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Replication Past the Butadiene Diepoxide-derived DNA Adduct S-[4-(*N*⁶-Deoxyadenosinyl)2,3-dihydroxybutyl]glutathione by DNA Polymerases

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Abstract

1,2,3,4-Diepoxbutane (DEB), a metabolite of the carcinogen butadiene, has been shown to cause glutathione (GSH)-dependent base-substitution mutations, especially A:T to G:C in *Salmonella typhimurium* TA1535 (*Chem. Res. Toxicol.* 23, 1544 (2010)) and *Escherichia coli* TRG8 cells (*Chem. Res. Toxicol.* 25, 1522 (2012)). We previously identified S-[4-(*N*⁶-deoxyadenosinyl)-2,3-dihydroxybutyl]GSH (*N*⁶dA-(OH)₂butyl-GSH) as a major adduct in the reaction of S-(2-hydroxy-3,4-epoxybutyl)glutathione (DEB-GSH conjugate) with nucleosides and calf thymus DNA and *in vivo* in livers of mice and rats treated with DEB (*Chem. Res. Toxicol.* 25, 706 (2012)). For investigation of the miscoding potential of the major DEB-GSH conjugate-derived DNA adduct (*N*⁶dA-(OH)₂butyl-GSH) and the effect of GSH conjugation on replication of DEB, extension studies were performed in duplex DNA substrates containing the site specifically-incorporated *N*⁶dA-(OH)₂butyl-GSH adduct, *N*⁶-(2,3,4-trihydroxybutyl)deoxyadenosine adduct (*N*⁶dA-butanetriol), or unmodified deoxyadenosine (dA) by human DNA polymerases (Pol) η , ι , and κ , bacteriophage polymerase T7, and *Sulfolobus solfataricus* polymerase Dpo4. Although dTTP incorporation was the most preferred addition opposite the *N*⁶dA-(OH)₂butyl-GSH adduct, *N*⁶dA-butanetriol adduct, or unmodified dA by all polymerases, dCTP misincorporation frequency opposite *N*⁶dA-(OH)₂butyl-GSH was significantly higher than that opposite the *N*⁶dA-butanetriol adduct or unmodified dA by Pol κ or Pol T7. LC-MS/MS analysis of full-length primer extension products confirmed that Pol κ or Pol T7 incorporated the incorrect base C opposite *N*⁶dA-

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ASSOCIATED CONTENT

Supporting Information

Scheme showing the two possible oligomers containing *N*⁶dA adducts from the reaction of S-(4-amino-2,3-dihydroxybutyl)GSH with 19-mer oligonucleotide containing 6-chloropurine; HPLC separation of oligomers containing *N*⁶dA adducts from the reaction of S-(4-amino-2,3-dihydroxybutyl)GSH with 19-mer oligonucleotide containing 6-chloropurine; ESI[−] mass spectra of oligomers containing 6-chloropurine, S-linked, and α -linked *N*⁶dA adducts from the reaction of S-(4-amino-2,3-dihydroxybutyl)GSH with 19-mer oligonucleotide containing 6-chloropurine; MS/MS spectra of deoxyribonucleosides generated from enzymatic digestion of oligomers containing S-linked and α -linked *N*⁶dA adducts from the reaction of S-(4-amino-2,3-dihydroxybutyl)GSH with 19-mer oligonucleotide containing 6-chloropurine; HPLC separation of oligomers containing 6-chloropurine and *N*⁶dA-butanetriol adducts from the reaction of 4-aminobutane-1,2,3-triol with 19-mer oligonucleotide containing 6-chloropurine; ESI[−] mass spectra of oligomers containing 6-chloropurine and *N*⁶dA-butanetriol adducts from the reaction of 4-aminobutane-1,2,3-triol with 19-mer oligonucleotide containing 6-chloropurine; observed and theoretical CID fragmentation of *m/z* 947.0 (−2) for Pol T7- and Pol κ -catalyzed extension; observed and theoretical CID fragmentation of *m/z* 939.2 (−2) for Pol T7- and Pol κ -catalyzed extension. This material is available free of charge via the Internet at <http://pubs.acs.org>.

(OH)₂butyl-GSH lesion. These results indicate the relevance of GSH-containing adducts for the A:T to G:C mutations produced by DEB.

INTRODUCTION

1,3-Butadiene (BD) is an important commodity chemical in industry, particularly in the production of synthetic rubber and other polymers, and is also an environmental pollutant found in cigarette smoke and automobile exhaust.^{1,2} BD is classified as a “probable human carcinogen” by the International Agency for Research on Cancer³ based on epidemiological association of occupational exposure to BD with increased lymphatic and hematopoietic cancer risk.^{4–6} BD was found to be carcinogenic in inhalation experiments with B6C3F1 mice and Sprague-Dawley rats, particularly the former species. The selectivity has been attributed to the increased formation of 1,2,3,4-diepoxybutane (DEB) and other DNA-reactive metabolites, related to species differences in epoxide hydrolase activity.^{7–9}

BD is relatively unreactive and requires metabolic activation for its mutagenicity, i.e. conversion to DNA reactive metabolites by P450 enzymes. The first oxidation of BD yields 3,4-epoxy-1-butene (EB). EB can be hydrolyzed to 1-butene-3,4-diol or undergo epoxidation to yield DEB, and both of these molecules can be further metabolized to 3,4-epoxy-1,2-butanediol.^{7,10} EB, DEB, and 3,4-epoxy-1,2-butanediol are capable of alkylating DNA and proposed to be responsible for the mutagenic properties of BD.^{11,12} Among the three epoxide metabolites of BD, DEB is the most mutagenic (50- to 100-fold > EB and 100- to 200-fold > 3,4-epoxy-1,2-butanediol),^{13,14} presumably because of its *bis* electrophilic properties and enhanced reactivity with DNA.^{14,15} Many DNA adducts derived from DEB have been reported, including mono-adducts and DNA-DNA intra- and inter-crosslinks adducts (~ 25 adducts, with the number being even higher when stereochemistry is considered),^{6–21} and some of these adducts have been found *in vivo*.^{16,17}

GSH conjugation is generally considered to be a detoxication process in the metabolism of xenobiotic chemicals.²² However, we previously reported significant enhancement of the base pair mutagenicity of DEB associated with the expression of rat GSH *S*-transferase (GST) 5-5²³ or human GST T1-1²⁴ in *Salmonella typhimurium* TA1535, and subsequently the synthetic *S*-(2-hydroxy-3,4-epoxybutyl)GSH (DEB-GSH) conjugate was found to be considerably more mutagenic than several other butadiene-derived epoxides—including DEB—in *S. typhimurium* TA1535²⁵ and *Escherichia coli* TRG8 cells.²⁶ In particular, A:T to G:C mutations were dominant in the *rpoB* gene of *E. coli* TRG8 treated with the DEB-GSH conjugate.²⁷

We previously identified six DNA adducts: *S*-[4-(*N*³-adenyl)-2,3-dihydroxybutyl]GSH (*N*³A-(OH)₂butyl-GSH), *S*-[4-(*N*⁶-deoxyadenosinyl)-2,3-dihydroxybutyl]GSH (*N*⁶dA-(OH)₂butyl-GSH), *S*-[4-(*N*⁷-guanyl)-2,3-dihydroxybutyl]GSH (*N*⁷G-(OH)₂butyl-GSH), *S*-[4-(*N*¹-deoxyguanosinyl)-2,3-dihydroxybutyl]GSH (*N*¹dG-(OH)₂butyl-GSH), *S*-[4-(*N*⁴-deoxycytidinyl)-2,3-dihydroxybutyl]GSH (*N*⁴dC-(OH)₂butyl-GSH), and *S*-[4-(*N*³-thymidinyl)-2,3-dihydroxybutyl]GSH (*N*³dT-(OH)₂butyl-GSH) in the reaction of the DEB-GSH conjugate with nucleosides and calf thymus DNA.²⁷ Two DNA adducts—*N*⁶dA-(OH)₂butyl-GSH (Scheme 1) and *N*⁷G-(OH)₂butyl-GSH—were identified as major adducts

in vivo in the livers of mice and rats treated with DEB²⁷ and in *E. coli* TRG8 cells treated with the DEB-GSH conjugate.²⁶ These results suggest that the DEB-GSH conjugate is a major mutagen with biological activity due to the cross-linking of GSH and DNA by DEB and therefore expected to contribute to the carcinogenicity of DEB.

In the present work, we investigated the miscoding potential of one of the two major DEB-GSH conjugate-derived major DNA adducts, *N*⁶dA-(OH)₂butyl-GSH, and the effect of GSH conjugation on replication of DEB. Extension studies were performed in duplex DNA substrates containing site specifically-incorporated *N*⁶dA-(OH)₂butyl-GSH, *N*⁶-(2,3,4-trihydroxybutyl)deoxyadenosine (*N*⁶dA-butanetriol), or unmodified deoxyadenosine (dA) by human DNA polymerase (Pol) η , ι , and κ and two model DNA polymerases, bacteriophage Pol T7 and *Sulfolobus solfataricus* polymerase Dpo4. The synthesis of oligonucleotides containing the site specific adducts was done based on a previously described general strategy.²⁸ In primer extension assays, Pol κ and Pol T7 exhibited high misinsertion frequency for dCTP misincorporation opposite *N*⁶-dA-(OH)₂butyl-GSH adduct. These results with human Pol κ indicate the relevance of GSH-containing adducts in enhanced A:T to G:C mutations produced by DEB.

EXPERIMENTAL PROCEDURES

Materials

DEB (racemic), GSH, and enzymes for digestion were purchased from Sigma Chemical Co. (St. Louis, MO). Unlabeled dNTPs, T4 polynucleotide kinase, and uracil DNA glycosylase (UDG) were purchased from New England Biolabs (Ipswich, MA). [γ -³²P]ATP (specific activity, 3×10^3 Ci mmol⁻¹) was purchased from PerkinElmer Life Sciences (Boston, MA). Micro Bio-Spin 6 columns were purchased from Bio-Rad Laboratories (Hercules, CA). Unmodified oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and were purified by the manufacturer using HPLC. Oligonucleotides containing a site-specific 6-chloropurine residue²⁸ were synthesized on a Perseptive Biosystems Model 8909 DNA synthesizer on a 1-Omol scale using Expedite reagents (Glen Research, Sterling, Virginia) using a standard synthetic protocol and were purified by HPLC. 1-Amino-2(*S*),3(*R*),4-butanetriol was obtained from Dr. C. Rizzo (Vanderbilt University). The amino-dihydroxybutyl-GSH conjugate (*S*-(4-amino-2,3-dihydroxybutyl)GSH) was synthesized as described previously.^{25,29} Human DNA Pol ι ,³⁰ Pol η ,³¹ and Pol κ ³² were purified following protocols described previously. Bacteriophage T7 DNA polymerase,³³ *E. coli* thioredoxin,³⁴ and *S. solfataricus* polymerase Dpo4³⁶ were expressed and purified as described previously.

Synthesis of Oligonucleotides Containing *N*⁶-dA-(OH)₂butyl-GSH or *N*⁶dA-butanetriol Adducts

Racemic *S*-(4-amino-2,3-dihydroxybutyl)GSH (with L-Cys and -Glu) (15 Omol) or 1-amino-2(*S*), 3(*R*),4-butanetriol (15 Omol) was dissolved in a mixture of DMSO (200 OL) and *N,N*-diisopropylethylamine (150 OL), followed by the addition of dry, 6-chloropurine-containing oligomer (3'-CCA CCA GGT ATT TGX CTC T, where X denotes 6-chloropurine, 50 nmol) for synthesis of oligonucleotides containing *N*⁶dA-(OH)₂butyl-GSH

or N^6 dA-butanetriol adducts, respectively. Following incubation at 37 °C for 24 h, the reaction mixtures were separated by HPLC with a Hitachi L-7100 pumping system and LDC Analytical SpectroMonitor 3200 variable wavelength detector using a Phenomenex Clarity Oligo-RP column (150 mm $\times$ 10 mm, 5 μ m). Mobile phase A was 10 mM $\text{NH}_4\text{CH}_3\text{CO}_2$ in H_2O (pH 6.5) and mobile phase B was CH_3CN . The following gradient program (v/v) was used with a flow rate of 3 mL min^{-1} : the gradient started with 2% B (v/v), increased to 20% B (v/v) at 23 min, to 70% B (v/v) at 25 min, and held at 70% B (v/v) for 5 min. The column was reequilibrated for 10 min with 2% B (v/v). The UV detector was set at a wavelength of 260 nm. The structures of the oligonucleotides containing N^6 dA-(OH) $_2$ butyl-GSH or N^6 dA-butanetriol adducts were confirmed by negative ion electrospray ionization (ESI)-MS and positive ESI-MS/MS of enzymatic digests (a Thermo-Finnigan LTQ mass spectrometer, ThermoElectron) (Supporting Information Figures S1–S6).

Enzymatic Hydrolysis

HPLC-purified synthetic oligonucleotides formed from the reaction of *S*-(4-amino-2,3-dihydroxybutyl)GSH with 6-chloropurine-containing oligomer were incubated with DNase I (5 units), alkaline phosphatase (1 units), and phosphodiesterase I type II (0.05 units) in 10 mM Tris-HCl buffer (pH 7.0, containing 10 mM MgCl_2) for 48 h at 37 °C. The digests were analyzed by positive ESI-MS/MS as described above.

^{32}P -labeled Primer Extension and Steady-state Kinetic Assays

A 12-mer oligomer (5'-GGTGGTCCATAA-3', for primer extension in the presence of four dNTPs) and 14-mer oligomer (5'-GGTGGTCCATAAAC-3', for primer extension in the presence of a single dNTP) were 5' [γ - ^{32}P]ATP end-labeled and annealed to a 19-mer template (5'-TCTCXGTTTATGGACCACC-3', X denotes dA, N^6 dA-butanetriol, or N^6 dA-(OH) $_2$ butyl-GSH). Primer extension experiments were performed in 50 mM Tris-HCl buffer (pH 7.5) containing 60 nM primer-template complex, 250 μM dNTPs, 5% (v/v) glycerol, 5 mM DTT, 50 mM NaCl, 5 mM MgCl_2 , 50 Og mL^{-1} bovine serum albumin (BSA), and 20 nM (for Dpo4, Pol T7, Pol η , and Pol κ) or 40 nM (for Pol ι) polymerase at 37 °C. Steady-state kinetic assays were performed under the same conditions except using 5–20 nM polymerase, various concentrations of a single dNTP, and 5–10 min incubation times so that maximal product formation was $\sim$ 20% of the substrate concentration. Reactions were quenched with 9 OL of 20 mM EDTA (pH 9.0) in 95% (v/v) formamide. Products were separated using 20% (w/v) acrylamide electrophoresis gels, and results were visualized using a phosphorimaging system (Bio-Rad, Molecular ImagerR FX) and analyzed by Quantity One software as described.³⁵ Steady-state kinetic results were fit to hyperbolic plots of v vs S .

Primer Extension Assays Analysis by LC-MS/MS

A 14-mer primer (5'-GGTGGTCCATAA(dU)C-3') was annealed to the same 19-mer oligomers as described above at a 1:1 molar ratio. Full-length extension was performed in 50 mM Tris-HCl buffer (pH 7.5) containing 5 OM primer-template complex, 5 mM dNTPs, 2% (v/v) glycerol, 5 mM DTT, 50 mM NaCl, 5 mM MgCl_2 , 50 Og mL^{-1} BSA, and 5 OM polymerase (for Pol T7 and Pol κ) at 37 °C for 3.5 h. The reactions were terminated by spin

column separations to extract dNTPs and Mg^{2+} . The resulting product was treated with 25 units of UDG and 0.25 M piperidine following a previous protocol.³⁵ To identify the replication products the resulting reactions were analyzed by LC-MS/MS, performed using 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 with a Waters Acquity UPLC BEH octadecylsilane (C_{18}) column (1.0 mm $\times$ 100 mm, 1.7 μm). LC conditions were as follows: Mobile phase A was 10 mM (aqueous) $\text{NH}_4\text{CH}_3\text{CO}_2$ and mobile phase B was 10 mM $\text{NH}_4\text{CH}_3\text{CO}_2$ in 95% CH_3CN (v/v). The following gradient program (v/v) was used with a flow rate of 200 OL min^{-1} : the gradient started with 2% B (v/v), increased to 10% B (v/v) at 5 min, to 20% B (v/v) at 9 min, and held at 30% B (v/v) for 1 min. The column was re-equilibrated for 3 min with 5% B (v/v). The temperature of the column was maintained at 50 $^\circ\text{C}$. The MS conditions were as follows: source voltage, 4 kV; source current 100 OA; capillary voltage, -49 V ; capillary temperature, 350 $^\circ\text{C}$; tube lens voltage, -90 V . Product ion spectra were acquired over the range m/z 300–2000 and the most abundant species (-2 charge) was used for collision-induced dissociation (CID) analysis.

RESULTS

Synthesis of Oligonucleotides Containing $N^6\text{dA}-(\text{OH})_2\text{butyl-GSH}$ or $N^6\text{dA-butanetriol}$ Adducts

In order to compare replication of the $N^6\text{dA}-(\text{OH})_2\text{butyl-GSH}$ adduct (a major DEB-GSH conjugate-derived DNA adduct) with the DEB-derived DNA adduct $N^6\text{dA-butanetriol}$ adduct, 19-mer oligonucleotides containing $N^6\text{dA}-(\text{OH})_2\text{butyl-GSH}$ adduct or $N^6\text{dA-butanetriol}$ adduct were synthesized.

In the reaction of *S*-(4-amino-2,3-dihydroxybutyl)GSH with 19-mer oligonucleotides containing a site-specific 6-chloropurine, two amino groups (the *S*-amino-dihydroxybutyl moiety and the α -amino group of Glu) are available for the displacement of the 6-chloro group (Supporting Information Figure S1). After the reaction, three oligomers (containing *S*- or α -linked $N^6\text{dA}$ adducts or unreacted 6-chloropurine) were separated by HPLC (Supporting Information Figure S2) and characterized by negative ESI-MS (Supporting Information Figure S3) and LC-MS/MS after enzymatic hydrolysis (Supporting Information Figure S4). The first peak (t_R 8.5 min, Supporting Information Figure S2) was judged to be the desired isomer due to its characteristic $[\text{M}-129]^+$ fragmentation ion (m/z 516 ion) for loss of Glu,²⁷ a characteristic GSH conjugate fragmentation that was not observed in the other product with the same parent ion (Supporting Information Figure S4).

The oligomer containing $N^6\text{dA-butanetriol}$ adduct was purified by HPLC (Supporting Information Figure S5) and also characterized by negative ESI-MS (Supporting Information Figure S6).

^{32}P -labeled Primer Extension and Steady-state Kinetic Assays

For investigation of the miscoding potential of the DEB-GSH conjugate-derived major DNA adduct (i.e. the $N^6\text{dA}-(\text{OH})_2\text{butyl-GSH}$ adduct) and the effect of GSH conjugation on

replication of DEB, ^{32}P -labeled primer extension studies were performed in duplex DNA substrates containing the site specifically-incorporated $N^6\text{dA}-(\text{OH})_2\text{butyl-GSH}$ adduct, $N^6\text{dA-butanetriol}$ adduct, or unmodified dA by human Pol η , ι , and κ , bacteriophage Pol T7, and *S. solfataricus* Dpo4.

In the presence of all four dNTPs, Pol T7 and Pol η were the most efficient of the five polymerases in terms of producing full-length products (7 bases extended), followed by Dpo4 (6 bases extended) and Pol κ (5 bases extended), and then Pol ι (3 bases extended) (Figure 1). For primer extension studies, higher concentrations of Pol ι (40 nM) were required for adequate sensitivity than other DNA polymerases (20 nM) (results not shown). Compared to unmodified dA, both $N^6\text{dA}-(\text{OH})_2\text{butyl-GSH}$ and $N^6\text{dA-butanetriol}$ adducts moderately blocked all six polymerases (Figure 1).

Extension studies were repeated in the presence of single dNTPs to obtain quantitative information about misincorporation (Figure 2). Although dTTP was preferentially incorporated opposite the $N^6\text{dA}-(\text{OH})_2\text{butyl-GSH}$ adduct and $N^6\text{dA-butanetriol}$ adduct (as well as unmodified dA) by all of the polymerases examined, dCTP misincorporation opposite $N^6\text{dA}-(\text{OH})_2\text{butyl-GSH}$ adduct was higher than that opposite $N^6\text{dA-butanetriol}$ adduct or unmodified dA by Pol κ or Pol T7 (Figure 2).

In order to determine the catalytic efficiency of incorporation of each dNTP and misinsertion frequency by different polymerases, steady-state kinetic analysis was performed (Table 1). Compared with the unmodified substrate (with dA), the catalytic efficiency of correct dTTP incorporation opposite $N^6\text{dA}-(\text{OH})_2\text{butyl-GSH}$ adduct was decreased by Pol T7 (1.6-fold) and Pol κ (15-fold). The catalytic efficiency of the incorrect dCTP incorporation opposite $N^6\text{dA}-(\text{OH})_2\text{butyl-GSH}$ adduct was significantly increased by Pol T7 (11-fold) and Pol κ (4.4-fold). Among the five polymerases examined, Pol T7 exhibited the highest misinsertion frequency (0.85) for dCTP misincorporation opposite the $N^6\text{dA}-(\text{OH})_2\text{butyl-GSH}$ adduct. The misinsertion frequency of dCTP misincorporation opposite the $N^6\text{dA}-(\text{OH})_2\text{butyl-GSH}$ adduct was highest for Pol κ among the human DNA translesion polymerases (0.45), significantly higher than that opposite the $N^6\text{dA-butanetriol}$ adduct (0.014) or unmodified dA (0.007) by Pol κ .

Primer Extension Analysis Using LC-MS/MS

Steady-state kinetic analysis provides useful information, including the catalytic efficiency and misinsertion frequency by various different polymerases. However, this analysis does not necessarily reflect the nucleotide incorporation conditions in cells (where DNA synthesis occurs in the presence of all four dNTPs) nor does it reflect rates of extension past mispaired bases. Therefore, primer extension experiments for the sequence analysis of extended oligonucleotides were performed in the presence of all four dNTPs using LC-MS/MS (Figure 3). For the primer extension experiments with LC-MS/MS, Pol T7 and Pol κ were used because the misinsertion frequencies by Pol T7 and Pol κ were higher than those by other polymerases (Table 1). The primer extension reactions were conducted using a uracil-containing primer (5'-GGTGGTCCATAA(dU)C-3'), and the product was cleaved using UDG followed by hot piperidine treatment to simplify the sequencing results obtained with CID fragmentation.³⁵ The most abundant species of product (−2 charge) were used for CID

analysis, and the products were identified by matching the fragmentation patterns to the theoretical patterns obtained from a program (The Mass Spectrometry Group, University of Utah) (Supporting Information Tables S1 and S2).³⁶ LC-MS/MS analysis of primer extension opposite the *N*⁶dA-butanetriol adduct or unmodified dAdo revealed the presence of only the correct full-length replication product and also products with T and C incorporated opposite *N*⁶dA-(OH)₂butyl-GSH adduct by Pol T7 or Pol κ (Table 2). The product with C incorporation was not observed with the substrate containing *N*⁶dA-butanetriol adduct in either case.

DISCUSSION

DEB is an important mutagenic metabolite of BD compared with mono-epoxide metabolites of BD.^{11,12} As a result of its bifunctional nature, DEB gives rise to DNA-DNA and DNA-protein cross-links.¹⁴ DNA-protein cross-links can be deleterious to cells because they are bulky, helix-distorting lesions that block the binding and progression of protein complexes and interfere with normal DNA metabolism, i.e. replication, transcription, and recombination.³⁷ DNA-protein cross-links induced by DEB were first observed in livers of B6C3F1 mice exposure to BD³⁸ and have been reported *in vitro* including *O*⁶-alkylguanine DNA alkyltransferase (AGT),⁴⁹ glyceraldehyde 3-phosphate dehydrogenase,⁴⁰ and histone H3.⁴¹ However, there is no evidence that DEB-derived DNA-protein cross-links generate mutations, aside from the case of these formed with AGT.^{39–41} We previously reported a dramatic enhancement of base pair mutagenicity of DEB following GST expression in *S. typhimurium* TA1535,^{23,24} and subsequently a synthetic DEB-GSH conjugate was shown to be considerably more mutagenic than DEB or several other butadiene-derived epoxides in *S. typhimurium* TA1535²⁵ and *E. coli* TRG8 cells.²⁶ Six DNA adducts were identified from the reaction of DEB-GSH conjugate with free deoxyribonucleosides or calf thymus DNA and among these six adducts, *N*⁶dA-(OH)₂butyl-GSH and *N*⁷G-(OH)₂butyl-GSH adducts were identified and quantitated *in vivo* in livers of mice and rats treated with DEB.²⁷

The oligonucleotide site chosen for this work was previously found to show considerably high A:T to G:C transitions or A:T to C:G transversions in the *rpoB* gene of *E. coli* TRG8 cells treated with the DEB-GSH conjugate.²⁶ The sequence of the *rpoB* gene including the major mutational hot spot (amino acid 512) induced by the DEB-GSH conjugate²⁶ was used for the 19-mer oligomer containing the *N*⁶dA-(OH)₂butyl-GSH or the *N*⁶dA-butanetriol adduct. For a qualitative comparison of the bypass efficiency past the *N*⁶dA-(OH)₂butyl-GSH adduct by various DNA polymerases, primer extension assays were performed in the presence of all four dNTPs (Figure 1). The order of bypass efficiency, Pol T7 ~ Pol η > Dpo4 > Pol κ > Pol ι (Figure 1), is similar to the reported bypass efficiency past *N*²,3-ethenoguanine by these polymerases.^{32,42} Compared with Pol κ and Pol ι, Pol η exhibited higher bypass efficiency past the adducts 1,*N*⁶-ethenoadenine^{43,44} and 1,*N*⁶-(2-hydroxy-3-hydroxymethylpropan-1,3-diyl)-2'-deoxyadenosine (1,*N*⁶-γ-HMHP-dA) adduct (formed from DEB),⁴⁵ which has been proposed to contribute to mutagenicity of BD.¹⁶ However, Pol ι showed some single base incorporation opposite 1,*N*⁶-γ-HMHP-dA,⁴⁵ and did not extend primer well opposite *N*²,3-ethenoguanine.⁴² The ability of Pol T7, a high-fidelity processive polymerase, to efficiently bypass these large adducts positioned at a Watson-Crick pairing site, is surprising, in light of previous work with *N*²dG adducts.^{33,46}

dTTP was the most preferentially incorporated opposite N^6 dA-(OH)₂butyl-GSH adduct, N^6 dA-butanetriol adduct, or unmodified dA with all polymerases examined in steady-state assays (Figure 2, Table 1). (Studies with REV1, which is highly selective for incorporation of C,⁴⁷ were not considered here.) High dCTP misincorporation was detected opposite the N^6 dA-(OH)₂butyl-GSH adduct with Pol T7 (0.85) or Pol κ (0.45) (Figure 2, Table 1), which is of interest in that Pol κ is known to insert mainly T (and some A and G) opposite 1, N^6 - γ -HMHP-dA (formed from DEB)⁴⁵ and mainly A opposite 7,8-dihydro-8-oxo-2'-deoxyguanosine.⁴⁸

LC-MS/MS analysis of the primer extension products for the sequence analysis was performed with Pol T7 or Pol κ (Figure 3) because these two polymerases exhibited high dCTP misinsertion frequency opposite N^6 dA-(OH)₂butyl-GSH adduct (Table 1). Roughly equal amounts of the products containing the correct base T and the incorrect base C opposite N^6 dA-(OH)₂butyl-GSH adduct were observed with both Pol T7 and Pol κ (Table 2). These results provide a possible mechanism for the enhanced induction of A:T to G:C transitions in the *rpoB* gene of *E. coli* TRG8 cells treated with the DEB-GSH conjugate²⁶ and the predominant A to G transitions of H-*ras* codon 61 in Harderian gland tumors of B6C3F1 mice treated with BD via inhalation.⁴⁹

The two N^6 dA DNA adducts (Scheme 1) used in this study differ in the respect that the triol adduct was a specific stereoisomer (2*S*, 3*R*) but the GSH adduct was a racemic mixture (four isomers). The GSH-derived adduct was specifically racemic because we intended to probe the entire scope of possible mutagenic adduct of this entity. Our prior mutation work was done with racemic *S*-(4-amino-2,3-dihydroxy)GSH²⁶ and also indicated that all GSTs examined had rather similar catalytic efficiencies in conjugating the different isomers of DEB.²⁷ Further, the different isomers of DEB had very similar mutagenicities when their GSH conjugates were prepared and used with *S. typhimurium*.²⁷ The N^6 dA-butanetriol adduct has been reported *in vivo* in humans exposed to BD⁵⁰ but the stereochemistry is unknown. Individual stereoisomers of the N^6 dA-butanetriol adduct have been examined and concluded to be only very weakly mutagenic,⁵¹ consistent with our findings with these polymerases.

GSH conjugation has been clearly shown to enhance the mutagenicity of DEB in bacteria.^{25,26} Six adducts containing GSH have been identified, but only two—the N^6 dA-(OH)₂butyl-GSH and the N^7 G-(OH)₂butyl-GSH adducts—were detected in mice and rats.²⁷ Detailed studies with the N^6 -dA adducts were possible, as presented here, but studies with the N^7 -dG adducts are not currently possible because of the instability of the glycosidic bond. The labile N^7 dG adducts, susceptible to spontaneous depurination, would be expected to produce G:C to T:A transversions.⁵² The studies with these N^6 dA adducts clearly show the role of the GSH moiety in miscoding (Scheme 1, Table 1, 2), and the molecular basis of this effect is under investigation. In that structures of Pol κ have been determined with DNA adducts,⁴⁸ it may be possible to understand these results with N^6 dA-(OH)₂butyl-GSH at a structural level.

In summary, we investigated the miscoding potential of the DEB-GSH conjugate-derived major DNA adduct, N^6 dA-(OH)₂butyl-GSH, in the context of the mechanism of mutations

induced by GSH conjugation. Both Pol T7 and, of more relevance, human Pol κ incorporated the correct T and incorrect C bases opposite N^6 dA-(OH)₂butyl-GSH adduct, and translesion bypass beyond the N^6 dA-(OH)₂butyl-GSH adduct by Pol T7 and Pol κ was error-prone. These results, coupled with our earlier findings,²⁶ indicate the relevance of GSH-containing adducts in A:T to G:C mutations produced by DEB, the oxidation product of BD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AGT	<i>O</i> ⁶ -alkylguanine DNA alkyltransferase
BD	1,3-butadiene
BSA	bovine serum albumin
CID	collision-induced dissociation
dA	deoxyadenosine
DEB	1,2,3,4-diepoxybutane (butadiene diepoxide)
DEB-GSH conjugate	<i>S</i> -(2-hydroxy-3,4-epoxybutyl)GSH
EB	3,4-epoxy-1-butene
ESI	electrospray ionization
GST	GSH <i>S</i> -transferase
1,<i>N</i>⁶-γ-HMHP-dA	1, <i>N</i> ⁶ -(2-hydroxy-3-hydroxymethylpropan-1,3-diyl)-2'-deoxyadenosine
<i>N</i>⁶dA-butanetriol	<i>N</i> ⁶ -(2,3,4-trihydroxybutyl)deoxyadenosine
<i>N</i>³A-(OH)₂butyl-GSH	<i>S</i> -[4-(<i>N</i> ³ -adenyl)2,3-dihydroxybutyl]GSH
<i>N</i>⁶dA-(OH)₂butyl-GSH	<i>S</i> -[4-(<i>N</i> ⁶ -deoxyadenosinyl)2,3-dihydroxybutyl]GSH
<i>N</i>⁷G-(OH)₂butyl-GSH	<i>S</i> -[4-(<i>N</i> ⁷ -guanyl)2,3-dihydroxybutyl]GSH
<i>N</i>¹dG-(OH)₂butyl-GSH	<i>S</i> -[4-(<i>N</i> ¹ -deoxyguanosinyl)2,3-dihydroxybutyl]GSH
<i>N</i>⁴dC-(OH)₂butyl-GSH	<i>S</i> -[4-(<i>N</i> ⁴ -deoxycytidinyl)2,3-dihydroxybutyl]GSH
<i>N</i>³dT-(OH)₂butyl-GSH	<i>S</i> -[4-(<i>N</i> ³ -thymidinyl)2,3-dihydroxybutyl]GSH
Pol	(DNA) polymerase

UDG

uracil DNA glycosylase

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Figure 1.

32 P-labeled primer extension in the presence of all four dNTPs by different DNA polymerases. Primer and template sequences where X is dA, N^6 dA-butanetriol, or N^6 dA-(OH)₂butyl-GSH (A). Reactions with Dpo4 (B), Pol T7 (C), Pol η (D), Pol ι (E), and Pol κ (F). Reactions were carried out for increasing times (as indicated) in 50 mM Tris-HCl buffer (pH 7.5) containing 60 nM primer-template complex, 250 μ M dNTPs, 5% (v/v) glycerol, 5 mM DTT, 50 mM NaCl, 5 mM MgCl₂, and 50 Og mL^{-1} BSA at 37 °C.

Figure 2.

³²P-Labeled primer extension in the presence of a single dNTP by different DNA polymerases. Primer and template sequences where X is dA, N⁶dA-butanetriol, or N⁶dA-(OH)₂butyl-GSH (A). Reactions with Dpo4 (B), Pol T7 (C), Pol η (D), Pol ι (E), and Pol κ (F). Reactions were carried out for 10 min (except for REV1, which was incubated for 20 min) in 50 mM Tris-HCl buffer (pH 7.5) containing 60 nM primer-template complex, 1 mM dNTPs, 5% (v/v) glycerol, 5 mM DTT, 50 mM NaCl, 5 mM MgCl₂, and 50 Og mL⁻¹ BSA at 37 °C.

Figure 3.

Extracted ion chromatograms and collision-induced dissociation (CID) mass spectra of m/z 939.2 (-2) (A and C) and 947.0 (-2) (B and D) from LC-MS/MS analysis of full-length extension product by Pol T7 formed with the sequence (template; 3'-CCACCAGGTATTTGXCTCT (X: N^6 dA-(OH)₂butyl-GSH), primer; 5'-GGTGGTCCATAAUC) in the presence of all four dNTPs.

Scheme 1.
Structures of dA and DNA Adducts Under Consideration

Table 1

Steady-State Kinetic Parameters for Polymerase-Catalyzed Single Base Insertion

Polymerase	Template	dNTP	k_{cat} (min ⁻¹)	K_m (μM)	k_{cat}/K_m (min ⁻¹ μM ⁻¹)	f_d
Dpo4	dA	C	0.056 ± 0.003	22 ± 3	0.003	0.07
		T	0.36 ± 0.02	10.3 ± 2.3	0.035	1
	N ⁶ dA-butanetriol	C	0.038 ± 0.002	20 ± 6	0.002	0.074
Pol T7		T	0.35 ± 0.02	13 ± 3	0.027	1
	N ⁶ dA-(OH) ₂ butyl-GSH	C	0.042 ± 0.002	18 ± 4	0.002	0.077
		T	0.28 ± 0.02	9.3 ± 3.7	0.030	1
Pol η	dA	C	0.19 ± 0.01	23 ± 10	0.008	0.05
		T	0.67 ± 0.03	4.1 ± 1.2	0.16	1
	N ⁶ dA-butanetriol	C	0.15 ± 0.01	25 ± 9	0.006	0.06
Pol ι		T	0.62 ± 0.04	6.4 ± 2.6	0.096	1
	N ⁶ dA-(OH) ₂ butyl-GSH	C	0.20 ± 0.02	2.4 ± 0.5	0.085	0.85
		T	0.60 ± 0.05	5.8 ± 2.1	0.10	1
Pol η	dA	C	0.31 ± 0.02	26 ± 13	0.012	0.037
		T	0.69 ± 0.05	2.1 ± 1.0	0.33	1
		A	0.37 ± 0.04	22 ± 9	0.017	0.052
N ⁶ dA-butanetriol		G	0.07 ± 0.01	13 ± 8	0.005	0.016
		C	0.20 ± 0.02	28 ± 14	0.007	0.03
		T	0.57 ± 0.05	2.4 ± 1.5	0.24	1
N ⁶ dA-(OH) ₂ butyl-GSH		A	0.23 ± 0.02	25 ± 13	0.010	0.04
		G	0.10 ± 0.01	11 ± 4	0.009	0.038
		C	0.18 ± 0.02	26 ± 12	0.004	0.017
Pol ι		T	0.61 ± 0.05	2.6 ± 0.8	0.23	1
		A	0.20 ± 0.02	28 ± 9	0.007	0.03
		G	0.19 ± 0.02	13 ± 5	0.015	0.06
N ⁶ dA-butanetriol	dA	C	0.006 ± 0.001	45 ± 11	0.0001	0.012
		T	0.321 ± 0.020	30 ± 13	0.011	1
		C	0.005 ± 0.001	42 ± 15	0.0001	0.015
		T	0.283 ± 0.015	35 ± 14	0.008	1

Polymerase	Template	dNTP	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($\text{min}^{-1} \mu\text{M}^{-1}$)	f^a
Pol κ	N ⁶ dA-(OH) ₂ butyl-GSH	C	0.004 $\pm$ 0.001	48 $\pm$ 23	0.00008	0.014
		T	0.243 $\pm$ 0.020	40 $\pm$ 19	0.006	1
	dA	C	0.23 $\pm$ 0.02	260 $\pm$ 80	0.0009	0.007
		T	1.07 $\pm$ 0.09	8.0 $\pm$ 2.7	0.133	1
Pol κ	N ⁶ dA-butanetriol	C	0.18 $\pm$ 0.02	310 $\pm$ 130	0.0006	0.014
		T	0.43 $\pm$ 0.04	10 $\pm$ 5	0.041	1
	N ⁶ dA-(OH) ₂ butyl-GSH	C	0.32 $\pm$ 0.02	83 $\pm$ 23	0.004	0.45
		T	0.28 $\pm$ 0.03	32 $\pm$ 10	0.009	1

^a Misinsertion frequency: $f = (k_{cat}/K_m(\text{dNTP}))_{\text{incorrect}}/(k_{cat}/K_m(\text{dTTP}))$.

Table 2

Fractions of Replication Products Produced from dA, *N*⁶-dA-butanetriol, and *N*⁶-dA-(OH)²-GSH-containing Substrates As Determined by LC-MS/MS

Polymerase	3'-CCACCAGGTATTTG <u>X</u> CTCT 5'-GGTGGTCCATAAUC		% of product	Base inserted
Pol T7	X: dA	C <u>T</u> GAGA	100	T
	X: <i>N</i> ⁶ dA-butanetriol	C <u>T</u> GAGA	100	T
	X: <i>N</i> ⁶ dA-(OH) ² butyl-GSH	C <u>T</u> GAGA	45	T
		C <u>C</u> GAGA	55	C
Pol κ	X: dA	C <u>T</u> GAGA	100	T
	X: <i>N</i> ⁶ dA-butanetriol	C <u>T</u> GAGA	100	T
	X: <i>N</i> ⁶ dA-(OH) ² butyl-GSH	C <u>T</u> GAGA	60	T
		C <u>C</u> GAGA	40	C