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Pre-Steady-State Kinetic Investigation of Bypass of a Bulky Guanine Lesion by Human Y-family DNA Polymerases

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Abstract

3-Nitrobenzanthrone (3-NBA), a byproduct of diesel exhaust, is highly present in the environment and poses a significant health risk. Exposure to 3-NBA results in formation of *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG^{C8-NABA}), a bulky DNA lesion that is of particular importance due to its mutagenic and carcinogenic potential. If not repaired or bypassed during genomic replication, dG^{C8-NABA} can stall replication forks, leading to senescence and cell death. Here we used pre-steady-state kinetic methods to determine which of the four human Y-family DNA polymerases (hPol η , hPol κ , hPol ι , or hRev1) are able to catalyze translesion synthesis of dG^{C8-NABA} *in vitro*. Our studies demonstrated that hPol η and hPol κ most efficiently bypassed a site-specifically placed dG^{C8-NABA} lesion, making them good candidates for catalyzing translesion synthesis (TLS) of this bulky lesion *in vivo*. Consistently, our publication (*Biochemistry* 53, 5323–31) in 2014 has shown that small interfering RNA-mediated knockdown of hPol η and hPol κ in HEK293T cells significantly reduces the efficiency of TLS of dG^{C8-NABA}. In contrast, hPol ι and hRev1 were severely stalled by dG^{C8-NABA} and their potential role *in vivo* was discussed. Subsequently, we determined the kinetic parameters for correct and incorrect nucleotide incorporation catalyzed by hPol η at various positions upstream, opposite, and downstream from dG^{C8-NABA}. Notably, nucleotide incorporation efficiency and fidelity both decreased significantly during dG^{C8-NABA} bypass and the subsequent extension step, leading to polymerase pausing and error-prone DNA synthesis by hPol η . Furthermore, hPol η displayed

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nucleotide concentration-dependent biphasic kinetics at the two polymerase pause sites, suggesting that multiple enzyme•DNA complexes likely exist during nucleotide incorporation.

Graphical Abstract

Keywords

3-nitrobenzanthrone; translesion synthesis; human DNA polymerase eta; DNA replication; DNA repair

1. INTRODUCTION

DNA damage caused by prevalent chemical agents in the environment poses significant threats to cell survival and proliferation and represents a major concern for occupational and public health. One molecule in particular, 3-nitrobenzanthrone (3-NBA)^a, a nitro-polycyclic aromatic hydrocarbon (nitroPAH), was discovered in diesel exhaust as bound to particulate air matter¹. 3-NBA is ubiquitously present in the environment, especially in areas exposed to high levels of diesel emissions, such as cities or areas of heavy construction². It has one of the most mutagenic profiles of known pollutants tested in the Ames *Salmonella typhimurium* (TA98) assay and is responsible for inducing mostly G→T transversions^{1;3}. Upon inhalation, 3-NBA is metabolized into various mutagenic and potentially carcinogenic

^a**Abbreviations:** 3-nitrobenzanthrone, 3-NBA; nitro-polycyclic aromatic hydrocarbon, nitroPAH; *Salmonella typhimurium* strain TA98, TA98; *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone, dG^{C8}-*N*-ABA; translesion DNA synthesis, TLS; human DNA polymerase eta, hPol η ; human DNA polymerase kappa, hPol κ ; human DNA polymerase iota, hPol ι ; human Rev1, hRev1; DNA polymerase IV from *Sulfolobus solfataricus*, PolIV; polyacrylamide gel electrophoresis, PAGE; electrophoretic mobility shift assay, EMSA; human embryonic kidney cell line 293T, HEK293T; aminobenzanthrone, ABA; trans 10*S*-benzo[a]pyrene-*N*²-deoxyguanosine, dG^BBPDE; *N*-(deoxyguanosin-8-yl)-1-aminopyrene, dG^{AP}; 8-(deoxyguanosin-*N*²-yl)-1-aminopyrene, dG^{1,8}; small interfering RNA, siRNA; proliferating cell nuclear antigen, PCNA; *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorine, dG^{AAF}.

metabolites that can react with purines in DNA ⁴. These reactions lead to the formation of bulky, aromatic DNA lesions which act as roadblocks to genomic replication. One such product, *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG^{C8-N-ABA}), is of particular interest because it is a major lesion formed in mice after intraperitoneal treatment with 3-NBA ⁵. The bulky lesion dG^{C8-N-ABA}, produced by the electrophilic attack of deoxyguanosine (dG) by a 3-NBA metabolite ⁴ (Figure S1), has been found to severely block replication fork progression ⁶.

During normal DNA replication, highly efficient and faithful DNA polymerases perform the bulk of the work. It is known that replicative DNA polymerases possess tight and selective active sites that allow them to stringently choose against incorrect nucleotides but also limit their abilities to catalyze translesion synthesis (TLS) through damaged template nucleotides ^{7; 8}. When a damaged site is encountered, replicative polymerases are stalled due to a significantly slowed nucleotide incorporation activity and a competing 3'→5' exonuclease activity, resulting in futile cycles of DNA synthesis and excision⁹. In contrast, Y-family DNA polymerases, which lack proofreading activity and possess comparatively flexible and solvent accessible active sites, are more suited to accommodate DNA lesions and catalyze TLS ¹⁰. Notably, the human genome encodes four Y-family members: DNA polymerase eta (hPol η), kappa (hPol κ), iota (hPol ι), and hRev1. These human polymerases vary in their lesion bypass capabilities and are differentially recruited to damaged DNA to catalyze TLS depending on the lesion that is present ¹⁰. In this study, we sought to determine which of the human Y-family DNA polymerase(s) are able to efficiently bypass dG^{C8-N-ABA} *in vitro*. Previously we utilized pre-steady-state kinetic methods to study the bypass of dG^{C8-N-ABA} catalyzed by a model Y-family polymerase, *Sulfolobus solfataricus* DNA Polymerase IV (Dpo4) ¹¹. We observed that Dpo4 is significantly less efficient and faithful during dG^{C8-N-ABA} bypass and the subsequent extension compared to undamaged DNA, implicating the mutagenic effect of this lesion *in vivo*. Here we applied similar methods to determine the most active human Y-family polymerase for dG^{C8-N-ABA} bypass and establish a kinetic mechanism for TLS of dG^{C8-N-ABA}. Initially, running start assays were performed to study the effect that a site-specifically placed, bulky dG^{C8-N-ABA} lesion had on each human Y-family polymerase. Our results indicate that hPol η is a likely candidate to catalyze TLS of dG^{C8-N-ABA} *in vivo*. Subsequently, we performed single-turnover kinetic assays to determine the DNA binding affinities (K_d , DNA), maximum nucleotide incorporation rate constants (k_p), apparent nucleotide binding affinities (K_d , dNTP), and the substrate specificities (k_p/K_d , dNTP) for nucleotide incorporations by hPol η at sites upstream, opposite, and downstream from the lesion. Finally, biphasic kinetic assays were performed to establish a minimal kinetic mechanism for nucleotide incorporation opposite dG^{C8-N-ABA} and the subsequent extension of the lesion bypass product.

2. MATERIALS AND METHODS

2.1 Materials

All reagents were purchased from the following companies: OptiKinase from United States Biochemical, [γ -³²P] ATP from Perkin-Elmer, Biospin-6 columns from Bio-Rad Laboratories, and dNTPs from Bioline. Full-length hPol η and truncated fragments of hPol κ

(9–518), hPol η (1–420), and hRev1 (341–829) were overexpressed and purified as described previously¹².

2.2 DNA Substrates

A DNA template (26-mer) containing a dG^{C8-N-ABA} lesion at the 21st position was synthesized as described previously¹¹. DNA containing the dG^{C8-N-ABA} lesion was kept in dark conditions to preserve the light-sensitive substrate. All other synthetic oligonucleotides were purchased from Integrated DNA Technologies (IDT) and gel purified by 17% denaturing polyacrylamide gel electrophoresis (PAGE). Primers were 5'-radiolabeled by incubating with [γ -³²P]-ATP and OptiKinase for 3 hr, followed by heat inactivation (95 °C for 2 min), and isolation of radiolabeled DNA using a Biospin-6 column to remove excess, unreacted [γ -³²P]-ATP. A primer was annealed to a template at a 1:1.35 ratio. Annealing solutions containing undamaged DNA and dG^{C8-N-ABA}-containing DNA were heated to 95 and 72 °C, respectively, for 5 minutes, followed by slow cooling to room temperature. All DNA substrates used in this study are listed in Table 1.

2.3 Reaction Buffers and Assay Analysis

All chemical quench assays were performed with a KinTek Rapid Chemical Quench-Flow apparatus at 37 °C in buffer R (50 mM HEPES, pH 7.5 at 37 °C, 50 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 5 mM DTT, and 0.1 mg/mL Bovine Serum Albumin (BSA)). Electrophoretic mobility shift assays (EMSAs) were performed at 23 °C in buffer S (50 mM Tris-HCl, pH 7.5 at 23 °C, 50 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 5 mM DTT, and 0.1 mg/mL BSA). All concentrations listed were final upon mixing and all gels were scanned using a TyphoonTrio (GE Healthcare). Quantification was carried out using ImageQuant (GE Healthcare) and Kaleidagraph (Synergy) software.

2.4 Running Start Assays

A pre-incubated solution of hPol η (100 nM), hPol κ (100 nM), hPol ι (1 μ M), or hRev1 (1 μ M) and 5'-[³²P]-17/26-mer (100 nM) was rapidly mixed with all four dNTPs (200 μ M each). After various incubation periods, the samples were quenched with the addition of 0.37 M EDTA and separated by 17% PAGE (8 M Urea, 1X TBE). Analysis and quantification of gels were performed as described in *Reaction Buffers and Assay Analysis*. The relative lesion bypass efficiencies (bypass %) were calculated for each polymerase as a function of reaction time. For each time point t , the bypass % was calculated as a ratio of bypass to encounter events (Eq. 1) as described previously^{12; 13; 14}.

$$\text{dG}^{\text{C8-N-ABA}} \text{ bypass \%} = (\text{B}/\text{E}) \times 100 = [\text{B}/([\text{20-mer}] + \text{B})] \times 100 \quad \text{Eq. 1}$$

Quantification of running start assays (Figure 1A–H) was achieved by treating the total encounter (E) events as equivalent to the concentration of 20-mer, and bypass (B) events as equivalent to the sum of the concentrations of all products with sizes greater than or equal to the 21-mer. The bypass % was calculated using Eq. 1 then plotted against reaction times, t . To compare the relative amount of time it took for each polymerase to bypass dG^{C8-N-ABA},

the t_{50} bypass was determined as the time to bypass of 50% of the total lesions encountered. In a similar fashion, the t_{50} was determined as the time to bypass 50% of the undamaged dG base.

2.5 Electrophoretic Mobility Shift Assays

hPol η (2–650 nM) was titrated into solutions containing 5'-[32 P]-labeled DNA (10 nM) and allowed to equilibrate in buffer S for 15 min at room temperature (23 °C). The samples were separated using 4.5% native PAGE which were analyzed and quantified as described in *Reaction Buffers and Assay Analysis*. The concentration of hPol η •DNA complex was plotted against the total concentration of hPol η and the data were fit to a quadratic equation (Eq. 2):

$$[E \bullet DNA] = 0.5(K_{d,DNA} + E_0 + D_0) - 0.5[(K_{d,DNA} + E_0 + D_0)^2 - 4E_0D_0]^{1/2} \quad \text{Eq. 2}$$

Where E_0 is the initial enzyme concentration, D_0 is the initial DNA concentration, and $K_{d,DNA}$ is the apparent equilibrium dissociation constant for the hPol η •DNA complex.

2.6 Standing Start Assay for hRev1

A pre-incubated solution of hRev1 (200 nM) and 5'-[32 P]-labeled undamaged (20/26-mer) or damaged (20/26-mer-dG^{C8-N-ABA}) DNA (200 nM) in buffer R was mixed with dATP, dCTP, dGTP, dTTP, or all four nucleotides (200 μ M each). After 10 min, the reaction was quenched with the addition of 0.37 M EDTA and the samples were separated using 17% denaturing PAGE.

2.7 Single-Turnover Assays

A pre-incubated solution of 5'-[32 P]-labeled DNA (20 nM) and hPol η (130 nM) in buffer R was rapidly mixed with varying concentrations of dNTP (10 – 1200 μ M). After various incubation times, the reaction was quenched with the addition of 0.37 M EDTA. The samples were then analyzed using 17% denaturing PAGE. Product concentration was plotted against time and the data were fit to a single-exponential equation (Eq. 3).

$$[\text{Product}] = A[1 - \exp(-k_{\text{obs}}t)] \quad \text{Eq. 3}$$

Where k_{obs} is the observed rate constant of nucleotide incorporation and A is the reaction amplitude. The k_{obs} values obtained using Eq. 3 were plotted against the respective dNTP concentration and the data were fit to a hyperbolic equation (Eq. 4)

$$k_{\text{obs}} = k_p[\text{dNTP}] / ([\text{dNTP}] + K_{d,\text{dNTP}}) \quad \text{Eq. 4}$$

Where k_p is the maximum nucleotide incorporation rate constant and $K_{d,\text{dNTP}}$ is the apparent equilibrium dissociation constant for the binding of dNTP to hPol•DNA.

2.8 Biphasic Assays

Biphasic assays were performed by rapidly mixing a pre-incubated solution of hPol η (120 nM) and 5'-[^{32}P]-labeled DNA (30 nM) with various concentrations of dNTP (25–1,000 μM) and excess, unlabeled DNA trap (5 μM). After various incubation periods, reactions were quenched with the addition of 0.37 M EDTA and samples were separated using 17% denaturing PAGE. Product concentration was plotted against time and the data were fit to a double-exponential equation (Eq. 5)

$$[\text{Product}] = A_f[1 - \exp(-k_f t)] + A_s[1 - \exp(-k_s t)] \quad \text{Eq. 5}$$

Where A_f and A_s are the reaction amplitudes of the fast and slow phases, respectively, and k_f and k_s are the rate constants of the fast and slow phases, respectively.

3. RESULTS

3.1 Measuring Relative Polymerase Efficiency during dG^{C8-N-ABA} Bypass

To test if each individual human DNA polymerase could bypass a single dG^{C8-N-ABA} lesion, running start assays were performed by rapidly mixing pre-incubated hPol η , hPol κ , hPol ι , or hRev1 and undamaged 17/26-mer or damaged 17/26-mer-dG^{C8-N-ABA} containing a site-specifically placed dG^{C8-N-ABA} (Table 1) with all four dNTPs. The lesion stalled DNA synthesis by each of the Y-family polymerases, but the degree of stalling as well as the positions at which stalling was observed varied between each polymerase. For hPol η , moderate pausing was observed at the 19-mer (–2), 20-mer (–1), 21-mer (0), and 22-mer (+1) positions. Significant accumulation of the 20-mer product indicated that incorporation of nucleotide at the bypass step (Position –1) was the most problematic for hPol η (Figure 1B). Despite this stalling, the full-length product appeared after 6 s, which was only 2-fold longer than the time required for hPol η to synthesize the full-length product with the undamaged 17/26-mer. hPol κ stalled significantly during the bypass and extension steps, indicated by the 20-mer and 21-mer accumulation at these positions (Figure 1D). hPol κ produced the full length products within 1 s with the undamaged 17/26-mer and took only 3-fold longer (3 s) with the 17/26-mer-dG^{C8-N-ABA} substrate, indicating that hPol κ was similarly efficient as hPol η when bypassing dG^{C8-N-ABA}. The observed accumulation of the 25-mer product is likely caused by polymerase slippage and template repositioning within the 5'-dC-rich region of the template (Table 1), resulting in the “–1” frameshift^{15; 16}. Additionally, the full length product (25-mer) produced by this mechanism was likely further extended via a blunt-end addition^{17; 18}. Interestingly, the bulky lesion caused significant stalling of hPol ι and hRev1 with no appearance of the full-length product even after long reaction periods (3 and 10.5 hr, respectively). With 17/26-mer-dG^{C8-N-ABA}, hPol ι synthesized the bypass and extension products after 300 and 3,900 s, respectively, which are 30- and 65-fold, respectively, longer than with the undamaged 17/26-mer (Figure 1E–F). In comparison, hRev1 synthesized the lesion bypass product within 1 hr but was unable to extend it even after 10.5 hr. The failure of hRev1, a dCTP polymerase, to efficiently catalyze TLS of dG^{C8-N-ABA} was likely caused by multiple template-independent misincorporations

of dCTP by hRev1¹⁹, resulting in DNA distortion. This is supported by the fact that hRev1 was not able to synthesize the full-length product even with the undamaged DNA substrate (Figure 1H). The results of the running start assay with hRev1 prompted us to perform standing start primer extension assays to qualitatively evaluate the efficiency and fidelity of hRev1 to incorporate a nucleotide directly opposite from dG^{C8-N-ABA}. A pre-incubated solution of hRev1 and 20/26-mer-dG^{C8-N-ABA} (or 20/26-mer) was mixed and reacted with a solution containing either dATP, dCTP, dGTP, dTTP, or all four dNTPs. Interestingly, hRev1 showed greater selectivity with damaged than with undamaged DNA by predominantly incorporating dCTP (Figure S1). With undamaged DNA, hRev1 incorporated all four nucleotides with a preference of dCTP > dATP = dGTP = dTTP.

To provide a comparison for the amount of time it took for each human Y-family polymerase to bypass dG^{C8-N-ABA}, gels from the running start assays (Figure 1 A–H) were quantified and Eq. 1 was used to calculate values for t_{50} and $t_{50\text{bypass}}$ which represent the times it took to elongate 50% of the 20-mer to the 21-mer or longer products with undamaged and damaged DNA, respectively. The t_{50} and $t_{50\text{bypass}}$ times measured for each of the Y-family polymerases is summarized in Table 2. Notably, hPol η and hRev1 did not bypass 50% of the dG^{C8-N-ABA} lesion in the allowed reaction time for the running start assays. In contrast, the relatively short $t_{50\text{bypass}}$ times for hPol η (13 s) and hPol κ (7 s) suggest a potential role involving these two polymerases in the bypass and/or extension of dG^{C8-N-ABA} lesions *in vivo*. Consistently, a recent publication reports ~25 and ~30% efficiency reductions in TLS of dG^{C8-N-ABA} in human embryonic kidney (HEK293T) cells when hPol η and hPol κ , respectively, were subject to gene knockdown²⁰. Based on the slightly smaller perturbation of DNA synthesis by hPol η in the presence of dG^{C8-N-ABA}, we decided to use pre-steady-state kinetic methods to initially characterize the efficiency and fidelity of TLS of dG^{C8-N-ABA} catalyzed by hPol η . However, it is apparent based on both the running start and HEK293T cell-based assays that hPol κ is also potentially involved in TLS of dG^{C8-N-ABA} *in vivo*. Accordingly, we are currently performing similar kinetic studies to determine the effect of dG^{C8-N-ABA} on nucleotide incorporation kinetics with hPol κ .

3.2 DNA Binding Affinity Estimated through Electrophoretic Mobility Shift Assays

To determine the apparent equilibrium dissociation constant for the binding of hPol η to DNA containing dG^{C8-N-ABA} ($K_{d, \text{DNA}}$), EMSAs were performed. A representative gel image and a plot for an EMSA experiment are shown in Figure S2 for hPol η binding to 21/26-mer-dG^{C8-N-ABA}. For all DNA substrates tested, the $K_{d, \text{DNA}}$ values were within a 2-fold range for the corresponding undamaged and damaged DNA substrates (Figure S3D and Table 3). Interestingly, the binding affinities of hPol η for 19/26-mer-dG^{C8-N-ABA} and 20/26-mer-dG^{C8-N-ABA} ($K_{d, \text{DNA}} = 12 \pm 2$ nM for both) were 2-fold tighter than the corresponding undamaged DNA substrates ($K_{d, \text{DNA}} = 25 \pm 5$ nM; 23 ± 2 nM). Based on our ternary crystal structure of hPol η in complex with dG^{C8-N-ABA}-DNA and dCTP²¹, the slight increase in binding affinity likely arises from positioning of the ABA moiety of dG^{C8-N-ABA} within a hydrophobic cleft in the hPol η active site thereby stabilizing the conformation of the damaged base. Similar effects are observed in the crystal structures of Dpo4 with dG^{BPDE} and dG^{AP}^{22; 23}. Overall, hPol η binds to dG^{C8-N-ABA}-containing DNA tightly enough such that it does not significantly contribute to the polymerase pausing pattern observed in the

running start assay (Figure 1B). Similar results have been obtained for dG^{AP} bypass by hPol η ¹³ as well as dG^{AP}, dG^{C8-N-ABA}, and abasic site bypass by Dpo4^{11; 24; 25}.

3.3 Determination of Kinetic Parameters for Nucleotide Incorporations upstream, opposite, and downstream from dG^{C8-N-ABA}

Since DNA binding did not significantly affect TLS of dG^{C8-N-ABA} by hPol η , it is possible that the stalling of hPol η during TLS (Figure 1B) was caused by decreased nucleotide binding affinity and incorporation efficiency. To examine this hypothesis, we utilized single-turnover assays to measure the apparent equilibrium dissociation constant for nucleotide binding (K_d , dNTP) and the maximum rate constant of nucleotide incorporation (k_p) at positions upstream, downstream, and opposite from dG^{C8-N-ABA}. By varying the primer length, we were able to measure the kinetic parameters at specific positions along the template strand relative to dG^{C8-N-ABA}, and thus determine the effect of dG^{C8-N-ABA} on the efficiency and fidelity of nucleotide incorporation during each step of TLS catalyzed by hPol η . Briefly, a pre-incubated solution of 5'-[³²P]-labeled DNA (20 nM, Table 1) and hPol η (130 nM) was rapidly mixed with a single dNTP at varying concentrations. Reactions were analyzed as described (Materials and Methods), and the data were fit to Eq. 3 to yield an observed rate constant of incorporation (k_{obs}). The k_{obs} values were then plotted against the respective dNTP concentrations and fit to a hyperbolic equation (Eq. 4) to yield the k_p and K_d , dNTP (Figure 2). hPol η showed a decrease in nucleotide incorporation efficiency (k_p/K_d , dNTP) at positions upstream, opposite, and downstream from the lesion (Position -3, -2, -1, 0, +1). With 18/26-mer-dG^{C8-N-ABA} at Position -3, hPol η correctly incorporated dCTP with a k_p of 66 ± 1 s⁻¹, although the dCTP binding affinity was weakened by 4-fold (K_d , dCTP = 200 ± 11 μ M) relative to undamaged 18/26-mer. As a result, the polymerase efficiency (k_p/K_d , dCTP = 3.3×10^{-1} s⁻¹ μ M⁻¹) was only slightly reduced compared to the value with 18/26-mer (k_p/K_d , dCTP = 1.5 s⁻¹ μ M⁻¹). Similar experiments were carried out with both undamaged and damaged DNA substrates containing primers of 19-mer (Position -2), 20-mer (Position -1), 21-mer (Position 0), and 22-mer (Position +1) and the kinetic parameters are listed in Tables 3 and S1. The most significant stalling occurred during incorporation opposite dG^{C8-N-ABA} (Position -1), caused by a 25-fold decrease in k_p (1.9 ± 0.1 s⁻¹) and a 4-fold increase in K_d , dCTP (325 ± 44 μ M) which resulted in an overall reduction in efficiency by approximately 100-fold (k_p/K_d , dCTP = 5.8×10^{-3} s⁻¹ μ M⁻¹) with 20/26-mer-dG^{C8-N-ABA} compared to undamaged 20/26-mer (k_p/K_d , dCTP = 5.6×10^{-1} s⁻¹ μ M⁻¹). At the extension step (Position 0), hPol η displayed a k_p that was 49-fold slower (0.8 ± 0.03 s⁻¹) but surprisingly bound the incoming dGTP more tightly (K_d , dGTP = 28 ± 5 μ M). With 21/26-mer-dG^{C8-N-ABA}, hPol η showed an approximate 5-fold increase in efficiency (k_p/K_d , dGTP = 2.8×10^{-2} s⁻¹ μ M⁻¹) compared to the lesion bypass step (Position -1). With 22/26-mer-dG^{C8-N-ABA} (Position +1), the efficiency of hPol η was similar (k_p/K_d , dCTP = 4.2×10^{-2} s⁻¹ μ M⁻¹) to that measured at position -2 (Table 4) which is indicated by similarly moderate accumulation at these positions (Figure 1B).

Previously, it has been shown that dG^{C8-N-ABA} bypass carried out by hPol η is error-prone in HEK293T cells²⁰. Thus, we investigated the effect of dG^{C8-N-ABA} on the fidelity of hPol η during dG^{C8-N-ABA} bypass and the subsequent extension step. The fidelity of hPol η , defined as $(k_p/K_d, \text{dNTP})_{\text{incorrect}} / [(k_p/K_d, \text{dNTP})_{\text{correct}} + (k_p/K_d, \text{dNTP})_{\text{incorrect}}]$, was calculated for the

bypass (20/26-mer-dG^{C8-N-ABA}) and extension (21/26-mer-dG^{C8-N-ABA}) steps by using the single-turnover kinetic data of both correct and three incorrect nucleotide incorporations (Table 4). At these two steps, the fidelity of hPol η (Table 4) was determined to be in the ranges of 4.8×10^{-2} to 1.6×10^{-1} and 4.9×10^{-2} to 1.9×10^{-1} , respectively. In comparison, the fidelity of hPol η with undamaged DNA was previously determined to be in the range of 10^{-2} to 10^{-3} (Table S1) by pre-steady-state kinetics¹³ and this range is consistent with the values determined using phage plaque assays^{26; 27}. Thus, the presence of dG^{C8-N-ABA} significantly reduced the fidelity of hPol η at both the lesion bypass and subsequent extension steps, especially during misincorporations of dGTP (29-fold) and dATP (33-fold) opposite dG^{C8-N-ABA} (Table 4). For these two TLS steps, nucleotide incorporation probabilities, given by $[(k_p/K_d)_{\text{damaged DNA}}/\Sigma(k_p/K_d)_{\text{damaged DNA}}] \times 100$, were also calculated. The probability of correct incorporation was reduced from 98% and 96% with undamaged DNA (Table S1) to 77% and 74% with damaged DNA (Table 4) for dG^{C8-N-ABA} bypass and the subsequent extension step, respectively. Notably, with damaged DNA, the probability of dATP misincorporation was 15 and 17% at the bypass and extension steps, respectively, and these probabilities are the highest among all misincorporations (Table 4). Consistently, the G→T transversion was found to occur at a high rate during TLS of dG^{C8-N-ABA} *in vivo*^{5; 20; 28}.

3.4 Determination of Biphasic Kinetic Parameters at Polymerase Pause Sites

Nucleotide incorporation during TLS has often been shown to follow biphasic kinetics. Thus, we performed biphasic kinetic assays as described previously^{13; 24; 25; 29} to test if hPol η followed the same trend during dG^{C8-N-ABA} bypass and the subsequent extension step. A pre-incubated solution of 5'-[³²P]-labeled DNA (30 nM) and hPol η (120 nM) was rapidly mixed with increasing concentrations of a correct nucleotide (25 to 1,000 μ M) and an unlabeled trap DNA substrate 20/26-mer (5 μ M). The trap DNA was added to a 150-fold molar excess over the labeled DNA so that any enzyme that had dissociated from the radiolabeled DNA substrate during the incubation time would be sequestered by the trap DNA. Thus, only labeled DNA product formed during a single enzyme binding event would be observed. The trap DNA concentration (5 μ M) was determined to be sufficiently high enough to sequester any hPol η that had dissociated from 5'-[³²P]-labeled DNA²⁴. During the incorporation of dCTP (1,000 μ M) onto 20/26-mer-dG^{C8-N-ABA}, hPol η indeed followed biphasic kinetics (Figure 3A). The plot of reaction time versus product concentration was fit to Eq. 5 (Materials and Methods) to yield reaction amplitudes of $A_f = 5.8 \pm 0.8$ nM (19%) and $A_s = 8.6 \pm 0.8$ nM (28%) with rate constants of $k_f = 7.2 \pm 1.4$ s⁻¹ and $k_s = 0.42 \pm 0.06$ s⁻¹ for the fast and slow phases, respectively (Table 5). To test if the kinetic parameters were affected by dCTP concentration, similar biphasic kinetic experiments were performed individually with 300 and 600 μ M dCTP. Indeed, as the dCTP concentration increased, the reaction rate constants (k_f and k_s) also increased while the reaction amplitudes remained relatively unchanged (Table 5). In addition, the biphasic kinetic experiments were also performed with 21/26-mer-dG^{C8-N-ABA}. As with 20/26-mer-dG^{C8-N-ABA}, a similar biphasic kinetic trend and a dNTP concentration-dependent increase in the rate constants were observed with 21/26-mer-dG^{C8-N-ABA} during the extension step (Figure 3B and Table 5). Interestingly, the dNTP concentration-dependent increase in the rate constants (k_f and k_s) had been observed previously with Dpo4 when it bypassed the dG^{C8-N-ABA}¹¹ and dG^{1,8}

lesions³⁰. However, our current study is the first one to report this concentration-dependence for lesion bypass catalyzed by a human Y-family DNA polymerase. Notably, kinetic parameters for the DNA trap experiments performed with undamaged 20/26-mer and 21/26-mer DNA are reported in Table S2, showing that even with undamaged DNA substrates, hPol η also displays biphasic kinetics of nucleotide incorporation.

4. DISCUSSION

In this paper, we performed running start assays to determine which of the four human Y-family DNA polymerases were able to most efficiently catalyze TLS of dG^{C8-N-ABA} *in vitro*. Both hPol η and hPol κ were able to bypass dG^{C8-N-ABA}, while hPol ι and hRev1 were completely stalled by the lesion (Figure 1). Consistently, small interfering RNA (siRNA)-mediated knockdown of hPol ι had a minimal effect on overall TLS of dG^{C8-N-ABA} in HEK293T cells, suggesting that hPol ι plays a very minor role during dG^{C8-N-ABA} bypass *in vivo*²⁰. Surprisingly, siRNA-mediated knockdown of hRev1 proved to be detrimental to dG^{C8-N-ABA} bypass in HEK293T cells. Based on its inability to catalyze the bypass of dG^{C8-N-ABA} *in vitro*, hRev1 likely serves as a scaffold protein that synergistically interacts with proliferating cell nuclear antigen (PCNA) and other TLS polymerases to aid in TLS of dG^{C8-N-ABA} and likely other DNA lesions *in vivo*^{13; 31; 32; 33}.

In contrast, hPol η and hPol κ were able to bypass dG^{C8-N-ABA} with similarly small $t_{50\text{bypass}}$ values (Table 2), indicating potential roles for these polymerases in dG^{C8-N-ABA} bypass *in vivo*. Consistently, knockdown of these two polymerases in HEK293T cells significantly reduced the efficiency of TLS of dG^{C8-N-ABA}²⁰. Knockdown of hPol κ resulted in no change in the percentage of mutagenic dG^{C8-N-ABA} bypass events, suggesting that hPol κ carries out relatively error-free TLS of dG^{C8-N-ABA}²⁰. On the other hand, knockdown of hPol η resulted in a 39% decrease in mutation frequency, indicating that the bypass of dG^{C8-N-ABA} by hPol η *in vivo* is error-prone²⁰. Taken together, the bypass of dG^{C8-N-ABA} by hPol η is likely a mechanism through which 3-NBA promotes lesion-induced mutagenesis and tumorigenesis. To determine a kinetic basis for mutagenic bypass of dG^{C8-N-ABA} by hPol η , we utilized pre-steady-state kinetic methods to investigate the effect of a site-specifically placed dG^{C8-N-ABA} on hPol η -catalyzed nucleotide incorporation at and near the lesion.

4.1 Kinetic basis for hPol η pausing caused by dG^{C8-N-ABA}

Overall, the binding affinity of hPol η to DNA (Table 3) is insignificantly affected by the presence of dG^{C8-N-ABA} and therefore does not significantly contribute to the polymerase stalling observed in Figure 1B. Instead, the decrease of nucleotide incorporation rate constants and the weakened nucleotide binding affinities are likely responsible for the observed accumulation of intermediate products. A similar kinetic trend is seen for the conversion of 18-mer→19-mer→20-mer as well as 19-mer→20-mer→21-mer. Based on the kinetic data in Table 4, the first conversion step has a higher rate constant (k_p) and also a higher efficiency ratio (k_p/K_d , dNTP) than the second, less efficient conversion step, leading to the observed accumulation of the 19-mer and 20-mer products (Figure 1B). The 19-mer and 20-mer accumulation was absent at later time points (30–1200 s) due to their eventual conversion to the 20-mer and 21-mer products, respectively. For the reaction series of the

conversion of 20-mer→21-mer→22-mer and 21-mer→22-mer→23-mer, the first conversion step was less efficient than the second conversion step. This kinetic pattern led to accumulation of 20-mer and 21-mer as seen in Figure 1B. Taken together, it is clear that the conversion of 20-mer→21-mer is less efficient than both the preceding and subsequent reactions leading to the strong accumulation of the 20-mer product. For 23-mer, 24-mer, and 25-mer, their accumulation appeared the same in Figure 1B, indicating that the nucleotide incorporation in the consecutive conversion steps associated with these intermediate products were similarly efficient, and that hPol η likely resumed its normal polymerase efficiency after being several positions downstream from the lesion. Thus, the conversion rate and efficiency of 23-mer→24-mer, although not measured here, are likely as high as the conversion of 18-mer→19-mer ($k_p = 66 \text{ s}^{-1}$, $k_p/K_{d, \text{dNTP}} = 0.33 \text{ }\mu\text{M}^{-1}\text{s}^{-1}$) but higher than the conversion of 22-mer→23-mer ($k_p = 9.1 \text{ s}^{-1}$, $k_p/K_{d, \text{dNTP}} = 0.042 \text{ }\mu\text{M}^{-1}\text{s}^{-1}$), contributing to the observed 22-mer accumulation (Figure 1B).

4.2 Kinetic mechanism for dG^{C8-N-ABA} bypass and subsequent extension of the lesion bypass product

Our biphasic kinetic assays revealed that two distinct phases exist when hPol η incorporated correct dNTPs opposite dG^{C8-N-ABA} (Position -1) and one base pair downstream (Position 0) from the lesion. The fast phases were characterized by relatively small reaction amplitudes and fast rate constants while the slow phases had larger reaction amplitudes and slower rate constants (Table 5). Individual contributions of the amplitudes (A_f and A_s) and rate constants (k_f and k_s) were used to calculate the overall nucleotide incorporation rate constant in the presence of the DNA trap. For example, in the presence of 20/26-mer-dG^{C8-N-ABA} and 1,000 μM dGTP (Table 5), the sum of the individual contributions of the fast phase [$(7.2 \text{ s}^{-1}) \times 19\%$] and slow phase [$(0.42 \text{ s}^{-1}) \times 29\%$] gave an overall rate constant of 1.5 s^{-1} . As expected, 1.5 s^{-1} is close to the k_{obs} of 1.4 s^{-1} , estimated using Eq. 4, 1.0 mM dCTP, and corresponding measured k_p and $K_{d, \text{dCTP}}$ values (Table 4), for dCTP incorporation opposite dG^{C8-N-ABA} under single-turnover reaction conditions. Similarly, in the presence of 21/26-mer-dG^{C8-N-ABA} and 50 μM dGTP, the sum of the individual contributions of the fast phase [$(5.2 \text{ s}^{-1}) \times 15\%$] and slow phase [$(0.13 \text{ s}^{-1}) \times 30\%$] gave an overall nucleotide incorporation rate constant of 0.8 s^{-1} , which is similar to the k_{obs} of 0.5 s^{-1} , calculated using Eq. 4, 50 μM dGTP, and corresponding measured k_p and $K_{d, \text{dGTP}}$ values (Table 4), for dGTP incorporation during the extension step. Thus, the biphasic kinetic feature of nucleotide incorporation at Positions -1 and 0 under single-turnover conditions was successfully deconvoluted into a fast and a slow phase through the DNA trap experiments (Figure 3). These two kinetic phases prompted us to propose a kinetic mechanism for dG^{C8-N-ABA} bypass and the subsequent extension by including the formation of productive ($\text{E} \cdot \text{DNA}_n^{\text{P}}$) and non-productive ($\text{E} \cdot \text{DNA}_n^{\text{N}}$) complexes (Scheme 1A). The portion of the hPol η population bound in the $\text{E} \cdot \text{DNA}_n^{\text{P}}$ will bind correct dNTP and rapidly elongate DNA with a fast rate constant of k_f . The fast phase k_f should be dNTP concentration-dependent and can reach its maximum value of k_1 . The remainder of hPol η bound in the $\text{E} \cdot \text{DNA}_n^{\text{N}}$ complex will elongate DNA only after the slow conversion (k_e) of $\text{E} \cdot \text{DNA}_n^{\text{N}} \rightarrow \text{E} \cdot \text{DNA}_n^{\text{P}}$. The slow phase rate constant of k_s is a function of both k_e and k_1 but should be limited by k_e . The bypass of a bulky dG^{AP} lesion by hPol η as well as the bypass of an abasic site, a cisplatin-d(GG) adduct, and a dG^{AP} lesion by Dpo4 have

previously been proposed to follow this mechanism^{13; 24; 25; 29}. If this mechanism is correct, the $E \cdot DNA_n^N \rightarrow E \cdot DNA_n^P$ conversion rate (k_e) and reaction amplitudes should not depend on dNTP concentration. However, both k_f and k_s increased with dNTP concentration at Positions -1 and 0 while both the fast and slow phase reaction amplitudes remained unchanged. To accommodate these observations, an alternative kinetic mechanism (Scheme 1B) was proposed for hPol η to bypass dG^{C8-N-ABA} and to subsequently extend the lesion bypass product. The same mechanism has been proposed by us for dG^{C8-N-ABA} bypass by Dpo4¹¹. This mechanism shows that k_s is actually a function of an exchange rate (k_e) and a slow nucleotide incorporation rate constant (k_2). If k_s had remained the same as dNTP concentration was increased, this would indicate that k_s were solely a function of k_e , and the mechanism could be simplified to reflect Scheme 1A. Because k_s increased with higher dNTP concentration, especially during the extension step, k_s was predominantly a function of k_2 during TLS of dG^{C8-N-ABA} by hPol η .

Notably, the total reaction amplitudes for the bypass and extension steps (48% and 46%, Table 5) are significantly smaller than the corresponding values with undamaged DNA (88% and 86%, Table S2). These differences indicate that hPol η may have bound to damaged DNA in a catalytically incompetent complex ($E \cdot DNA_n^D$) where conformational change(s) of hPol η would not be able to produce a catalytically competent complex (Scheme 1B). In instances when any molecules of the $E \cdot DNA^D$ complex dissociated, the enzyme would bind to excess, unlabeled trap DNA resulting in no detectable product formation. Thus, the presence of such a dead-end binary complex can only be inferred by the sub-maximal product formation observed over the course of TLS of dG^{C8-N-ABA} by hPol η . Similar results have been observed in previous kinetic studies of lesion bypass under comparable conditions^{13; 24; 25; 29}.

Although the hPol η •dG^{C8-N-ABA}-DNA binary structures are not available yet, some of their binding conformations could be inferred from an NMR structure of a 12-mer DNA duplex containing an embedded dG^{C8-N-ABA}:dC basepair³⁴ and crystal structures of yeast Pol η (yPol η) in complex with DNA containing a bulky 2-acetylaminofluorine lesion (dG^{AAF})³⁵. In the NMR structure, the ABA moiety of dG^{C8-N-ABA} is intercalated in the DNA duplex and takes the place of a base pair, with the damaged guanine in the *syn* conformation and extruded into the major groove, and with the opposing cytosine also pushed into the major groove³⁴. Similarly, one yPol η •dG^{AAF}-DNA structure shows that the bulky adduct stacks on the primer/template junction base pair, occupies the polymerase active site, and precludes the incoming nucleotide from binding³⁵. If such a binding conformation also exists in hPol η •dG^{C8-N-ABA}-DNA, it likely represents $E \cdot DNA_n^D$ in Scheme 1. Interestingly, in the other structure of yPol η •dG^{AAF}-DNA³⁵, the AAF adduct is partially rotated out of the DNA helix to allow some excess for incoming dCTP and the structure likely represents the binding conformation of $E \cdot DNA_n^N$ (Scheme 1). Lastly, our ternary crystal structure of hPol η •dG^{C8-N-ABA}-DNA•dCTP shows that the adducted guanine forms a standard Watson-Crick pair with the incoming dCTP, with the ABA moiety is accommodated inside a hydrophobic cleft to the side of the active site of hPol η ²¹. Such a binding conformation of hPol η •dG^{C8-N-ABA}-DNA is considered to be $E \cdot DNA_n^P$ (Scheme 1).

4.3 Error-prone bypass of dG^{C8-N-ABA} and subsequent extension of the lesion bypass product

During dG^{C8-N-ABA} bypass, hPol η showed a 10-fold reduced fidelity (4.8×10^{-2} – 1.6×10^{-1}) relative to undamaged DNA (2.0×10^{-3} – 1.2×10^{-2} , Table S1) with nucleotide incorporation preference following the order of dCTP > dATP > dGTP > dTTP (Table 4). Thus, the presence of dG^{C8-N-ABA} decreased the ability of hPol η to discriminate against incorrect nucleotides, especially dATP. Our kinetic studies show that hPol η will misincorporate dATP 15% of the time when bypassing dG^{C8-N-ABA} (Table 4). Interestingly, our previous studies have shown that in NER-proficient HEK293T cells, G→T transversions were the most common mutation resulting from dG^{C8-N-ABA} bypass, followed by G→A transitions and G→C transversions, albeit at much lower rates²⁰. Furthermore, our previous studies have also demonstrated that siRNA-mediated knockdown of hPol η leads to a 39% decrease in mutation frequency in HEK293T cells, and strongly suggest that hPol η plays a major role in the error-prone bypass of dG^{C8-N-ABA} *in vivo*²⁰. Therefore, these observations from our published cell-based assays²⁰ are consistent with the results of our kinetic studies with hPol η (Table 4).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- hPol η and hPol κ efficiently bypassed a site-specifically placed dG^{C8-*N*-ABA} lesion
- The fidelity of hPol η decreased during dG^{C8-*N*-ABA} bypass and extension
- hPol η predominantly misincorporated dATP during dG^{C8-*N*-ABA} bypass
- Nucleotide incorporation at hPol η pause sites followed biphasic kinetics
- A kinetic basis was established for mutagenic bypass of dG^{C8-*N*-ABA} *in vivo*

Figure 1. Running start assays at 37 °C

A pre-incubated solution of 100 nM 5'-[³²P]-labeled 17/26-mer DNA (A, C, E, and G) or 17/26-mer-dG^{C8}-N-ABA (B, D, F, and H) and 100 nM hPolη (A and B), 100 nM hPolκ (C and D), 1 μM hPolη (E and F), or 1 μM hRev1 (G and H) was rapidly mixed with all four dNTPs (200 μM each) for various incubation periods before being quenched with the addition of 0.37 M EDTA. Sizes of important products are indicated. The dG^{C8}-N-ABA lesion is located at the 21st position from the 3' terminus of the DNA template.

Figure 2. Determination of the kinetic parameters for dCTP incorporation into 20/26-mer-dG^{C8-N}-ABA at 37 °C

(A) A pre-incubated solution of hPol η (120 nM) and 5'-[³²P]-labeled 20/26-mer-dG^{C8-N}-ABA (20 nM) was rapidly mixed with increasing concentrations of dCTP (25 μ M, ●; 50 μ M, ○; 100 μ M, ■; 200 μ M, □; 400 μ M, ◆; 600 μ M, ◇; and 800 μ M, ▲) before being quenched by the addition of 0.37 M EDTA at the indicated time points. Product formation was plotted against time and the data were fit to Eq. 3 to yield the observed rate constant of product formation (k_{obs}). (B) Each k_{obs} value was plotted against its respective dCTP concentration and the data were fit to Eq. 4 to yield the maximum dCTP incorporation rate constant (k_p) of $1.9 \pm 0.1 \text{ s}^{-1}$ and dCTP equilibrium dissociation constant ($K_{d, \text{dCTP}}$) of $325 \pm 44 \text{ }\mu\text{M}$.

Figure 3. Biphasic kinetics of dNTP incorporation in the presence of a DNA trap at 37 °C

A pre-incubated solution of 120 nM hPol η and 30 nM 20/26-mer-dG^{C8-N-ABA} (A) or 21/26-mer-dG^{C8-N-ABA} (B) was rapidly mixed with 5 μ M unlabeled 20/26-mer trap DNA and an indicated concentration of dCTP (A) or dGTP (B). After various incubation periods, the reaction was quenched with the addition of 0.37 M EDTA. Product formation was plotted against time and the data were fit to Eq. 5 to yield A_f and A_s , which are the reaction amplitudes of the fast and slow phases, respectively, as well as k_f and k_s , which are the rate constants of the fast and slow phases, respectively. The insets are magnifications of shorter time points to show the fast phase of product formation.

Scheme 1.

Proposed kinetic mechanism for dG^{C8-N-ABA} bypass and subsequent extension.

Table 1

Sequences of DNA oligonucleotides

Primers (Position) ^a	Sequence
17-mer (-4)	5'-AACGACGGCCAGTGAAT-3'
18-mer (-3)	5'-AACGACGGCCAGTGAATT-3'
19-mer (-2)	5'-AACGACGGCCAGTGAATTC-3'
20-mer (-1)	5'-AACGACGGCCAGTGAATTCG-3'
21-mer (0)	5'-AACGACGGCCAGTGAATTCGC-3'
22-mer (+1)	5'-AACGACGGCCAGTGAATTCGCG-3'
Templates	
26-mer	3'-TTGCTGCCGGTCACTTAAGCGCGCCC-5'
26-mer-dG ^{C8-N-ABA}	3'-TTGCTGCCGGTCACTTAAGCGCGCCC-5'

G indicates the dG^{C8-N-ABA} lesion at the 21st position in the template.

^aPosition of primer 3' terminus relative to the templating dG^{C8-N-ABA}.

Table 2dG^{C8-N-ABA} bypass halftimes of human Y-family polymerases at 37 °C

Polymerase	$t_{50}(s)^a$	$t_{50}^{bypass}(s)^b$	t_{50}^{bypass}/t_{50}
hPol η	0.7	13	19
hPol κ	0.3	7	23
hPol ι	200	>10,800	>55
hRev1	1,800	>37,000	>20

^aCalculated as the time required for traversing 50% of undamaged dG.^bCalculated as the time required for bypassing of 50% of dG^{C8-N-ABA}.

Table 3DNA binding affinity of hPol η at 23 °C

DNA Substrate	K_d , undamaged DNA (nM)	K_d , damaged DNA (nM) ^a	Affinity Ratio ^b
18/26-mer	28 ± 3	32 ± 5	1.1
19/26-mer	25 ± 5	12 ± 2	0.5
20/26-mer	23 ± 2	12 ± 2	0.5
21/26-mer	26 ± 1	26 ± 7	1.0
22/26-mer	17 ± 6	24 ± 4	1.4

^aDamaged DNA refers to template 26-mer containing a dG^{C8-N}-ABA lesion at the 21st position.^bCalculated as K_d , damaged DNA/ K_d , undamaged DNA.

Table 4

Kinetic parameters for dNTP incorporation into dG^{C8-N}ABA-containing DNA at 37 °C

dNTP	K _d , dNTP (μM)	k _p (s ⁻¹)	k _p /K _d , dNTP (μM ⁻¹ s ⁻¹)	Efficiency Ratio ^{a,b}	Fidelity ^c	Fidelity ratio ^d	Probability ^e (%)
18/26-mer-dG ^{C8-N} ABA - Template dG (Position -3)							
dCTP	200 ± 11	66 ± 1	3.3 × 10 ⁻¹	5	-	-	-
dATP	369 ± 48	2.1 ± 0.1	5.7 × 10 ⁻³	0.6	1.7 × 10 ⁻²	7.4	-
19/26-mer-dG ^{C8-N} ABA - Template dC (Position -2)							
dGTP	150 ± 34	6.6 ± 0.4	4.4 × 10 ⁻²	23	-	-	-
dCTP	90 ± 19	0.093 ± 0.0001	1.0 × 10 ⁻³	5	2.2 × 10 ⁻²	4.8	-
20/26-mer-dG ^{C8-N} ABA - Template dG ^{C8-N} ABA (Position -1)							
dCTP	325 ± 44	1.9 ± 0.1	5.8 × 10 ⁻³	97	-	-	77
dATP	161 ± 38	0.18 ± 0.01	1.1 × 10 ⁻³	3	1.6 × 10 ⁻¹	33	15
dGTP	39 ± 5	0.014 ± 0.0003	3.6 × 10 ⁻⁴	3	5.8 × 10 ⁻²	29	5
dTTP	475 ± 85	0.14 ± 0.01	2.9 × 10 ⁻⁴	23	4.8 × 10 ⁻²	4	4
21/26-mer-dG ^{C8-N} ABA - Template dC (Position 0)							
dGTP	28 ± 5	0.8 ± 0.03	2.9 × 10 ⁻²	29	-	-	74
dATP	473 ± 25	3.2 ± 0.10	6.8 × 10 ⁻³	2	1.9 × 10 ⁻¹	11	17
dCTP	201 ± 20	0.4 ± 0.02	2.0 × 10 ⁻³	9	6.4 × 10 ⁻²	3	5
dTTP	392 ± 108	0.6 ± 0.01	1.5 × 10 ⁻³	3	4.9 × 10 ⁻²	9	4
22/26-mer-dG ^{C8-N} ABA - Template dG (Position +1)							
dCTP	215 ± 67	9.1 ± 1.0	4.2 × 10 ⁻²	4	-	-	-
dGTP	41 ± 8	0.028 ± 0.001	6.8 × 10 ⁻⁴	0.6	1.6 × 10 ⁻²	6.5	-

^aCalculated as [(k_p/K_d, dNTP)undamaged DNA/(k_p/K_d, dNTP)damaged DNA].

^bKinetic parameters for undamaged DNA from Table S1 of Sherrer *et al.*¹³

^cCalculated as (k_p/K_d, dNTP)_{incorrect}/[(k_p/K_d, dNTP)_{incorrect} + (k_p/K_d, dNTP)_{correct}].

^dCalculated as Fidelity_{damaged DNA}/Fidelity_{undamaged DNA}.

^eCalculated as [(k_p/K_d, dNTP)damaged DNA/Σ(k_p/K_d, dNTP)damaged DNA] × 100.

Table 5Kinetic parameters from biphasic kinetic assays with hPol η at 37 °C

[dNTP]	A_f (nM)	k_f (s ⁻¹)	A_s (nM)	k_s (s ⁻¹)
20/26-mer-dG ^{C8-N} -ABA				
300 μ M dCTP	5.6 $\pm$ 0.8 (19%)	4.6 $\pm$ 1.3	8.3 $\pm$ 0.8 (28%)	0.32 $\pm$ 0.06
600 μ M dCTP	5.5 $\pm$ 0.7 (18%)	6.3 $\pm$ 1.5	8.5 $\pm$ 0.7 (28%)	0.36 $\pm$ 0.05
1000 μ M dCTP	5.8 $\pm$ 0.8 (19%)	7.2 $\pm$ 1.4	8.6 $\pm$ 0.8 (29%)	0.42 $\pm$ 0.06
21/26-mer-dG ^{C8-N} -ABA				
25 μ M dGTP	4.4 $\pm$ 0.7 (15%)	3.8 $\pm$ 1.5	9.2 $\pm$ 0.8 (31%)	0.082 $\pm$ 0.002
50 μ M dGTP	4.4 $\pm$ 0.8 (15%)	5.2 $\pm$ 1.7	9.1 $\pm$ 0.6 (30%)	0.13 $\pm$ 0.02
90 μ M dGTP	4.3 $\pm$ 0.6 (14%)	11 $\pm$ 4	9.3 $\pm$ 0.7 (31%)	0.16 $\pm$ 0.03

A_f and A_s are the reaction amplitudes of the fast and slow phases, respectively, while k_f and k_s are the rate constants of the fast and slow phases, respectively.