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Formation of S-[2-(N⁶-Deoxyadenosinyl)ethyl]glutathione in DNA and Replication Past the Adduct by Translesion DNA Polymerases

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Abstract

1,2-Dibromoethane (DBE, ethylene dibromide) is a potent carcinogen due at least in part to its DNA crosslinking effects. DBE crosslinks glutathione (GSH) to DNA, notably to sites on 2'-deoxyadenosine and 2'-deoxyguanosine (Cmarik, J. L. et al. (1991) *J. Biol. Chem.* 267, 6672–6679). Adduction at the N6 position of 2'-deoxyadenosine (dA) had not been detected, but this is a site for the linkage of O⁶-alkylguanine DNA alkyltransferase (Chowdhury, G., et al. (2013) *Angew. Chem. Int. Ed.* 52, 12879–12882). We identified and quantified a new adduct, S-[2-(N⁶-deoxyadenosinyl)ethyl]GSH, in calf thymus DNA using LC-MS/MS. Replication studies were performed in duplex oligonucleotides containing this adduct with human DNA polymerases (hPols) η , ι , and κ , as well as with *Sulfolobus solfataricus* Dpo4, *Escherichia coli* polymerase I Klenow fragment, and bacteriophage T7 polymerase. hPols η and ι , Dpo4, and Klenow fragment were able to bypass the adduct with only slight impedance; hPol η and ι showed increased misincorporation opposite the adduct compared to unmodified 2'-deoxyadenosine. LC-MS/MS analysis of full-length primer extension products by hPol η confirmed the incorporation of dC opposite S-[2-(N⁶-deoxyadenosinyl)ethyl]GSH and also showed the production of a –1 frameshift. These results reveal the significance of N⁶-dA GSH-DBE adducts in blocking replication, as well as producing mutations, by human translesion synthesis DNA polymerases.

Graphical abstract

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Associated Content

Supporting Information. Figure showing LC-MS characterization of the purified S-[2-(N⁶-deoxyadenosinyl)ethyl]GSH-containing oligonucleotide, displaying the chromatogram, mass spectrum, and deconvoluted mass spectrum of the product. Figure showing primer extension past the S-[2-(N⁶-deoxyadenosinyl)ethyl]GSH adduct by bacteriophage Pol T7 and *E. coli* Pol I Klenow fragment. This material is available free of charge at <http://pubs.acs.org>.

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Introduction

Halogenated hydrocarbons are widely used in commerce and industry, e.g. as solvents, pesticides, and propellants. 1,2-Dibromoethane (DBE, ethylene dibromide) has been historically used as an anti-knock additive in fuel and also has had uses as a pesticide in soil and in the organic synthesis of various compounds such as vinyl bromide. DBE is a known carcinogen^{1, 2} and had the highest hazard score for carcinogens in the Human Exposure/Rodent Potency Index prior to changes in the restrictions on the compound.³ DBE causes tumors in rats and mice in several different tissues, including nasal cavity, blood vessels, skin, lung, kidney, and liver.^{4–7}

DBE is not particularly reactive itself. It can be oxidized to 2-bromoacetaldehyde, but there is considerable evidence that the more important reaction in genotoxicity is conjugation with GSH, facilitated by GSH transferases.^{8–12} The resulting product *S*-(2-bromoethyl)GSH, a half-mustard, then reacts with DNA through an episulfonium ion intermediate (Scheme 1).^{13, 14} *S*-(2-Chloroethyl)GSH can be synthesized and used as a substitute, generating the same products.^{15, 16} Multiple DNA adducts arising from the GSH-DBE conjugate include those formed at the N7, N2, and O6 positions of 2'-deoxyguanosine (dG) as well as the N1 position of 2'-deoxyadenosine (dA).^{17, 18} Mutations of a transgene in mouse liver were attenuated by an inhibitor of GSH synthesis, indicating that these adducts strongly contribute to DBE genotoxicity *in vivo*.¹⁹

A similar chemical mechanism is involved in the DBE modification of the DNA repair protein O⁶-alkylguanine DNA alkyltransferase (AGT, MGMT)^{20–25} and other proteins that become crosslinked to DNA.^{26, 27} After calf thymus DNA was treated with DBE in the presence of AGT, we identified adducts at the N7, N1, N2, and O6 positions of dG^{24, 28} and an N⁶-dA adduct, which had not been seen in the earlier work with GSH. This adduct was reported at levels similar to those of the other non-labile adducts (the N⁷-dG adduct is labile and can depurinate).^{24, 28}

In this work, we investigated the ability of DBE, and more specifically the GSH conjugate *S*-(2-chloroethyl)GSH (an analogue of *S*-(2-bromoethyl)GSH, with similar biological properties^{15, 16}) to react at the N6 position of deoxyadenosine to form *S*-[2-(N⁶-deoxyadenosinyl)ethyl]GSH in calf thymus DNA. Extension studies were performed in duplex oligonucleotides containing the adduct by human translesion synthesis DNA polymerases (hPols) η , ι , and κ and with *Sulfolobus solfataricus* DNA polymerase Dpo4, *Escherichia coli* polymerase (Pol) I Klenow fragment, and bacteriophage T7 polymerase.

Experimental Procedures

Materials

GSH, *N*-(*tert*-butoxycarbonyl) (BOC)-protected 2-bromoethylamine, 1-bromo-2-chloroethane, phosphodiesterase I, DNase 1, and other chemical reagents were purchased from Sigma-Aldrich. Uracil-DNA glycosylase (UDG) was purchased from New England Biolabs (Ipswich, MA). 6-Chloropurine deoxyriboside was obtained from Alfa Aesar (Haverhill, MA). Alkaline phosphatase was purchased from Promega Corporation (Madison, WI). Unmodified oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and were HPLC-purified by the manufacturer. The 6-chloropurine phosphoramidite was obtained from Prof. Carmelo Rizzo (Vanderbilt University, Nashville, TN). Oligonucleotides containing the 6-chloropurine residue were synthesized on a Perspective Biosystems model 8909 DNA synthesizer on a 1- μ mol scale using Expedite reagents (Glen Research, Sterling, VA), utilizing a standard synthetic protocol and were purified by HPLC using a Phenomenex Alumina RP octadecylsilane (C₁₈) column (250 mm $\times$ 4.6 mm, 5 μ m). Buffers consisted of Mobile Phase A (0.10 M NH₄HCO₂) and Mobile Phase B (CH₃CN). The following gradient program (v/v) was used with a flow rate of 1.5 mL/min: starting point 0% B, increased to 10% B over 15 minutes, then increased to 20% B at 20 min, held at 20% B for 5 min, increased to 80% B for 3 minutes, held at 80% B for 4 minutes, and re-equilibrated for 5 min at 0% B (all v/v). The UV detector was set at 240 nm. Human DNA Pol η , ι , and κ (catalytic core—hPol η : 1–432 amino acids, hPol ι : 1–420 amino acids, hPol κ : 19–526) and *S. solfataricus* DNA polymerase Dpo4 were expressed and purified as described previously.^{29–32}

Synthesis of S-(2-Aminoethyl)GSH

GSH (50 mg, 163 μ mol) was dissolved in 10 mL of CH₃OH along with 20 mg (0.86 mmol) of Na⁺. A solution of *N*-BOC-protected 2-bromoethylamine (72.2 mg, 322 μ mol), in 5 mL CH₃OH, was added dropwise. The reaction mixture was stirred for 24 h before quenching with 0.7 mL glacial CH₃CO₂H. The reaction product was dried under a stream of N₂ gas. The resulting white solid was redissolved in H₂O before purification by HPLC (mobile phase A: 0.01% CH₃CO₂H in H₂O, mobile phase B: 95% CH₃CN in H₂O with 0.01% CH₃CO₂H, all v/v) using a semi-preparative Beckman Ultrasphere RP octadecylsilane (C₁₈) column (250 mm $\times$ 10 mm, 5 μ m). The following gradient program (v/v) was used with a flow rate of 3 mL/min: starting point 2% B, increased to 10% B over five minutes, then increased to 30% B at 10 min, held at 30% B for 5 min, and re-equilibrated for 5 min at 2% B (all v/v). The UV detector was set at 240 nm. The *N*-BOC-protected S-(2-aminoethyl)GSH was collected and its structure was confirmed by positive ion ESI-MS/MS. The compound was then redissolved in a mixture of 2.5 mL of CH₂Cl₂ and 0.3 mL CF₃CO₂H and stirred for 1 h to remove the N-BOC. The reaction mixture was dried under a stream of N₂ before repurification by HPLC using the same protocol as above.

Synthesis of S-[2-(N⁶-Deoxyadenosinyl)ethyl]GSH

S-(2-Aminoethyl)GSH (180 μ mol) was dissolved in a mixture of DMSO (225 μ L) and *N,N*-diisopropylethylamine (25 μ L) and added to dry 6-chloropurine deoxyribonucleoside (1 μ mol). Following the incubation at 60 °C for 24 h, the reaction mixture was purified by

HPLC as shown above, identified by $^1\text{H-NMR}$ (Supporting Information, Figure S1), and quantified by UV absorbance at 260 nm.³³

Synthesis of *S*-[2-(*N*⁶-Deoxyadenosinyl)ethyl]GSH-Containing Oligonucleotide

S-(2-Aminoethyl)GSH (750 nmol) was dissolved in a mixture of $(\text{CH}_3)_2\text{SO}$ (100 μL) and *N,N*-diisopropylethylamine (25 μL) and added to a dry 6-chloropurine-containing oligonucleotide (5'-TCTCXGTTTATGGACCACC-3', where X is 6-chloropurine) (15 nmol). Following incubation at 60 °C for 24 h, the reaction product was purified by 15% (w/v) polyacrylamide gel electrophoresis, visualized using a UV lamp (to locate bands), cut out of the gel, and extracted using 200 mM NaCl/1 mM EDTA (pH 8.0); the identity was confirmed by negative ion ESI-MS (Supporting Information, Figure S2).

Alkylation of Calf Thymus DNA

S-(2-Chloroethyl)GSH was synthesized by dissolving 0.5 g (1.6 mmol) of GSH in 12 mL of dry CH_3OH in which 0.12 g of Na^+ had been dissolved.¹⁸ A solution of 2.3 g (16 mmol) of 1-bromo-2-chloroethane in 12 mL of CH_3OH was then added dropwise to the GSH solution and stirred for 1 h before quenching with 0.25 mL glacial $\text{CH}_3\text{CO}_2\text{H}$. The resulting precipitate (*S*-(2-chloroethyl)GSH) was collected by centrifugation (2000 $\times$ g, 10 min). It was redissolved in 0.5 M Tris-HCl buffer (pH 7.7) containing 10% CH_3OH (v/v) to a final concentration of 10, 50, or 250 mM and immediately added to a solution of calf thymus DNA (2 mg/mL) in the same buffer (final volume for the reaction was 250 μL). The reaction was incubated at 37 °C for 30 minutes. After the incubation the DNA was precipitated by the addition of 2.5 volumes of cold $\text{C}_2\text{H}_5\text{OH}$ and digested using a nuclease cocktail consisting of phosphodiesterase I from *Crotalus adamanteus* venom (0.02 units), bovine pancreatic DNase 1 (20 units), and calf intestinal alkaline phosphatase (5 units) for 24 h at 37 °C. The resulting solution was filtered through a Microcon 10K Centrifugal filter before drying using centrifugal lyophilization. The samples were redissolved in 100 μL of running buffer (0.1% HCO_2H in H_2O , v/v) and analyzed by UPLC-MS. The solvents used were Mobile Phase A (0.1% HCO_2H in H_2O , v/v) and Mobile Phase B (0.1 % HCO_2H in 95% CH_3CN in H_2O , v/v). The following gradient was used at a flow rate of 0.3 mL/min: started at 0% B, held for 5 min, increased to 30% B at 13 min, then increased to 95% B at 15 min, and maintained there for 2 min before re-equilibrating to 0% B at 19 min (all v/v). The temperature of the column was 40 °C. Product ion spectra were collected over the m/z range of 100–1000, and MS/MS fragmentation spectra were collected over the m/z range of 160–700. The MS conditions were as follows: capillary temperature, 350 °C; source voltage, 5 kV; capillary voltage, 13 V; tube lens voltage, 60 V. MS fragmentation analysis was performed with a collision energy of 35%, m/z isolation width of 2, activation Q of 0.25, and an activation time of 30 ms.

6-Carboxyfluorescein (FAM)-Labeled Primer Extension and Steady-State Kinetic Assays

A 12-mer oligomer (FAM-5'-GGTGGTCCATAA-3', for full primer extension in the presence of four dNTPs) and a 14-mer oligomer (FAM-5'-GGTGGTCCATAAAC-3', for single base primer extension and steady-state kinetics in the presence of single dNTPs) were annealed to the 19-mer oligomer template (5'-TCTCXGTTTATGGACCACC-3', where X is dA or *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH). Primer extension was performed with 120 nM

oligonucleotide complex in 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 5 mM MgCl₂, 500 μM dNTPs, 2% (v/v) glycerol, 50 μg/mL bovine serum albumin (BSA), and 20 nM polymerase (with the exception of hPol α , 40 nM) at 37 °C. Steady-state kinetics were performed under the same conditions except using polymerase concentrations from 1–40 nM, varying dNTP concentrations (0.1–500 μM), and incubation times from 5–30 min so that the maximum incorporation was < 20% of the substrate concentration. Reactions were quenched with 7 μL of 20 mM EDTA (pH 9.0) in 95% (v/v) formamide. Products were separated using 18% (w/v) polyacrylamide gel electrophoresis and visualized using a Typhoon imaging system (GE Healthcare).

LC-MS Primer Extension Assays

A 14-mer oligomer (FAM-5'-GGTGGTCCATAA(dU)C-3') was annealed to the 19-mer oligomers as described above. Full-length primer extension was performed with 2.5 μM oligonucleotide complex in 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 5 mM MgCl₂, 1 mM dNTPs, 2% (v/v) glycerol, 50 μg/mL BSA, and 5 μM hPol η at 37 °C for 1 h. The reactions were stopped by spin column filtration to remove the salts and buffer before redissolving in UDG stock buffer (1 mM DTT and 1 mM EDTA in 20 mM Tris-HCl, pH 8.0) and treating with 25 units of UDG for 37 °C for 4 h, then adding 0.25 M piperidine and heating at 95 °C for 1 h. To examine reaction products, samples were analyzed by LC-MS/MS, performed with a Waters Acquity UPLC system (Waters, Milford, MA) interfaced to a Thermo-Finnigan LTQ mass spectrometer (Thermo Scientific Corp., San Jose, CA) equipped with an ESI source. Chromatographic separation was achieved using a Waters Acquity UPLC BEH octadecylsilane (C₁₈) column (1.0 mm × 10 mm, 1.7 μm) at a flow rate of 0.3 mL/min. Mobile Phase A consisted of 10 mM NH₄CH₃CO₂, and Mobile Phase B was 10 mM NH₄CH₃CO₂ in 95% CH₃CN in H₂O (v/v). The gradient started at 2% B, increased to 10% B at 5 min, 30% B at 9 min, and was held there for 2 min before returned to 2 % B for 2 minutes (all v/v). The column was maintained at 50 °C. The MS conditions were as follows: capillary temperature, 350 °C; source voltage, 4kV; source current, 100 μA; capillary voltage, -35 V; tube lens voltage, -93 V. MS fragmentation analysis was performed using data dependent scanning with a collision energy of 35%, *m/z* isolation width of 2, activation *Q* of 0.25, and an activation time of 30 ms. Product ion spectra were taken over an *m/z* range of 300–2000, and the most abundant species (-2 charge) was used for collision-induced dissociation (CID).

Results

Synthesis of *S*-[2-(*N*⁶-Deoxyadenosinyl)ethyl]GSH and an Oligonucleotide Containing the Lesion

S-[2-(*N*⁶-Deoxyadenosinyl)ethyl]GSH was synthesized as a standard to quantitate the adduct in calf thymus DNA and characterized by positive ESI-MS/MS (Figure 1) and ¹H-NMR (Supporting Information, Figure S1). Additionally, an 19-mer oligonucleotide containing *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH was prepared for *in vitro* replication assays (Supporting Information, Figure S2).

GSH was treated with *N*-BOC-protected 2-bromoethylamine and deprotected to release the free amino group. The product was used for coupling with 6-chloropurine, either for the synthesis of a free nucleoside or a 19-mer oligonucleotide for the standard and replication studies, respectively. In the reaction, the amino group from 2-aminoethyl GSH displaced the 6-chloro group, resulting in the formation of dA with a thioethylene linkage to GSH. The site of attachment at the dA N6 atom is at the Cys-ethylamino nitrogen and not the Glu α -amino group, as revealed by the M-129 plus M-116 losses in the mass spectra; the former is indicative of a GSH adduct (Figure 1).³⁴ This is the same result seen with the synthesis of the *N*²-dG adduct previously, established in a different way.³⁵

The *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH standard was purified by HPLC and characterized by positive ESI-MS. The oligonucleotide containing *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH was purified by gel electrophoresis and characterized by negative ESI-MS (Supporting Information Figure S1).

Formation of *S*-[2-(*N*⁶-Deoxyadenosinyl)ethyl]GSH in DNA

Calf thymus DNA was treated with *S*-(2-chloroethyl)GSH at 37 °C for 30 minutes. The DNA was then extracted, digested with nucleases and alkaline phosphatase, and desalted prior to LC-MS analysis. A peak corresponding to the correct retention time and mass of the adduct and fragmenting to *m/z* 340 (loss of γ -glutamine plus loss of deoxyribose) was observed in the chromatogram (Figure 1A and C). Similar fragmentation patterns for the parent ion were found in both the standard and the reaction product (Figure 1B and D). The levels of the adduct were quantified in relation using the standard. Using a concentration of 250 mM *S*-(2-chloroethyl)GSH for the reaction, 2.5 *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH adducts/10⁴ bases were detected.

Primer Extension Assays

To investigate the ability of DNA polymerases to bypass the *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH adduct, FAM-labeled primer extension assays were performed in duplex oligonucleotide substrates containing the site-specifically incorporated *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH adduct in the template strand. Replication experiments were done with the human translesion polymerases hPol κ , η , and ι , as well as with *S. solfataricus* Dpo4, bacteriophage Pol T7, and *E. coli* Klenow fragment.

To determine the replication bypass of the GSH adduct, primer extension assays were performed in the presence of all four dNTPs (Figure 2 and Figure S3 of the Supporting Information). hPol η , Dpo4, and Klenow fragment were the most efficient of the four polymerases in terms of producing full-length primer products (P+7). hPol ι showed slight bypass of the adduct (P+2) but required higher enzyme concentrations (40 nM) and longer reaction incubations for extended bypass. However, hPol κ and Pol T7 were strongly blocked by the adduct. These polymerases were able to insert the two bases prior to the adduct site, but only low levels of bypass were observed after a 60 minute incubation at 37 °C. Overall, the *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH adduct considerably blocked replication by each of the three human DNA polymerases.

Primer extension assays were also performed in the presence of single dNTPs to study misincorporation opposite the GSH adduct (Figure 3). hPol κ incorporated the correct dTTP in the control oligonucleotide but was completely blocked by the adduct. hPols η and ι each had similar or lower extents of misincorporation opposite the DNA adduct compared to the control template. Interestingly, Dpo4 showed less misincorporation opposite the *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH adduct compared to the control template.

To better understand the catalytic efficiency of the incorporation of each dNTP and the frequency of misinsertion, steady-state kinetic analysis was performed with hPols κ , η , and ι (Table 1). Compared to the unmodified substrate with dA, the *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH adduct decreased the catalytic efficiency of correct dTTP incorporation by each of the polymerases. hPol κ was strongly hindered by the adduct, with the catalytic efficiency for correct incorporation decreased $>10^3$ -fold, while hPol η (5.6-fold) and pol ι (1.5-fold) had less attenuation. The catalytic efficiency of misincorporation was also low for the various polymerases. With hPol η , there was an increase in misinsertion frequency of dATP, dCTP, and dGTP of 3.2-, 5.5-, and 5.4-fold, respectively. The misinsertion frequency of dATP was also increased with hPol ι (2.7-fold) while the misinsertion of dATP by hPol κ was below the limit of quantitation ($k_{\text{cat}} < 0.004 \text{ min}^{-1}$).

Primer Extension Analysis by LC-MS

While steady-state kinetic measurements provide useful information about the catalytic efficiency of the correct versus incorrect incorporation opposite the DNA adduct, this analysis does not provide any information about the patterns of extension nor other types of mistakes such as frame-shift events. Therefore, full primer extension assays were performed, in the presence of all four dNTPs, using LC-MS/MS analysis to sequence nucleotide incorporations opposite and after the *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH adduct. This analysis utilized a uracil-containing primer (5'-GGTGGTCCATAA(dU)C-3'), and the product is cleaved using UDG followed by hot piperidine treatment to create a shorter DNA oligonucleotide for MS analysis.^{29, 36} Analysis was performed with hPol η because its misinsertion frequencies were the highest of the three DNA polymerases. The product species were isolated and fragmented using CID (Figure 4). These fragment ions were then compared to theoretical ions calculated using an Oligo Mass Calculator (The RNA Institute, University of Albany SUNY, Albany, NY; <http://mods.rna.albany.edu/masspec/Mongo-Oligo>) to identify the products. Analysis of the unmodified oligonucleotide showed only the correct full-length replication product, whereas the modified oligonucleotide revealed an n-1 frameshift mutation (3%) and a dCTP misinsertion opposite the adduct (6%) in the full-length replication product (Table 2).

Discussion

Mutagenic DNA-peptide crosslinks can be formed from DBE with the tripeptide GSH and the DNA repair protein AGT.³⁷ We first reported a crosslink formed with GSH following the reaction of GSH and DBE with DNA in the presence of GSH transferase.¹³ The major adduct was identified at the N7 position of dG^{13, 38, 39} with lesser amounts of adduction located at the N2 and O6 positions of dG¹⁷ as well as the N1 position of dA.¹⁸ Our studies

on the sites of adduction of AGT later identified the N6 position of dA as another adduction site that had not been seen using GSH.²⁸ Therefore, we were interested in whether or not the GSH adduct was also formed at the N6 position of dA and, if so, what its biological properties are.

S-[2-(*N*⁶-Deoxyadenosinyl)ethyl]GSH was detected in calf thymus DNA treated with *S*-(2-chloroethyl)GSH (Figure 1). Higher concentrations of *S*-(2-chloroethyl)GSH were used for DNA modification compared to our previous studies, which may explain why the adduct was not detected previously. However, the result can also be explained by advances in analytical chemistry, particularly in mass spectrometry, since 1992. Adjusting the concentration of *S*-(2-chloroethyl)GSH used to that in our earlier work would predict the extent of modification for the *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH adduct to be ~0.1% of the total adducts (Scheme 1). The levels of the *N*⁶-dA adduct formed with GSH are in the range of those formed at the dG N2 and O6 and the dA N1 positions.¹⁷ We had previously reported that *S*-[2-(*N*¹-deoxyadenosinyl)ethyl]GSH did not convert to *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH in a Dimroth rearrangement unless the sample was subjected to high pH.¹⁸

Replication studies were done with the *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH adduct placed in a 19-mer oligonucleotide, utilizing a sequence that has been used previously in this laboratory for GSH crosslinking studies.⁴⁰ Qualitative analysis of the bypass efficiency of various translesion polymerases (Figure 2) showed that Dpo4 was the most efficient polymerase for replicating past the adduct, followed by hPol η and hPol ι . hPol κ was strongly blocked by *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH, showing no replication past the adduct. These results were similar to previous studies showing hPol η to be the most efficient for bypass of bulky adducts in the major groove of DNA.^{41–43} Dpo4, although sometimes considered to be a homolog of hPol κ , had similar activity as hPol η .⁴⁴

In single-base insertion assays, dTTP was the nucleoside triphosphate most readily incorporated opposite the adduct (Figure 3). Steady-state kinetic analysis showed that misincorporation was somewhat higher than in controls for hPol η and hPol ι , with less than a five-fold increase in misinsertion frequency for the dNTPs (Table 1). This increase was similar to our previous results with hPol η and hPol ι with the *S*-[4-(*N*⁶-deoxyadenosinyl)-2,3-dihydroxybutyl]GSH adduct.⁴⁰ hPol κ was severely blocked by the adduct, and no misincorporation was detected opposite the adduct in the work with hPol κ .

In order to better understand the insertion events opposite and past the adduct, LC-MS/MS analysis was performed to sequence the synthesized extension product. This protocol²⁹ utilizes a uracil residue in the primer strand, which allows the extension product to be cut using UDG/piperidine. Following cleavage, the products can be analyzed by HPLC-ESI-MS/MS, identified, and quantified. This experiment was performed only with hPol η , due to its high level of misinsertion of each of the dNTPs (Table 2). The major products formed included the correct base T inserted opposite the *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH with or without a blunt-end addition. A minor product corresponding to 6% of the overall product represented a misinsertion of C opposite the adduct, approximately the same percentage as the misinsertion predicted by steady-state kinetic analysis. An additional product was a –1

frameshift deletion, accounting for 3% of the product. The -1 frameshift product corresponds to the addition of a G opposite the C 5' to the adduct and is consistent with the preferential insertion of G in the steady-state kinetic analyses (Table 1), in which a frameshift would not have been detected. Apparently if A is inserted (Table 1), it is not extended to generate full-length product.

Other N^6 -adenyl adducts are known in the literature. The role of N^6 -methyldeoxyadenosine is well-established in epigenetic mechanisms but to our knowledge no information is available regarding miscoding. Treatment of DNA with classical small alkylating agents does not yield much in the way of N^6 -alkyl dA products.⁴⁵ An adduct was found to be formed from endogenous *N*-nitroso compounds to generate the lesion N^6 -carboxymethyl-2'-deoxyadenosine.⁴⁶ However, this lesion did not block DNA replication or induce mutations *in vitro* or in *E. coli*.^{47, 48} However, some larger electrophiles do generate N^6 -dA DNA adducts,⁴⁹ including some polycyclic hydrocarbons and aryl amines, which have been studied in some detail.^{50, 51} In a reference from our group cited earlier,⁴⁰ the N^6 -adenyl adduct formed by conjugation of butadiene diepoxide (and hydrolysis), the DNA polymerases studied here (η , ι , κ , Dpo4) were not very affected, but this entity linked to GSH caused hPol κ to insert C. Studies with two other butadiene-related adducts showed that N^6 -(2-hydroxy-3-buten-1-yl)2'-deoxyadenosine retarded polymerization but did not affect fidelity, but one isomer of N^6,N^6 -(2,3-hydroxybutan-1,4-diyl)-2'-deoxyadenosine caused lack of selectivity in nucleotide incorporation with hPols κ and η .⁵² The adduct N^6 -oxopropenyl 2'-deoxyadenosine retarded the human translesion synthesis DNA polymerases but did not lead to error-prone polymerization.⁵³

One open question is how misincorporation at N^6 -[2-[deoxyadenosinyl]ethyl]GSH compares with the other DBE-GSH adducts we have characterized (Scheme 1). Three of these (the corresponding N^7 -, N^2 -, and O^6 -dG adducts) had been synthesized earlier in this laboratory³⁵ but had only been examined with two *Escherichia coli* DNA polymerases, pol I (Klenow fragment) and pol II exo.⁵⁴ (The human translesion synthesis DNA polymerases were yet unknown, and we do not currently have these three modified oligonucleotides in hand.) However, all three of the adducts showed some blockage and miscoding with *E. coli* Pol I and Pol II.⁵⁴ We are still interested in establishing the relative abilities of the different adducts to retard polymerization and miscode with the human DNA polymerases, as well as their functions in human cells.

In conclusion, we identified the S -[2-(N^6 -deoxyadenosinyl)ethyl]GSH adduct formed in calf thymus DNA. hPols η and ι , *S. solfataricus* Dpo4, and *E. coli* Pol I Klenow fragment were capable of bypassing the adduct without significant obstruction. The incorporation proceeds with relatively low but definite misincorporation by hPols η and ι and *S. solfataricus* Dpo4. These results help elucidate the relevance of individual GSH-coupled adducts in relation to the overall mutagenic effects of DBE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BOC	<i>N</i> -(<i>tert</i> -butoxycarbonyl)
BSA	bovine serum albumin
CID	collision-induced dissociation
DBE	1,2-dibromoethane
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
ESI	electrospray ionization
FAM	6-carboxyfluorescein
GSH	glutathione
hPol	human DNA polymerase
Pol	DNA polymerase
UDG	uracil DNA glycosylase

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Figure 1.

Detection of *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH in calf thymus DNA treated with *S*-(2-choroethyl)GSH. UPLC chromatogram of synthetic *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH (A) and reaction product (C), observing precursor ion m/z 585 fragmenting to m/z 340.

MS/MS data of precursor ion m/z 585 splitting into fragment ions for synthetic standard (t_R 8.19 min) (B) and reaction product (t_R 8.21 min) (D).

Figure 2.

Primer extension by DNA polymerases in the presence of all four dNTPs. (A) Primer and template sequences where X is dA or *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH (*N*⁶-dA-ethyl-GSH). Reactions were done with FAM-labeled primer in the presence of hPol κ (B), hPol η (C), hPol ι (D), and Dpo4 (E). Reactions were conducted for increasing times (indicated in minutes) in the presence of 20 nM enzyme with the exception of hPol ι (40 nM).

Figure 3.

Primer extension in the presence of single dNTPs by Y-family DNA polymerases. (A) Primer and template sequences where X is dA or *S*-[2-(N⁶-deoxyadenosinyl)ethyl]GSH (N⁶-dA-ethyl-GSH). Reactions were done with FAM-labeled primers in the presence of hPol κ (B), hPol η (C), hPol ι (D), and Dpo4 (E). Reactions were conducted for 5 minutes in the presence of 20 nM enzyme with the exception of hPol ι (40 nM).

Figure 4.

Extracted ion chromatograms and MS/MS spectra of ions at m/z 947.3 (–2) (A and C) and 939.2 (–2) (B and D) from full-length primer extension products formed by hPol η in the presence of all four dNTPs.

Scheme 1.

Structures and relative quantities of glutathione-DNA crosslinks formed from ethylene dibromide and *S*-(2-bromoethyl)GSH.¹⁷ *S*-[2-(*N*⁶-deoxyadenosinyl)ethyl]GSH is shown in bold. dR: 2'-deoxyribose.

Table 1

Steady-State Kinetic Analysis of DNA Primer Single-Base Insertion Reaction

polymerase	template	dNTP	$k_{cat}(min^{-1})$	$K_m(\mu M)$	$k_{cat}/K_m(min^{-1}\mu M^{-1})$	f^a
hPol κ	dA	T	5.0 ± 0.4	25 ± 7	0.20	1
		A	0.013 ± 0.002	170 ± 62	0.000079	0.00040
	N ⁶ -dA-ethyl-GSH	T	0.044 ± 0.014	230 ± 140	0.00019	1
		A	n/d^b	n/d^b	n/d^b	n/a
hPol η	dA	T	0.59 ± 0.02	0.67 ± 0.12	0.89	1
		A	0.49 ± 0.03	21 ± 5	0.023	0.026
		C	0.88 ± 0.07	91 ± 18	0.0097	0.011
		G	0.49 ± 0.04	20 ± 5	0.025	0.028
	N ⁶ -dA-ethyl-GSH	T	0.89 ± 0.07	5.6 ± 1.2	0.16	1
		A	1.6 ± 0.2	120 ± 28	0.013	0.083
		C	0.89 ± 0.03	92 ± 7	0.0096	0.060
		G	0.12 ± 0.01	4.9 ± 1.0	0.024	0.15
hPol ι	dA	T	2.3 ± 0.2	33 ± 8	0.069	1
		A	0.076 ± 0.004	79 ± 10	0.00096	0.014
	N ⁶ -dA-ethyl-GSH	T	1.9 ± 0.1	42 ± 9	0.046	1
		A	0.062 ± 0.003	36 ± 5	0.0017	0.038

^aMisinsertion frequency $f = [k_{cat}/K_m]_{\text{incorrect}} / [k_{cat}/K_m]_{\text{correct}}$ ^bDNA incorporation was below limits of quantitation ($k_{cat} < 0.004 \text{ min}^{-1}$)

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Table 2

LC-MS/MS Analysis of DNA Primer Extension
5'6FAM-GGTGGTCCATAA **UC**
3'-CCACCAGGTATTG **XCTCT**

product sequence	m/z (-2)	peak area	% frameshift	%C	%T
CTGAGA	947.1	60842			91
CTGAGAA	1103.3	125955			
CCGAGA	939.6	13057		6	
C_GAGA	795.0	5746	3		