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Polymerase Bypass of *N*⁶-Deoxyadenosine Adducts Derived from Epoxide Metabolites of 1,3-Butadiene

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Abstract

*N*⁶-(2-Hydroxy-3-buten-1-yl)-2'-deoxyadenosine (*N*⁶-HB-dA I) and *N*⁶,*N*⁶-(2,3-dihydroxybutan-1,4-diyl)-2'-deoxyadenosine (*N*⁶,*N*⁶-DHB-dA) are exocyclic DNA adducts formed upon alkylation of the *N*⁶ position of adenine in DNA by epoxide metabolites of 1,3-butadiene (BD), a common industrial and environmental chemical classified as a human and animal carcinogen. Since the *N*⁶-H atom of adenine is required for Watson-Crick hydrogen bonding with thymine, *N*⁶-alkylation can prevent adenine from normal pairing with thymine, potentially compromising the accuracy of DNA replication. To evaluate the ability of BD-derived *N*⁶-alkyladenine lesions to induce mutations, synthetic oligodeoxynucleotides containing site-specific (*S*)-*N*⁶-HB-dA I and (*R,R*)-*N*⁶,*N*⁶-DHB-dA adducts were subjected to *in vitro* translesion synthesis in the presence of human DNA polymerases β , η , ι , and κ . While (*S*)-*N*⁶-HB-dA I was readily bypassed by all four enzymes, only polymerases η and κ were able to carry out DNA synthesis past (*R,R*)-*N*⁶,*N*⁶-DHB-dA. Steady-state kinetic analyses indicated that all four DNA polymerases preferentially incorporated the correct base (T) opposite (*S*)-*N*⁶-HB-dA I. In contrast, hPol β was completely blocked by (*R,R*)-*N*⁶,*N*⁶-DHB-dA, while hPol η and κ inserted A, G, C, or T opposite the adduct with similar frequency. HPLC-ESI-MS/MS analysis of primer extension products confirmed that while translesion synthesis past (*S*)-*N*⁶-HB-dA I was mostly error-free, replication of DNA containing (*R,R*)-*N*⁶,*N*⁶-DHB-dA induced significant numbers of A, C, and G insertions and small deletions. These results indicate that singly substituted (*S*)-*N*⁶-HB-dA I lesions are not miscoding, but exocyclic (*R,R*)-*N*⁶,*N*⁶-DHB-dA adducts are strongly mispairing, probably due to their inability to form stable Watson-Crick pairs with dT.

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Supporting Information available: Extension of ³²P-end labeled 9-mer primer annealed to 18-mer template containing site specific dA, (*S*)-*N*⁶-HB-dA I, or (*R,R*)-*N*⁶,*N*⁶-DHB-dA by hpol η under running-start conditions; and co-operativity between human TLS polymerases hpol ι and hpol η during the bypass of the (*R,R*)-*N*⁶,*N*⁶-DHB-dA lesion. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

DNA damage; DNA polymerase; mutagenesis mechanism; nucleic acid chemistry; enzyme kinetics; mass spectrometry (MS)

INTRODUCTION

Genomic DNA is under continuous attack by reactive species present in our diet, our environment, and generated endogenously as a result of oxidative stress, immune response, and normal cellular metabolism.^{1,2} The resulting structurally modified DNA nucleobases (DNA adducts) can compromise the accuracy of DNA replication, leading to heritable genetic damage. Mutations in genes responsible for genomic stability, signal transduction, and cell death can lead to the initiation of cancer.³

Many carcinogen-induced DNA adducts block the progression of replicative DNA polymerases (Pols) α , δ , and ε as a result of their steric bulk, their effects on DNA structure, and their inability to form standard Watson-Crick base pairs.⁴ A specialized group of translesion synthesis (TLS) polymerases including hPols η , ι , κ , and ζ are recruited to stalled replication forks; this is followed by polymerase switching during which the replicative polymerases are replaced by the TLS polymerases.^{5,6} These TLS polymerases are characterized by open and flexible active sites which can accommodate the bulky DNA adducts, allowing for replication to continue in the presence of nucleobase damage.^{5–10} For example, Pol η accurately bypasses thymine dimers, thereby suppressing the mutagenic effects of UV-induced DNA damage, while Pol κ bypasses oxidation-induced thymine glycol and N^2 -guanine adducts induced by benzo[*a*]pyrene diolepoxide.^{11,12} Following lesion bypass, another polymerase switching occurs, and replicative DNA polymerases take over again, completing chromosomal replication. Although lesion bypass polymerases alleviate the genotoxicity of bulky DNA lesions, they generally have lower fidelity compared to replicative DNA polymerases as a result of the relaxed active site geometry and the lack of exonuclease proofreading.^{13,14} These increased error rates during replication may lead to miscoding and mutations, potentially contributing to multi-stage carcinogenesis.¹⁵

One important carcinogen ubiquitously present in cigarette smoke, cooking fires, and urban air is 1,3-butadiene (BD).^{16,17} BD is a major industrial chemical used in the manufacture of rubber and plastics, with annual US production of 9 metric tons.¹⁸ BD induces tumors at multiple organ sites of laboratory mice and rats^{19,20} and increases the risk of leukemia in occupationally exposed workers.²¹ BD is metabolically activated to 3,4-epoxy-1-butene (EB)^{22,23} and 1,2,3,4-diepoxybutane (DEB).²⁴ Both EB and DEB are directly acting genotoxic agents that can induce large numbers of mutations at G:C and A:T base pairs and deletions.^{25,26} DEB induced A→T transversions and partial deletions, while EB caused G→A point mutations and A→T transversions at the *hprt* of TK6 human lymphoblastoid cells exposed to the BD epoxides.²⁷ *In vivo* studies in B6C3F1 *lacI* transgenic mice exposed to BD showed that BD induced A→G transitions and A→T transversions.²⁷

EB and DEB react with DNA to form a range of DNA adducts including adenine and guanine monoadducts,^{28,29} DNA-DNA cross links,^{30–32} and exocyclic DNA adducts.^{33,34}

Among nucleobase adducts induced by BD epoxides, deoxyadenosine adducts are of significant interest because they are likely to contribute to A→T, A→C, and A→G mutations observed upon exposure to BD and its epoxides.^{25,27} EB modifies the exocyclic amine group of adenine to form *N*⁶-(2-hydroxy-3-buten-1-yl)-2'-deoxyadenosine (*N*⁶-HB-dA I) and *N*⁶-(1-hydroxy-3-buten-2-yl)-2'-deoxyadenosine (*N*⁶-HB-dA II) (Scheme 1).^{35–38} *N*⁶-HB-dA I and *N*⁶-HB-dA II adducts have been observed in DNA isolated from several tissues of laboratory mice and rats exposed to BD by inhalation.^{39,40} EB can sequentially react at two sites within the adenine heterocycle to form exocyclic deoxyadenosine lesions: *N*⁶,*N*⁶-(2,3-dihydroxybutan-1,4-diyl)-2'-deoxyadenosine (*N*⁶,*N*⁶-DHB-dA), *N*⁶-(2-hydroxy-3-hydroxymethylpropan-1,3-diyl)-2'-deoxy-adenosine (1,*N*⁶- γ HMHP-dA), and 1,*N*⁶-(1-hydroxymethyl-2-hydroxypropan-1,3-diyl)-2'-deoxyadenosine (1,*N*⁶- α HMHP-dA) (Scheme 1).^{32,39–42} 1,*N*⁶-HMHP-dA and *N*⁶,*N*⁶-DHB-dA adducts were found in calf thymus DNA treated with DEB,³³ while 1,*N*⁶-HMHP-dA adducts were also detected in DNA of mice exposed to BD.⁴¹ While it has been reported that *N*⁶-HB-dA II adducts are not mispairing in *Escherichia coli*,⁴³ polymerase bypass past *N*⁶-HB-dA I and *N*⁶,*N*⁶-DHB-dA lesions has not yet been examined.

The primary goal of this study was to investigate the influence of *N*⁶-HB-dA I and *N*⁶,*N*⁶-DHB-dA adducts on DNA replication by human DNA polymerases, with an ultimate goal of establishing the mechanisms of BD-induced A → T, A→C, and A→G mutations and deletions. Primer extension studies were conducted using synthetic DNA templates containing site specific (*S*)-*N*⁶-HB-dA I and (*R,R*)-*N*⁶,*N*⁶-DHB-dA in the presence of recombinant human Pol β , Pol κ , Pol η , and Pol ι . Our results reveal significant differences between the outcomes of replication past *N*⁶-HB-dA I and *N*⁶,*N*⁶-DHB-dA lesions. While *N*⁶-HB-dA I was not miscoding, *N*⁶,*N*⁶-DHB-dA induced large numbers of misincorporations and deletions, probably as a result of its inability to form a Watson-Crick base pair with dT.

EXPERIMENTAL PROCEDURES

Materials

Full-length recombinant human polymerase κ (hPol κ) used for gel electrophoresis experiments was purchased from Enzymax (Lexington, KY). Human Pol β was obtained from Trevigen (Gaithersburg, MD). Recombinant human DNA polymerases hPol η (amino acids 1–437), hPol ι (amino acids 1–420), and hPol κ (amino acids 19–526) (active core enzymes) were expressed and purified according to previously published procedures.^{44–46} T4 polynucleotide kinase (T4-PNK) and *E. coli* uracil DNA glycosylase (UDG) were purchased from New England Biolabs (Beverly, MA). [γ -³²P]ATP was purchased from Perkin-Elmer Life Sciences (Boston, MA). Acrylamide/bis-acrylamide solution (40% 19:1, w/w) and Bio-spin columns were obtained from Bio-Rad laboratories (Hercules, CA). All other chemicals were purchased either from Sigma-Aldrich or Fisher Scientific.

Oligonucleotide Synthesis, Labeling, and Annealing

Synthetic 18-mer oligodeoxynucleotides (5'-TCATXGAATCCTTCCCCC-3') containing 6-chloropurine at position X were prepared by standard solid phase synthesis and coupled with

(*S*)-*N*-Fmoc-1-aminobut-3-en-2-ol⁴⁷ and (*R,R*)-pyrrolidine-3,4-diol⁴⁸ to yield the corresponding strands containing site- and stereospecific (*S*)-*N*⁶-HB-dA I and (*R,R*)-*N*⁶,*N*⁶-DHB-dA adducts. Detailed methodologies for oligodeoxynucleotide synthesis have been previously reported.^{47,48} The corresponding unmodified 18-mer template containing native dA (5'-TCATAGAAT CCTTCCCCC-3'), 13-mer primers (5'-GGGGGAAGGATTC-3' and 5'-GGGGGAAGGAUTC-3') and a 9-mer primer (5'-GGGGGAAGG-3') were purchased from Integrated DNA Technologies (Coralville, IA). All DNA oligomers were purified by semi-preparative HPLC, characterized by HPLC-ESI[−] MS/MS, and quantified by UV spectroscopy.^{47,48} The 13-mer primer (5'-GGGGGAAGGATTC-3') and the 9-mer primer (5'-GGGGGAAGG-3') were radiolabeled and subsequently annealed to the corresponding 18-mer templates to obtain primer-template complexes for *in vitro* replication studies (Scheme 2).

Primer Extension Assays

Primer extension studies were performed using the previously published methods,⁴⁹ with a few modifications. For standing start experiments, ³²P-endlabeled 13-mer:18-mer primer-template complexes (Scheme 2A) containing unmodified dA, (*S*)-*N*⁶-HB-dA I, or (*R,R*)-*N*⁶,*N*⁶-DHB-dA at the 14th position of the template strand (50 nM) were incubated at 37 °C in the presence of individual human DNA polymerases (hPol β, 12.5 nM; hPol η, 10 nM; hPol ι, 20 nM; hPol κ, 5 nM) in a buffer containing 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 5 mM dithiothreitol, 100 μg/ml BSA, and 10% glycerol (v/v). Primer extension reactions were initiated by the addition of dNTP mix (500 μM) and MgCl₂ (5 mM). Aliquots of the reaction mixture (4 μL) were taken at 0, 5, 15, 30, 45, and 60 min and quenched with stop solution (95% formamide (w/v), 10 mM EDTA, 0.03% bromophenol blue (w/v), 0.03% xylene cyanol (w/v), 36 μL). “Running start” reactions were conducted analogously using ³²P-endlabeled 9-mer primer/template duplexes (50 nM, Scheme 2B) in the presence of higher enzyme concentrations (hPol β, 25 nM; hPol η, 25 nM; hPol κ, 12.5 nM). Primer extension products were resolved by gel electrophoresis using a 20% (w/v) denaturing polyacrylamide gel containing 7 M urea. The radioactive primer extension products were visualized on a GE Typhoon FLA 7000 phosphorimager (GE Healthcare Life Sciences, Piscataway, NJ).

Steady-state Kinetic Analyses

The kinetics of incorporation of various nucleotides opposite (*S*)-*N*⁶-HB-dA I or (*R,R*)-*N*⁶,*N*⁶-DHB-dA were determined by conducting polymerization reactions in the presence of increasing concentrations of individual dNTPs (0–800 μM) for different time periods (0–180 min). DNA polymerase concentrations used were: hPol β, 12.5 nM; hPol η, 10 nM; hPol ι, 20 nM; hPol κ, 2.5–3.5 nM; the DNA concentration was 50 nM. The radioactive product bands were visualized on a GE Typhoon FLA7000 phosphorimager and quantified using ImageQuant software (GE Healthcare Life Sciences, Piscataway, NJ). Nonlinear regression analysis (one-site hyperbolic fits in Prism (GraphPad, La Jolla, CA)) was employed to determine the steady-state kinetic parameters for nucleotide incorporation.

HPLC-ESI-MS/MS Analysis of Primer Extension Products from DNA Polymerase Reactions

Uracil-containing primer/template complexes (100 pmol) (Scheme 2C) were incubated with hPol η or hPol κ (40 pmol) in a buffer containing 50 mM Tris-HCl (pH 7.8), 5% glycerol (v/v), 5 mM dithiothreitol, 5 mM MgCl₂, 100 μ g/mL bovine serum albumin and 1 mM each of the four dNTPs at 37 °C for 5 hours. Excess dNTPs were removed by size-exclusion using Bio-Spin 6 columns (Bio-Rad, Hercules, CA), and appropriate buffers were added to restore the concentrations in the filtrate to 50 mM Tris-HCl (pH 7.8), 5 mM dithiothreitol and 1 mM EDTA. The mixture was incubated with uracil DNA glycosylase (UDG) (6 units, 37 °C for 6 h), and the resulting abasic sites were cleaved with hot piperidine (0.25 M, 95 °C for 1 h) to reduce the size of primer extension products for sequencing by HPLC-ESI-MS/MS.⁴⁹ The final reaction mixture was dried under vacuum and reconstituted in 25 μ L of 15 mM ammonium acetate buffer containing a 14-mer oligonucleotide used as an internal standard for mass spectrometry (5'-p-CTTCACGAGCCCC-3', 40 pmol). Primer extension products were resolved on a Agilent Zorbax SB 300 C18 (0.5 mm $\times$ 150 mm, 5 μ m) column using an Eksigent HPLC system (Eksigent, Dublin, CA) coupled to a Thermo LTQ Orbitrap Velos mass spectrometer (ThermoFisher Scientific, Waltham, MA).⁴⁹ Relative quantification and MS/MS sequencing of primer extension products were conducted as described previously.⁴⁹

RESULTS

Primer Extension under Standing Start and Running Start Conditions

“Standing start” experiments were conducted with 13-mer primer/18-mer template complexes, where the primer 3' terminus was positioned immediately upstream from the adducted base (Scheme 2A). Control experiments with the unmodified template showed that under these experimental conditions, hPol β , hPol κ , and hPol η produced full length 18-mer products (Figures 1A, 2A and 2B, first panel), while hPol ι reactions generated primarily 14–16-mer products (Figure 1B, first panel). The presence of (*S*)-*N*⁶-HB-dA I in the template strand did not prevent hPol β , hPol κ or hPol η from extending the primer to the terminus to yield 18-mer products (Figures 1A, 2A, 2B, second panels). However, the efficiency of hPol β , hPol κ , and hPol η -mediated DNA synthesis was reduced in the presence of (*S*)-*N*⁶-HB-dA, as evidenced by the observation of incomplete primer extension products (14–17-mers in the second panels of Figures 1A, 2A, and 2B). hPol ι formed 14–16-mer products, as was the case for the control template (Figure 1B, second panel). These results suggest that hPol ι -dependent TLS pathways would require the participation of an additional TLS polymerase that could extend from the nucleotide inserted by Pol ι .

Polymerase bypass of (*R,R*)-*N*⁶,*N*⁶-DHB-dA was significantly less efficient. DNA synthesis by hPol β was completely blocked by this lesion (Figure 1A, third and fourth panels), while hPol ι incorporated a single base opposite the adduct but did not extend the primer any further, even at increased enzyme concentrations (Figure 1B). Under standard conditions, primer extension by hPol κ was stalled after single nucleotide incorporation opposite (*R,R*)-*N*⁶,*N*⁶-DHB-dA to produce 14-mer products (Figure 1C, third panel), but full extension was achieved at increased enzyme concentrations (Figure 1C, fourth panel). Similarly, hPol η was able to bypass (*R,R*)-*N*⁶,*N*⁶-DHB-dA to form 18-mer full extension products, but only

when polymerase concentrations were increased significantly (Figure 1D). These observations indicate that the rate of postlesion synthesis is slower than the rate of single nucleotide incorporation opposite the damaged base.

To determine whether polymerase bypass of BD-dA adducts is more efficient under running start conditions, primer extension were repeated with -5 primers (Scheme 2B). As was the case in standing start experiments, hPol β , hPol κ , and hPol η were able to extend the primer past (S)- N^6 -HB-dA I and completely to the terminus (central panels of Figures 2A, 2B, and S1). In contrast, the presence of (R,R)- N^6,N^6 -DHB-dA led to replication stalling at the site of damage and the formation of 13–14-mer incomplete extension products (right panels in Figures 2A, 2B, and S1) due to inefficient postlesion synthesis.

Possible Co-operativity of TLS Polymerases During Replication through (R,R)- N^6,N^6 -DHB-dA Lesions

To determine whether TLS polymerases can potentially work together to help bypass strongly blocking (R,R)- N^6,N^6 -DHB-dA adducts, additional primer extension experiments were conducted in the presence of both hPol ι and hPol η . As shown in Figure 1B, hPol ι incorporated a single nucleotide opposite (R,R)- N^6,N^6 -DHB-dA but was unable to catalyze further primer extension. When the incomplete extension products from hPol ι reactions were incubated with hPol η , the primer was extended completely to the terminus to form 18-mer products (Figure S2A). Full extension was also achieved when hPol ι and hPol η were added simultaneously (Lane 3 in Figure S2B). The amounts of 18-mer full extension products generated following extension reactions with both enzymes (12%, Lane 3) were higher than in control experiments where extension reactions were performed with hPol η alone (8%, Lane 2) or when extension products from reactions performed with hPol ι and hPol η alone were combined following protein inactivation (2%, lane 4). These observations provide preliminary evidence that hPol ι mediated TLS pathway past N^6,N^6 -DHB-dA lesions requires Pol η for extending from the nucleotide inserted by Pol ι opposite the adduct.

Steady-state Kinetic Analysis of Incorporation of Individual dNTPs Opposite the BD-dA Lesions

In order to investigate the fidelity of nucleotide incorporation opposite (R,R)- N^6,N^6 -DHB-dA and (S)- N^6 -HB-dA I, primer extension experiments were repeated in the presence of individual dNTPs (0–800 μ M). The kinetics of nucleotide insertion was evaluated by conducting polymerization experiments in the presence of increasing concentrations of each nucleotide for specified time periods (0–180 min). Steady-state kinetic parameters (k_{cat} and K_{m}) for nucleotide incorporation, catalytic specificity constants ($k_{\text{cat}}/K_{\text{m}}$) and the misinsertion frequencies for nucleotide insertion (f) were determined as described previously.^{49,50}

hPol β was less efficient at incorporating the correct nucleotide (dTTP) opposite (S)- N^6 -HB-dA I as compared to unmodified dA, as reflected by the 20-fold decrease in the $k_{\text{cat}}/K_{\text{m}}$ values (Table 1). No kinetic analysis was possible for dTTP incorporation opposite (R,R)- N^6,N^6 -DHB-dA by this polymerase, as product formation was too low to be quantified. In

the case of hPol ι , dTMP incorporation opposite (*S*)- N^6 -HB-dA I and (*R,R*)- N^6,N^6 -DHB-dA was 5- and 33- fold less efficient than opposite unmodified dA, respectively. As shown in Table 1, the k_{cat} values for these reactions were similar, while the K_m values were markedly increased in the presence of either BD-dA lesion.

hPol κ preferentially incorporated dTMP opposite dA in the unmodified template, with dAMP and dGMP added 50–400 fold less efficiently (Table 1). Similarly, hPol κ preferentially inserted the correct nucleotide (dTMP) opposite (*S*)- N^6 -HB-dA I, although nucleotide incorporation was 3-fold less efficient as compared to the unmodified template ($k_{\text{cat}}/K_m = 0.38$ vs $1.0 \mu\text{M}^{-1} \text{min}^{-1}$, see Table 1). The efficiency of addition of incorrect nucleotides (dCMP, dAMP, and dGMP) opposite (*S*)- N^6 -HB-dA I was 100-fold lower (Table 1), suggesting that this adduct retains the ability to form correct Watson-Crick base pair with dT. In contrast, hPol κ catalyzed the incorporation of all four nucleotides opposite (*R,R*)- N^6,N^6 -DHB with similar efficiency ($k_{\text{cat}}/K_m = 2.3$ (T), 1.2 (A), 1.1 (G), and $1.7 \times 10^{-3} \mu\text{M}^{-1} \text{min}^{-1}$ (C), Table 1). Nucleotide incorporation opposite (*R,R*)- N^6,N^6 -DHB-dA by hPol κ was ~ 450 – 1040 fold less efficient than opposite unmodified dA (Table 1), indicating that hPol κ has a low tolerance for this adduct.

Similar kinetic experiments were conducted with hPol η . We found that the efficiency of error-free replication opposite (*S*)- N^6 -HB-dA I was ~ 60 -fold higher than that of dAMP or dCMP and ~ 200 -fold higher than that of dGMP (Table 1). In contrast, the preference order of hPol η -mediated nucleotide insertion opposite (*R,R*)- N^6,N^6 -DHB-dA by hPol η was $G > T \sim A \sim C$, with ~ 1.6 -fold higher efficiency for dGMP incorporation as compared to the other three bases. k_{cat}/K_m values for insertion of all four dNTPs opposite (*R,R*)- N^6,N^6 -DHB-dA were similar for both hPol η and hPol κ (Table 1). Overall, these results indicate that translesion synthesis past (*S*)- N^6 -HB-dA I is quite efficient and is essentially error-free. In contrast, the presence of (*R,R*)- N^6,N^6 -DHB-dA in the template strand creates a strong replication block and introduces polymerase errors.

HPLC-ESI-MS/MS Analysis of Primer Extension Products Formed by hPol η and hPol κ

HPLC-ESI-MS/MS sequencing of hPol κ and hPol η -mediated primer extension products was used to confirm the results of gel electrophoresis based experiments (Table 1, Figs 1–2) and to identify additional primer extension products not detectable by gel electrophoresis (e.g. deletion products). In brief, polymerase primers were constructed to contain a uracil residue three bases upstream from the lesion site (Scheme 2C). Following *in vitro* replication in the presence of human polymerases, primer extension products were cleaved with UDG/piperidine to yield 5-, 6-, or 7-mer products, which could be readily sequenced by tandem mass spectrometry.^{49,50} The relative abundance of each primer extension product was calculated by comparing their HPLC-ESI-MS peak areas to that of a 14-mer internal standard, while their identity was established by MS/MS sequencing on an Orbitrap Velos mass spectrometer.⁴⁹

HPLC-ESI-MS/MS analysis of primer extension products generated using the (*S*)- N^6 -HB-dA I-containing template and hPol κ revealed oligodeoxynucleotide products at m/z 825.1, 1078.7, 1086.2, and 1090.7, corresponding to 5'-p TCTTATGA-3', 5'-p TCCATGA-3', 5'-p TCTATGA-3', and 5'-p TCAATGA-3', respectively. The error-free extension product (5'-p

TCTATGA-3') accounted for 83% of the total extension products (Scheme 3). The relative contributions of the three additional extension products, 5'-p TCTTATGA-3', 5'-p TCCATGA-3', and 5'-p TCAATGA-3', were 6, 3, and 8 %, respectively (Scheme 3). These results are consistent with the gel electrophoresis data shown in Figures 1 and 2 and Table 1, indicating that the correct nucleotide (dTTP) is preferentially inserted opposite (S)-N⁶-HB-dA I.

In contrast, HPLC-ESI-MS/MS sequencing of primer extension products originating from hPol κ catalyzed replication of the (R,R)-N⁶,N⁶-DHB-dA containing template resulted in a complex mixture of products, including those with *m/z* 777.6, 922.1, 934.1, 942.1, 1078.7, 1086.2, 1090.7 and 1098.7, which correspond to 5'-p TC__TGA-3', 5'-p TCC_TGA-3', 5'-p TCA_TGA-3', 5'-p TCG_TGA-3', 5'-p TCCATGA-3', 5'-p TCTATGA-3', 5'-p TCAATGA-3', and 5'-p TCGATGA-3', respectively (Figure 3, Scheme 3). The products formed by misinsertion of A opposite the lesion (5'-p TCAATGA-3') accounted for 20% of the total products (Scheme 3). Approximately 38% of total polymerization products corresponded to -1 and -2 deletions. 5'-p TCA_TGA-3' and 5'-p TC__TGA-3' were the major deletion products, corresponding to 18 and 9% of total products, respectively (Scheme 3). The product of error-free extension (5'-p TCTATGA-3') accounted for only 37% of total extension products.

Parallel reactions were conducted for hPol η . We found that *in vitro* replication of the (S)-N⁶-HB-dA I containing template produced only 5'-p TCTATGA-3' (error free, 92%) and 5'-p TCCATGA-3' (8%) (Scheme 4). In contrast, hPol η catalyzed bypass of (R,R)-N⁶,N⁶-DHB-dA generated seven different oligomers (Scheme 4). The major product (5'-p TCCATGA-3') was formed by misinsertion of C opposite (R,R)-N⁶,N⁶-DHB-dA (46%). Error-free product (5'-p TCTATGA-3') accounted for 19% of the total extension products, while 20% corresponded to the T $\rightarrow$ A transversion product (5'-p TCAATGA-3'). Deletion products accounted for 6% of *in vitro* replication products past (R,R)-N⁶,N⁶-DHB-dA by hPol η (Scheme 4).

Nucleotide sequences of the primer extension products were determined from their MS/MS spectra. Good sequence coverage of a-B and w ion series was observed, allowing for accurate identification. For example, the MS/MS spectrum of *m/z* 1078.7 showed product ions at *m/z* 1059.3 and 1276.3, corresponding to the a₄-B₄ and w₄ ions for 5'-p TCCATGA-3', respectively (Figure 4C). MS/MS spectra of the major primer extension products (5'-p TCTATGA-3', 5'-p TCAATGA-3', 5'-p TCCATGA-3', and 5'-p TCA_TGA-3') are shown in Figure 4. Overall, the HPLC-ESI-MS/MS results confirmed that polymerase bypass of (S)-N⁶-HB-dA I by human polymerases is mainly error-free, while (R,R)-N⁶,N⁶-DHB-dA adducts are highly mispairing, resulting in predicted A $\rightarrow$ G transitions, A $\rightarrow$ T and A $\rightarrow$ C transversions, and deletions (Schemes 3 and 4). In general, HPLC-ESI-MS/MS results were consistent with kinetic results shown in Table 1, although several differences have been noted. This is not surprising because the steady-state kinetic experiments shown in Table 1 characterize the kinetics of single nucleotide incorporation opposite a lesion, while the formation of full-length extension products detected by HPLC-

ESI⁻-MS/MS requires post-lesion synthesis steps. As discussed above, these extension steps can be rate-limiting, affecting the identity of the products detected by mass spectrometry.

DISCUSSION

BD and its epoxide metabolites induce A→G, A→T, and A→C mutations,^{25,27,51–54} but the structural origins of these genetic changes remain to be established. BD gives rise to a large range of DNA adducts including 1-hydroxy-3-buten-2-yl and 2,3,4-trihydroxybut-1-yl monoadducts, exocyclic lesions, DNA-DNA cross-links, and DNA-peptide and DNA-protein conjugates. Polymerase bypass of synthetic DNA templates containing N⁶-THB-adenine monoadducts showed that these adducts induced very low levels of A → G and A → C base substitutions (< 0.3%).⁴³ N¹-Trihydroxybutyl-dI and N¹-(1-hydroxy-3-buten-2-yl)-dI lesions originating from deamination of the corresponding N¹-dA adducts and 1,4-*bis*-(2'-deoxyadenosin-N⁶-yl)-butane-2,3-diol (*bis*-N⁶A-BD) cross-links were strongly mispairing,^{43,55} but have not yet been detected *in vivo*. Glutathione-derived S-[4-(N⁶-deoxyadenosinyl)-2,3-dihydroxybutyl]glutathione induced A→G mutations.^{56,57} N⁶-HB-dA II adducts (Scheme 1) were not miscoding in either *in vitro* and *in vivo* assays.⁴³

Recent studies in our laboratory identified several novel exocyclic adducts of DEB, including N⁶,N⁶-(2,3-dihydroxybutan-1,4-diyl)-2'-deoxyadenosine (N⁶,N⁶-DHB-dA), N⁶-(2-hydroxy-3-hydroxymethyl-propan-1,3-diyl)-2'-deoxy-adenosine (1,N⁶-γHMHP-dA), and 1,N⁶-(1-hydroxymethyl-2-hydroxypropan-1,3-diyl)-2'-deoxyadenosine (1,N⁶-α HMHP-dA). Primer extension past (R,S)-1,N⁶-γHMHP-dA in the presence of human lesion bypass Pols η and κ gave rise to large numbers of A and G insertions (corresponding to A→T and A→C transversions) and deletions.⁴⁹ In the present study, *in vitro* translesion synthesis studies were extended to another exocyclic adduct of DEB ((R,R)-N⁶,N⁶-DHB-dA). To our knowledge, this is the first example of an exocyclic *bis*-N⁶,N⁶-dA DNA adduct, and therefore its effects on DNA replication were of significant interest. For comparison, the corresponding monoalkylated (S)-N⁶-HB-dA I adducts were also included in the study.

Our primer extension studies included recombinant human DNA polymerases β, η, ι, and κ. Among these, hPol β belongs to the X family of DNA Polymerases and is characterized by relatively high fidelity.¹⁵ Polymerases η, ι, and κ are Y-family polymerases involved in translesion synthesis. These three enzymes are structurally distinct and exhibit different adduct specificities.¹⁰ For example, bulky O⁶-[4-oxo-4-(3-pyridyl)butyl] guanine (O⁶-POB-G) adducts are bypassed by hPol η, but not by hPol ι and κ.⁵⁸ Bulky *anti*-N²-BPDE (7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene) adducts are bypassed by hPol κ.¹² (R,S)-1,N⁶-γHMHP-dA adducts examined in our earlier work were bypassed by all three polymerases, but with different efficiency and fidelity.⁴⁹ Additionally, S-[4-(N⁶-deoxyadenosinyl)-2,3-dihydroxybutyl] glutathione (N⁶dA-(OH)₂ butyl-GSH) and N⁶-(2,3,4-trihydroxybutyl) deoxyadenosine (N⁶-THB-dA) adducts were also bypassed by hpols η, ι, and κ.⁵⁷ In the present work, we were interested in evaluating whether human TLS polymerases η, ι, and κ have the ability to bypass (S)-N⁶-HB-dA I and (R,R)-N⁶,N⁶-DHB-dA adducts induced by BD in an error-free or error-prone manner.

We found that all four polymerases examined (β , η , ι , and κ) catalyzed error-free replication past monosubstituted N^6 -HB-dA I lesions (Figures 1–2), with the correct nucleobase (dT) incorporated preferentially opposite the lesion (Table 1). Among the three TLS polymerases evaluated, the efficiency of primer extension past N^6 -HB-dA I was hPol κ > hPol η > hPol ι (Figure 1), while the efficiency of incorporation of correct base (T) opposite N^6 -HB-dA I was hPol κ > hPol ι > hPol η (Table 1). HPLC-ESI[−]-MS/MS sequencing of primer extension products confirmed that replication past N^6 -HB-dA I was largely error free (Schemes 3 and 4). These results are consistent with previous studies for structurally analogous adducts such as N^6 -HB-dA II, N^6 -THB-dA, and N^6 -dA adducts of styrene oxide.^{43,59} Additionally, hpols η , ι , and κ ; Pol T7, and Dpo4 were able to bypass N^6 -THB-dA adduct in an efficient and error-free manner.⁵⁷

Among all N^6 -adenine adducts derived from epoxide metabolites of BD (Scheme 1), N^6 -HB-dA I and II are the least mutagenic, probably because they retain one of the N^6 -hydrogens, allowing for correct hydrogen bonding with dT. Structural studies of (*S*)- N^6 -HB-dA I in 5'-d(C¹G²G³A⁴C⁵Y⁶A⁷G⁸A⁹A¹⁰G¹¹)-3':5'-d(C¹²T¹³T¹⁴C¹⁵T¹⁶T¹⁷G¹⁸T¹⁹C²⁰C²¹C²²)-3' using high field NMR (Y⁶ = (*S*)- N^6 -HB-dA I)⁶⁰ have revealed that the adduct is accommodated in the major groove of the DNA, maintaining standard Watson-Crick base pairing with T¹⁷.⁶⁰ Although (*S*)- N^6 -HB-dA I (Y⁶) did not stack well with its 5' neighbor (C⁵), it stacked normally with its 3' neighbor (A⁷), thereby leading to only minor perturbations in the overall DNA structure. Structurally analogous N^6 -THB-dA and N^6 -dA adducts are also readily accommodated in the DNA major groove, maintaining Watson-Crick base pairing.^{61–63}

In contrast, none of the DNA polymerases examined in this study were able to catalyze efficient replication past (*R,R*)- N^6,N^6 -DHB-dA (Table 1). (*R,R*)- N^6,N^6 -DHB-dA adducts completely blocked DNA replication by hPol β (Figures 1–2) and required high concentrations of hPol η and κ to achieve lesion bypass. However, our experiments shown in Figure S2 provide preliminary evidence that hPol ι and hPol η may act cooperatively to allow for bypass of (*R,R*)- N^6,N^6 -DHB-dA lesions, with hPol ι acting as an inserter polymerase and hPol η catalyzing post-lesion synthesis.

Steady-state kinetic studies revealed that hPol η and κ incorporated both correct and incorrect nucleotides opposite N^6,N^6 -DHB-dA (Table 1). The efficiency of insertion of correct base (T) opposite N^6,N^6 -DHB-dA by hPol η and κ was reduced by ~400- to 800-fold as compared to unmodified dA (Table 1). Similar decreases in efficiency (up to 1000-fold) have been previously reported for the incorporation of dTMP opposite 1, N^6 -ethenodeoxyadenosine by hPol κ .⁶⁴ The order of efficiency for incorporation of correct base (T) opposite (*R,R*)- N^6,N^6 -DHB-dA was hPol ι > hPol κ > hPol η (Table 1). HPLC-ESI[−]-MS/MS sequencing of the extension products generated in the presence of hpols η and κ identified a number of misincorporation and -1 and -2 deletion products (Schemes 3 and 4). If these events also take place *in vivo*, they would result in A→G, A→T, A→C, and frameshift mutations.

An NMR structure of (*R,R*)- N^6,N^6 -DHB-dA in the 5'-d(C¹G²G³A⁴C⁵Y⁶A⁷G⁸A⁹A¹⁰G¹¹)-3' : 5'-d(C¹²T¹³T¹⁴C¹⁵T¹⁶T¹⁷G¹⁸T¹⁹C²⁰C²¹C²²)-3'

duplex where $\mathbf{Y}^6 = (R,R)\text{-}N^6,N^6\text{-DHB-dA}$ adduct opposite $\mathbf{T}^{17}$ reveals that the adduct was accommodated in the major groove of DNA, with the 3,4-dihydroxypyrrrolidine (DHB) moiety adopting an out-of-plane rotation about the C6- N^6 bond.⁶⁵ The adduct maintained an *anti* conformation about the glycosidic bond. The DHB moiety was out of plane, and the complementary $\mathbf{T}^{17}$ remained stacked within the duplex, forming a single hydrogen between the N1-imine nitrogen of $(R,R)\text{-}N^6,N^6\text{-DHB-dA}$ and the N3H imino proton of $\mathbf{T}^{17}$.⁶⁵ There was no hydrogen bonding between the N^6 of $(R,R)\text{-}N^6,N^6\text{-DHB-dA}$ and the O4 atom of the complementary thymine, leading to decreased thermal stability of the duplex. Furthermore, there was no evidence for the presence of any other hydrogen bonding between the hydroxyl groups of DHB and the complementary $\mathbf{T}^{17}$. The presence of an $N^6,N^6\text{-DHB}$ substituent on adenine led to the loss of base stacking interactions with the 5' neighboring C^5 and interrupted the base stacking interaction between the complementary $\mathbf{T}^{17}$ and 3' neighbor G^{18} . Because $N^6,N^6\text{-DHB-dA}$ is able to form only one hydrogen bond with dT, TLS polymerases cannot discriminate between individual dNTPs, leading to incorporation of any of the four dNTPs opposite this lesion. This is consistent with studies by Zhang et al. showing that double alkylation of the exocyclic amino group of $N^2,N^2\text{-dimethyl G}$ results in mispairing with dA, dT, and dG.⁶⁶ Interestingly, hpol κ and Pol T7 misincorporated dC opposite bulky $N^6\text{dA-(OH)}_2$ butyl-GSH adducts, while the polymerase bypass by hpol η and ι opposite this lesion was error-free.⁵⁷

By comparison with $(R,S)\text{-}1,N^6\text{-}\gamma\text{HMHP-dA}$ investigated in our earlier study,⁴⁹ $(R,R)\text{-}N^6,N^6\text{-DHB-dA}$ represents a stronger block to DNA replication. If bypassed, both $1,N^6\text{-}\gamma\text{HMHP-dA}$ and $N^6,N^6\text{-DHB-dA}$ adducts can direct the incorporation of G, A, or T in the presence of hPol η and κ ; but can be replicated in an error-free manner by hPol ι (Table 1). It should be noted, however, that hPol ι incorporated only a single T opposite the exocyclic DEB-dA lesions, requiring another polymerase to complete primer extension.⁴⁹ Our preliminary experiments (Figure S2) suggest that hPol ι may collaborate with hpol κ or hpol η to allow for error-free bypass of $(R,R)\text{-}N^6,N^6\text{-DHB-dA}$ *in vivo*. However, further studies incorporating additional protein factors (e.g. proliferating cell nuclear antigen, PCNA), are needed to establish whether such polymerase switching takes place in cells.⁶⁷

In summary, our primer extension results indicate that monoalkylated $(S)\text{-}N^6\text{-HB-dA}$ I adducts are not mispairing and are unlikely to be responsible for the toxicity and mutations observed following exposure to BD. In contrast, doubly alkylated $(R,R)\text{-}N^6,N^6\text{-DHB-dA}$ lesions are strongly blocking and are capable of inducing A→G transitions, A→T and A→C transversions, and small deletions. Therefore, if formed *in vivo*, $(R,R)\text{-}N^6,N^6\text{-DHB-dA}$ may contribute to BD-mediated toxicity and mutations, along with previously characterized $1,N^6\text{-}\gamma\text{HMHP-dA}$ and $S\text{-}[4\text{-(}N^6\text{-deoxyadenosinyl)-2,3-dihydroxybutyl}]$ glutathione adducts.^{49,57} Future structural studies of ternary complexes involving specific DNA polymerases and primer-template duplexes containing BD-dA adducts are required to improve our understanding of the mechanisms of lesion bypass.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

1,N⁶-α HMHP-dA	1,N ⁶ -(1-hydroxymethyl-2-hydroxypropan-1,3-diyl)-2'-deoxyadenosine
1N⁶-γ HMHP-dA	N ⁶ -(2-hydroxy-3-hydroxymethylpropan-1,3-diyl)-2'-deoxyadenosine
BD	1,3-butadiene
DEB	1,2,3,4-diepoxybutane
EB	3,4-epoxy-1-butene
hPol	human Polymerase
N⁶N⁶-DHB-dA	N ⁶ ,N ⁶ -(2,3-dihydroxybutan-1,4-diyl)-2'-deoxyadenosine
N⁶-HB-dA I	N ⁶ -(2-hydroxy-3-buten-1-yl)-2'-deoxyadenosine
N⁶-HB-dA II	N ⁶ -(1-hydroxy-3-buten-2-yl)-2'-deoxyadenosine
T4-PNK	T4 Polynucleotide kinase
TLS Polymerases	translesion synthesis Polymerases
UDG	uracil DNA glycosylase

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Figure 1.

Standing start primer extension past (*S*)- N^6 -HB-dA I and (*R,R*)- N^6,N^6 -DHB-dA adducts by hPol β (A), hPol ι (B), hPol κ (C) and hPol η (D). ^{32}P -13-mer primer/18-mer template complexes containing the adduct or unmodified dA at the 14th position of the template (50 nM) were incubated in the presence of hPol β (12.5 nM), hPol ι (20 nM), hPol κ (5 nM), or hPol η (10 nM) in the presence of all four dNTPs. The reactions were quenched at designated time points (0–60 minutes), and the products were analyzed by 20% (w/v) PAGE and visualized by phosphorimaging.

Figure 2.

Running start bypass of (*S*)- N^6 -HB-dA I and (*R,R*)- N^6,N^6 -DHB-dA in the presence of hPol β (A) and hPol κ (B). Primer/template complexes (50 nM) were incubated with hPol β (25 nM) and hPol κ (12.5 nM) in the presence of all 4 dNTPs. The reactions were stopped by adding quench solution at specific time points (0–60 minutes). The radiolabeled extension products were separated using 20% (w/v) PAGE and visualized by phosphorimaging. Similar results for hPol η are shown in Supporting Information Figure S1.

Figure 3.

Extracted ion chromatograms of primer extension products formed upon *in vitro* primer extension opposite 18-mer template containing (*R,R*)-*N*⁶,*N*⁶-DHB-dA by hPol κ . 13-mer dU primer/18-mer template duplex (100 pmol) containing (*R,R*)-*N*⁶,*N*⁶-DHB-dA was incubated with hPol κ (40 pmol) and a mixture of all four dNTPs in a reaction buffer for 5 hours. Excess dNTPs were removed by size exclusion chromatography, and the primer was cleaved by UDG followed by piperidine treatment. The cleaved extension products were separated and detected using capillary HPLC-ESI⁺-MS/MS as described.

Figure 4.

MS/MS spectra of the major products formed upon *in vitro* primer extension opposite 18-mer template containing (*R,R*)-*N*⁶,*N*⁶-DHB-dA by hPol κ and hPol η . (A) CID spectra of 5'-p TCTATGA-3', the major product of hPol κ incubation. (B) CID spectra of 5'-p TCAATGA-3', the second major product in both the reaction mixtures (C) CID spectra of 5'-p TCCATGA-3', the major product of hPol η catalyzed primer extension. (D) CID spectra of 5'-p TCA_TGA-3', -1 deletion product that accounted for 18% of replication products following incubation with hPol κ .

Scheme 1.

Formation of BD-induced *N*⁶-deoxyadenosine adducts *N*⁶-HB-dA, *N*⁶,*N*⁶-DHB-dA, and 1,*N*⁶-HMHP-dA from 3,4-epoxy-1-butene (EB) and 1,2,3,4-diepoxybutane (DEB), respectively.

Gel electrophoresis experiments

A. Standing start:

5'-TCA T**X**G AAT CCT TCC CCC-3'
 3'-   C TTA GGA AGG GGG-5'

5'-TCA T**A**G AAT CCT TCC CCC-3'
 3'-   C TTA GGA AGG GGG-5'

B. Running start:

5'-TCA T**X**G AAT CCT TCC CCC-3'
 3'-   GGA AGG GGG-5'

5'-TCA T**A**G AAT CCT TCC CCC-3'
 3'-   GGA AGG GGG-5'

C. LC-MS/MS experiments

5'-TCA T**X**G AAT CCT TCC CCC-3'
 3'-   C T**U**A GGA AGG GGG-5'

5'-TCA T**A**G AAT CCT TCC CCC-3'
 3'-   C T**U**A GGA AGG GGG-5'

Scheme 2.

Oligonucleotide sequences employed in this study.

hPol κ	TTATGA 3'	6%
	CATGA 3'	3%
	AATGA 3'	8%
	TATGA 3'	83%

5' GGGGGAAGGAUTC
 3' CCCCCTTCCTAAG**X**TACT 5'

X=N⁶-HB-dA

__TGA 3'	9%
G_TGA 3'	5%
C_TGA 3'	6%
A_TGA 3'	18%
GATGA 3'	1%
CATGA 3'	4%
AATGA 3'	20%
TATGA 3'	37%

5' GGGGGAAGGAUTC
 3' CCCCCTTCCTAAG**X**TACT 5'

X=N⁶,N⁶-DHB-dA

Scheme 3.

Percentages of primer extension products formed upon in vitro primer extension of (*S*)-N⁶-HB-dA I and (*R,R*)-N⁶,N⁶-DHB-dA containing DNA templates by hPol κ .

hPol η

CATGA	3'	8%
TATGA	3'	92%

5' GGGGGAAGGAUTC
 3' CCCCCTTCCTAAG**X**TACT 5'

X=N⁶-HB-dA

___TGA	3'	<1%
G_TGA	3'	2%
A_TGA	3'	4%
GATGA	3'	8%
TATGA	3'	19%
AATGA	3'	20%
CATGA	3'	46%

5' GGGGGAAGGAUTC
 3' CCCCCTTCCTAAG**X**TACT 5'

X=N⁶,N⁶-DHB-dA

Scheme 4.

Relative percentages of primer extension products formed upon *in vitro* primer extension of (*S*)-N⁶-HB-dA I and (*R,R*)-N⁶,N⁶-DHB-dA containing DNA templates by hPol η .

Table 1

Steady-state kinetic parameters for incorporation of individual nucleotides opposite dA, (S)-N⁶-HB-dA I and (R,R)-N⁶,N⁶-DHB-dA lesions by the four human DNA polymerases hPol κ , η , ι and β . f = misincorporation frequency = $(k_{cat}/K_m)_{incorrect} dNTP / (k_{cat}/K_m)_{correct} dNTP$ (dITP). Fold decrease in efficiency calculated as $(k_{cat}/K_m)_{dNTP} / ((k_{cat}/K_m)_{dITP} \text{ opposite X=dA})$.

Polymerase	Template	dNTP	k_{cat} (min ⁻¹)	K_m (μ M)	k_{cat}/K_m (μ M ⁻¹ min ⁻¹)	f	Fold decrease efficiency
hPol β	dA	T	2.6 $\pm$ 0.5	28 $\pm$ 7	0.09	1	1
	N ⁶ -HB-dA I	T	2.4 $\pm$ 0.4	511 $\pm$ 140	4.8 $\times 10^{-3}$	1	19
	N ⁶ ,N ⁶ -DHB-dA	T	NA	NA	NA	NA	NA
hPol ι	dA	T	1.0 $\pm$ 0.1	21 $\pm$ 2	0.05	1	1
	N ⁶ -HB-dA I	T	0.88 $\pm$ 0.06	83 $\pm$ 13	0.01	1	5
	N ⁶ ,N ⁶ -DHB-dA	T	0.74 $\pm$ 0.08	481 $\pm$ 117	1.5 $\times 10^{-3}$	1	33
hPol κ	dA	T	5.7 $\pm$ 0.6	5.5 $\pm$ 1.8	1.0	1	1
		A	0.87 $\pm$ 0.05	355 $\pm$ 45	2.4 $\times 10^{-3}$	2.4 $\times 10^{-3}$	430
		G	4.3 $\pm$ 0.7	197 $\pm$ 18	0.02	0.02	52
hPol η		T	5.2 $\pm$ 0.5	14 $\pm$ 3	0.38	1	3
		A	0.8 $\pm$ 0.02	153 $\pm$ 12	5.4 $\times 10^{-3}$	0.01	190
		G	1.0 $\pm$ 0.08	180 $\pm$ 35	5.4 $\times 10^{-3}$	0.01	190
hPol κ		C	0.08 $\pm$ 0.01	15 $\pm$ 7	5.5 $\times 10^{-3}$	0.01	190
hPol η		T	0.35 $\pm$ 0.04	153 $\pm$ 37	2.3 $\times 10^{-3}$	1	450
		A	0.35 $\pm$ 0.03	286 $\pm$ 67	1.2 $\times 10^{-3}$	0.53	870
		G	0.45 $\pm$ 0.06	455 $\pm$ 119	1.0 $\times 10^{-3}$	0.43	1040
hPol η		C	0.29 $\pm$ 0.03	170 $\pm$ 38	1.7 $\times 10^{-3}$	0.76	610
hPol η		T	2.4 $\pm$ 0.19	1.8 $\pm$ 0.53	1.3	1	1
		A	0.60 $\pm$ 0.02	51 $\pm$ 7	0.01	8.8 $\times 10^{-3}$	130
		G	0.52 $\pm$ 0.07	85 $\pm$ 27	6.0 $\times 10^{-3}$	4.6 $\times 10^{-3}$	220
hPol η		T	2.60 $\pm$ 0.19	31 $\pm$ 7	0.08	1	16
		A	0.15 $\pm$ 0.02	112 $\pm$ 31	1.4 $\times 10^{-3}$	0.02	940

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Polymerase	Template	dNTP	k_{cat} (min ⁻¹)	K_m (μM)	k_{cat}/K_m (μM ⁻¹ min ⁻¹)	f	Fold decrease efficiency
N ⁶ ,N ⁶ -DHB-dA		G	0.07 ± 0.01	177 ± 36	0.4 × 10 ⁻³	4.6 × 10 ⁻³	3300
		C	0.25 ± 0.02	178 ± 30	1.4 × 10 ⁻³	0.02	940
		T	0.13 ± 0.02	79 ± 17	1.7 × 10 ⁻³	1	780
		A	0.12 ± 0.01	72 ± 14	1.6 × 10 ⁻³	0.98	830
		G	0.31 ± 0.02	115 ± 19	2.7 × 10 ⁻³	1.6	490
		C	0.25 ± 0.02	140 ± 32	1.8 × 10 ⁻³	1.1	730