cat} (min⁻¹)	K_m (μM)	k_{cat}/K_m (μM⁻¹ min⁻¹)	F_{inc}[a]
5'-GTGC**G***TGTT TGTATC GCTTGCAGGGG-3' ACAAACATAGCGAACp32				
Undamaged substrate				
dCTP	8.1 ± 1.7	0.04 ± 0.01	198	1
dATP	1.7 ± 0.3	159 ± 13	0.01	5.1×10^{-5}
dTTP	1.9 ± 0.3	101 ± 8	0.02	1.0×10^{-4}
dGTP	2.3 ± 0.5	35 ± 6	0.065	3.3×10^{-4}
S-cdG–containing substrate				
dCTP	6 ± 3	4 ± 2	1.5	7.6×10^{-3}
dATP	0.50 ± 0.09	349 ± 119	0.001	0.5×10^{-5}
dTTP	0.30 ± 0.04	39 ± 1	0.008	4.0×10^{-5}
dGTP	0.06 ± 0.03	46 ± 16	0.001	0.5×10^{-5}
5'-GTGC**A***TGTT TGTATC GCTTGCAGGGG-3' ACAAACATAGCGAACp32				
Undamaged substrate				
dCTP	1.6 ± 0.2	113 ± 44	0.014	1.8×10^{-4}
dATP	3.2 ± 0.6	45 ± 10	0.07	8.8×10^{-4}
dTTP	6.4 ± 0.4	0.08 ± 0.03	80	1
dGTP	0.71 ± 0.1	25 ± 8	0.03	3.8×10^{-4}
S-cdA–containing substrate				
dCTP	0.37 ± 0.01	46 ± 6	0.008	1.0×10^{-4}
dATP	2.9 ± 0.3	84 ± 16	0.035	4.4×10^{-4}
dTTP	2.4 ± 0.8	1.6 ± 1.2	1.5	2.0×10^{-2}
dGTP	1.5 ± 0.2	76 ± 28	0.02	2.5×10^{-4}

[a]Fidelity (F) of incorporation or extension was determined by the following equation: $(k_{cat}/K_m)_{incorrect}/(k_{cat}/K_m)_{correct}$.

so, they exhibit distinct biological properties as well. In terms of similarities, S-cdG and S-cdA are highly genotoxic in *E. coli*, with viability less than 1% relative to control, which increased several-fold with SOS (Table 1 and ref 8). Furthermore, they are mutagenic in *E. coli*, but S-cdG is considerably more mutagenic than S-cdA (Table 2 and ref 8). TLS of both lesions is entirely dependent on pol V since in pol V-deficient strains viability dropped to nearly 0%. With respect to their differences, in *E. coli*, the major class of mutations induced by S-cdG is G → A transitions,[8,9] whereas S-cdA induced both A → T and A → G base substitutions (Table 2). S-cdA induced A → T transversions and A → G transitions in equal frequency in cells with normal repair and replication functions, which remained the same with SOS induction, but A → T was the dominant mutation in pol II-deficient and SOS-induced pol IV-deficient strains. The A → G mutations dropped considerably in pol IV-deficient strains, suggesting that pol IV may be able to incorporate dCMP opposite S-cdA, but it needs pol V for further extension. In contrast, pol II seems to play a role in error-free replication, but it also is dependent on pol V.

It is worth noting that another study of TLS of S-cdG and S-cdA in *E. coli* has been published.[9] However, this investigation concentrated only on SOS-induced *E. coli*, whereas our study also determined the bypass and mutagenicity in the absence of SOS. While the pattern of bypass efficiency and mutagenicity in SOS-induced *E. coli* of the earlier report and our results show similar trend, there are significant quantitative differences between the results of these investigations. We believe that the

difference stems from the differences between the two experimental systems. The reported study exhibited a high background on both the bypass efficiency and error rate,[9] whereas the background in our approach has been very low.[8] As a result, we could determine the absolute requirement of pol V in TLS of S-cdA (this study) and S-cdG (ref 8), which the other study was unable to ascertain.[9] The bypass efficiency of S-cdA in the pol V-deficient strain was 13% in this earlier work,[9] whereas we determined it to be less than 1%. Furthermore, the substantial reduction in MF of A → G mutations in pol IV-deficient strain noted in the current work was not identified, presumably due to the high background MF. However, some of the differences between the studies may also have been due to the location of the lesions in different sequence contexts.

The cellular results of the current study prompted us to investigate in vitro replication by three different DNA polymerases to determine if these lesions are strong replication blocks and if they are error-prone when a purified polymerase replicates past them. We chose pol IV DinB, which seems to play critical roles in TLS of cyclopurine lesions in *E. coli*. As anticipated from the cellular studies, S-cdG and S-cdA were major blocks to pol IV, and only very sluggishly it could incorporate a nucleotide opposite these lesions. Because there are many more copies of pol IV compared to pol V in SOS-induced cells,[20] we hypothesize that pol IV forms many unproductive primer–template–protein complexes, and only a fraction of the primer–template complexes are further extended by pol V. Since pol IV incorporated dTMP and

Table 6. Kinetic Parameters for dNTP Incorporation by Dpo4 on an Undamaged and *S*-cdG or *S*-cdA Containing Substrate

dNTP	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})	F_{inc}[a]
5'-GTGC*G**TGTT TGTATC GCTTGCAGGGG-3'				
ACAAACATAGCGAACp32				
Undamaged substrate				
dCTP	3.6 ± 0.3	0.26 ± 0.08	14	1
dATP	0.6 ± 0.1	20 ± 0.4	0.03	2.1×10^{-3}
dTTP	2.0 ± 0.0	32 ± 8	0.06	4.3×10^{-3}
dGTP	2.2 ± 0.2	61 ± 21	0.04	2.9×10^{-3}
S-cdG–containing substrate				
dCTP	0.8 ± 0.3	102 ± 9	0.008	5.7×10^{-4}
dATP	1.1 ± 0.3	187 ± 57	0.006	4.3×10^{-4}
dTTP	2.4 ± 0.3	51 ± 15	0.05	3.6×10^{-3}
dGTP	0.05 ± 0.01	73 ± 11	0.0007	5.0×10^{-5}
5'-GTGC*A**TGTT TGTATC GCTTGCAGGGG-3'				
ACAAACATAGCGAACp32				
Undamaged substrate				
dCTP	0.24 ± 0.01	35 ± 8	0.007	0.7×10^{-3}
dATP	1.1 ± 0.1	59 ± 5	0.02	2.0×10^{-3}
dTTP	0.6 ± 0.2	0.06 ± 0.01	10	1
dGTP	1.21 ± 0.02	17 ± 7	0.07	7.0×10^{-3}
S-cdA–containing substrate				
dCTP	0.270 ± 0.002	161 ± 66	0.002	0.2×10^{-3}
dATP	0.9 ± 0.2	194 ± 99	0.005	0.5×10^{-3}
dTTP	4.7 ± 0.4	269 ± 120	0.017	1.7×10^{-3}
dGTP	0.30 ± 0.09	33 ± 9	0.009	0.9×10^{-3}

[a]Fidelity (*F*) of incorporation or extension was determined by the following equation: $(k_{cat}/K_m)_{incorrect}/(k_{cat}/K_m)_{correct}$.

Table 7. Kinetic Parameters for One-Base Extension Past the Lesion (i.e., dGTP Incorporation Opposite the 5′C to the Lesion or Control) with a Correct or Mismatched Base Opposite It by KF (exo⁻)

Template (X)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})
5'-GTGCXTGT TTG TATCGCT TGCAGGGG-3'			
CACAAACATAGCGAACp32			
G	3.1 ± 0.2	0.02 ± 0.012	155
S-cdG	0.020 ± 0.001	65 ± 21	0.0003
5'-GTGCXTGT TTGTATCGCT TGCAGGGG-3'			
TACAAACATAGCGAACp32			
G	1.13 ± 0.05	57 ± 4	0.02
S-cdG	0.014 ± 0.002	40 ± 19	0.0004
5'-GTGCXTGT TTGTATCGCT TGCAGGGG-3'			
TACAAACATAGCGAACp32			
A	24 ± 11	0.18 ± 0.06	133
S-cdA	0.46 ± 0.02	127 ± 20	0.004
5'-GTGCXTGT TTG TATCGCT TGCAGGGG-3'			
CACAAACATAGCGAACp32			
A	6.2 ± 0.5	34 ± 6	0.2
S-cdA	0.061 ± 0.002	4 ± 1	0.015

Table 8. Kinetic Parameters for One-Base Extension Past the Lesion (i.e., dGTP incorporation Opposite the 5′C to the Lesion or Control) with a Correct or Mismatched Base Opposite It by Dpo4

Template (X)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})
5'-GTGCXTGT TTG TATCGCT TGCAGGGG-3' CACAAACATAGCGAACp32			
G	0.70 ± 0.06	0.006 ± 0.001	117
S-cdG	0.10 ± 0.01	32 ± 2	0.003
5'-GTGCXTGT TTGTATCGCT TGCAGGGG-3' TACAAACATAGCGAACp32			
G	0.78 ± 0.16	104 ± 20	0.008
S-cdG	0.03 ± 0.02	70 ± 29	0.0004
5'-GTGCXTGT TTGTATCGCT TGCAGGGG-3' TACAAACATAGCGAACp32			
A	0.06 ± 0.02	0.055 ± 0.02	1
S-cdA	0.05 ± 0.01	28 ± 12	0.0018
5'-GTGCXTGT TTG TATCGCT TGCAGGGG-3' CACAAACATAGCGAACp32			
A	2.0 ± 0.1	59 ± 9	0.03
S-cdA	0.053 ± 0.012	36 ± 18	0.0014

dCMP opposite S-cdA with nearly comparable efficiency, A → G mutation may be the result of nucleotide incorporation by this enzyme. In pol IV-deficient strain, this mutation was nearly eliminated. It is conceivable that pol V is responsible for most A → T mutations (Table 2). Another consequence of the absence of pol IV in the pol IV-knockout strain is that pol V can bypass more lesion-containing templates, resulting in the increased viability, as revealed in Table 1 for S-cdA and ref 8 for S-cdG.

For additional in vitro TLS studies, we have employed KF since for many years KF has served as a model DNA polymerase for studying the mechanism of template-directed DNA synthesis. In the early 1980s, studies of KF showed the first structural view of a DNA polymerase whose essential form was found to be conserved in all other polymerases.[21] Extensive kinetic studies have established the kinetic mechanism for nucleotide incorporation by KF.[22] In the current study, both running-start and standing-start experiments with KF (exo⁻) showed that S-cdA and S-cdG are indeed very strong blocks of replication. Even with a high concentration of the enzyme and longer incubation time, detectable full-length extension product was not formed. As in the case of pol IV, KF (exo⁻) was able to incorporate a nucleotide opposite the cyclopurine lesions, albeit much more slowly than opposite the control. Further extension was even more inefficient. In fact, the mismatched unmodified base-pairs were extended more efficiently than the pairs with a cyclopurine lesion. Yet, opposite both S-cdG and S-cdA, KF (exo⁻) preferentially incorporated the correct dNMP.

We compared the kinetic data from KF (exo⁻) and pol IV with that of Dpo4. The DNA polymerase Dpo4 from *Sulfolobus solfataricus* has been studied biochemically and structurally as a model Y-family DNA polymerase.[23−25] Like *E. coli* pol IV, both Dpo4 and eukaryotic pol κ are members of the DinB subfamily and possess similar error rates.[26,27] Crystal structures of Dpo4 and other Y-family DNA polymerases show that the Y-family

enzymes have the signature right-hand shape with palm, finger, and thumb domains found in all known DNA polymerases.[28,29] In addition, a fourth "little finger" domain tethered to the thumb domain is present only in the Y-family enzymes.[23,30] The little finger domain of Dpo4 fits into the DNA major groove upstream of the active site and interacts mainly with the backbone of both strands to increase the binding affinity between Dpo4 and DNA.[23,30,31] The Dpo4 active site is relatively loose and accessible to solvent when compared to the closed active sites of replicative DNA polymerases in the presence of DNA and a nucleotide. This may allow Dpo4 to tolerate bulky or distorted DNA lesions.

Unlike KF (exo⁻) and pol IV, Dpo4 was able to extend a small fraction of the primer to a full-length 23-mer product on both S-cdG and S-cdA templates. The major fraction of the primer, however, extended only up to the lesion site. The kinetic studies showed that Dpo4 is highly error-prone opposite S-cdG and S-cdA. For S-cdA, although dTMP was incorporated preferentially over the other nucleotide monophosphates, the discrimination was only 2- to 8-fold more proficient. Also, the kinetics of one-base extension of the S-cdA:T and S-cdA:C were not significantly different, indicating that some erroneous replication is likely to occur. For S-cdG, however, the wrong nucleotide, dTMP, was incorporated much more efficiently than dCMP. However, compared to the S-cdG:T mispair, one-base extension was more efficient for the S-cdG:C pair. Nevertheless, S-cdG would be expected to cause G → A transition, an outcome already reported to take place in *E. coli*.[8,9]

The nucleotide incorporation pattern by the three DNA polymerases opposite S-cdG and S-cdA was compared in Figure 5. Of the three enzymes, KF (exo⁻) was largely accurate and incorporated the correct dNMP opposite these lesions with high selectivity. However, the two DinB subfamily polymerases
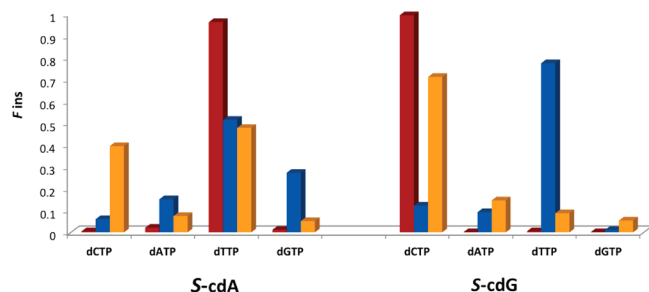
Figure 5. Comparison of the relative nucleotide incorporation efficiencies opposite *S*-cdA and *S*-cdG by pol IV (orange), KF (exo⁻) (red), and Dpo4 (blue), based on their fidelity of incorporation (F_{inc}).

not only were less selective but also showed distinct pattern of nucleotide incorporation opposite each lesion. Dpo4 was highly error-prone opposite *S*-cdG, incorporating dTMP preferentially over dCMP. It was not very accurate opposite *S*-cdA as well, despite a somewhat higher preference for the correct dTMP over other dNMPs. pol IV, however, was more error-prone opposite *S*-cdA. It is interesting to note that a third member of the DinB enzyme human pol κ has been recently reported to incorporate opposite *S*-cdG and *S*-cdA inefficiently.[32] This study also determined that pol η, pol ι, and pol ζ, but not pol κ, play important roles in replicating through these lesions in human cells.[32]

In a previous in vitro study, replication of yeast and human pol η past these cyclopurine lesions was investigated, which showed that pol η can accurately and efficiently bypass *S*-cdA and *S*-cdG.[13] In contrast, our results with KF (exo⁻), pol IV, and Dpo4 indicate that the cyclopurine lesions are very strong blocks of replication. This difference can be rationalized when a unique characteristic of pol η, part of which may also be shared by the *E. coli* pol V,[33] is considered. A distinction between pol η and other Y-family DNA polymerases is that the polymerase core is rotated away from the little finger domain to create even a larger binding pocket that can accommodate two nucleotides (as in the case of pyrimidine—pyrimidine dimer) into the pol η binding site.[34,35] This feature of pol η may also allow it to read through the rigid nucleobases such as *S*-cdG and *S*-cdA. Since Dpo4 and pol IV do not share this characteristic of pol η, they are much less efficient in bypassing the cyclopurine lesions. These data parallel the report that pol IV and Dpo4 are very inefficient in bypassing TT dimer, whereas pol η replicates through it efficiently and accurately.[36,37] On the basis of the current study, it is conceivable that, with the exception of pol η and pol V, *S*-cdG and *S*-cdA are strong replication blocks to other Y-family DNA polymerases. Even so, bypass of blocking lesions in mammalian cells is often carried out by two polymerases.[38] Our results suggest that it may also occur in *E. coli*.

ASSOCIATED CONTENT

Ⓢ Supporting Information

SDS-PAGE profile of purified HT-DinB protein and representative steady-state kinetic assay results are shown. 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.

DEDICATION

This manuscript is dedicated to the fond memory of Victor Fung, Ph.D., a former Program Officer at NCI and former Scientific Review Officer of the Cancer Etiology study section of CSR, NIH, for his wisdom, compassion, integrity, his love of sciences and the arts, his incredible culinary skills, and above all, his contributions to the career development of so many investigators during his own distinguished career.

ABBREVIATIONS

S-cdA, (5′*S*)-8,5′-cyclo-2′-deoxyadenosine; *S*-cdG, (5′*S*)-8,5′-cyclo-2′-deoxyguanosine; pol, DNA polymerase; NER, nucleotide excision repair; KF (exo⁻), exo-free Klenow fragment of DNA polymerase I; MF, mutation frequency; TLS, translesion synthesis; TKO, *E. coli* strain with triple knockouts (i.e., knockouts in pol II, pol IV, and pol V)

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