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Mechanistic Investigation of the Bypass of a Bulky Aromatic DNA Adduct Catalyzed by a Y-family DNA Polymerase

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Abstract

3-Nitrobenzanthrone (3-NBA), a nitropolyaromatic hydrocarbon (NitroPAH) pollutant in diesel exhaust, is a potent mutagen and carcinogen. After metabolic activation, the primary metabolites of 3-NBA react with DNA to form dG and dA adducts. One of the three major adducts identified is *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG^{C8-N-ABA}). This bulky adduct likely stalls replicative DNA polymerases but can be traversed by lesion bypass polymerases *in vivo*. Here, we employed running start assays to show that a site-specifically placed dG^{C8-N-ABA} is bypassed *in vitro* by *Sulfolobus solfataricus* DNA polymerase IV (Dpo4), a model Y-family DNA polymerase. However, the nucleotide incorporation rate of Dpo4 was significantly reduced opposite both the lesion and the template position immediately downstream from the lesion site, leading to two strong pause sites. To investigate the kinetic effect of dG^{C8-N-ABA} on polymerization, we utilized pre-steady-state kinetic methods to determine the kinetic parameters for individual nucleotide incorporations upstream, opposite, and downstream from the dG^{C8-N-ABA} lesion. Relative to the replication of the corresponding undamaged DNA template, both nucleotide incorporation efficiency and fidelity of Dpo4 were considerably decreased during dG^{C8-N-ABA} lesion bypass and the subsequent extension step. The lower nucleotide incorporation efficiency caused by the lesion is a result of a significantly reduced dNTP incorporation rate constant and modestly weaker dNTP binding affinity. At both pause sites, nucleotide incorporation followed biphasic kinetics with a fast and a slow phase and their rates varied with nucleotide concentration. In contrast, only the fast

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SUPPORTING INFORMATION AVAILABLE

A table of the kinetic parameters for the 18/26-mer control substrate (Table S1), denaturing PAGE of ³²P-labeled control and adducted 26-mer (Fig. S1), and ESI-MS analysis of the adducted 26-mer-dG^{C8-N-ABA} (Fig. S2) are available in the Supporting Information. This information is available free of charge via the Internet at <http://www.journals.elsevier.com/dna-repair/>.

phase was observed with undamaged DNA. A kinetic mechanism was proposed for the bypass of dG^{C8-N-ABA} bypass catalyzed by Dpo4.

Keywords

Sulfolobus Solfataricus Dpo4; Pre-steady-state kinetics; 3-Nitrobenzanthrone; Mutation; Y-family DNA polymerase; NitroPAH

1. Introduction

Combustion of every form of fossil fuel produces potent environmental pollutants that are known to affect human health at the molecular level.[1] Once metabolized, many of these pollutants become mutagenic and carcinogenic by damaging cellular genomes in a variety of ways, such as forming bulky DNA adducts, causing oxidative damage, and producing single and double stranded DNA breaks.[1] If the DNA adducts are not recognized and repaired by various cellular DNA repair pathways, they will stall replication machinery and eventually induce apoptosis.[2] To rescue stalled DNA replication, cells switch from a replicative polymerase to a DNA lesion bypass polymerase at a lesion site.[2] Notably, most of these lesion bypass polymerases belong to the Y-family, one of the six families (A, B, C, D, X, and Y) of DNA polymerases.[3] The Y-family enzymes possess relatively flexible and solvent-accessible active sites to accommodate bulky DNA lesions. However, these attributes of the Y-family enzymes also lead to their error-prone manner of DNA synthesis with both undamaged and damaged DNA.[4, 5] Interestingly, the Y-family DNA polymerases have been identified in all three domains of life, *e.g.* four in humans (DNA polymerases η , κ , ι , and Rev1), three in *Schizosaccharomyces pombe* (DNA polymerases η , κ , and Rev1), two in *Escherichia coli* (DNA polymerases IV and V), and one in *Sulfolobus solfataricus* (DNA polymerases IV). Being the lone Y-family enzyme in *S. solfataricus*, DNA polymerases IV (Dpo4) likely bypasses various lesions *in vivo* and thus, has been intensely studied as a model enzyme *in vitro*. With an undamaged DNA template, Dpo4 catalyzes DNA synthesis with a fidelity of one error per 1,000–10,000 nucleotide incorporations based on our pre-steady-state kinetic studies from 37 to 56 °C.[6, 7] Dpo4 has been found to bypass various DNA lesions, *e.g.* abasic sites [8, 9] and *N*-(deoxyguanosin-8-yl)-1-aminopyrene (dG^{AP}). [10] The latter lesion is a bulky adduct resulting from a dG base reacting with the metabolites of 1-nitropyrene, a product of incomplete diesel and gasoline combustion.[11]

Another interesting product of diesel exhaust, albeit present at a lower concentration than 1-nitropyrene, is 3-nitrobenzanthrone (3-NBA, Scheme 1).[12] 3-NBA, one of the most mutagenic compound ever tested on the Ames *Salmonella typhimurium* (TA98) assay, is also found in ambient air particulate matter.[12] The mutagenicity of 3-NBA is comparable to 1,8-dinitropyrene, [13] and has been suspected to be carcinogenic to humans.[14–16] This possibility is supported by cellular and animal model data.[17–20] For example, 3-NBA and its metabolites are found to induce micronuclei and DNA adducts in mouse and human cells, [12, 19] and form DNA adducts and tumors in rats.[18–21] Bioactivation of 3-NBA is initiated by an essential nitroreduction step, forming *N*-hydroxy arylamine (*N*-OH-ABA)

(Scheme 1).[17] This step is catalyzed by cytochrome P450 and cytochrome P450 reductase in rats,[17] and NAD(P)H:Quinone Oxidoreductase in humans.[22] *N*-OH-ABA is then metabolized to either *N*-AcO-ABA or *N*-OSO₃H-ABA by *N,O*-acetyltransferases or sulfotransferases, respectively.[17, 23] Finally, either of these highly reactive derivatives reacts with DNA to form bulky aromatic DNA adducts including *N*-(deoxyguanosin-8-yl)-3-aminobenzanthrone (dG^{C8-N-ABA}, Scheme 1).

The DNA adducts derived from 3-NBA, if not repaired, are likely bypassed by lesion bypass polymerases *in vivo*. [2] In this paper, we kinetically investigated the potential bypass of a site specifically placed dG^{C8-N-ABA}, one of the three major adducts formed by 3-NBA,[24] catalyzed by Dpo4. Our results show that the prototype Y-family polymerase was indeed capable of bypassing dG^{C8-N-ABA} but its polymerase efficiency and fidelity were significantly affected by the bulky lesion.

2. Materials and Methods

2.1. Chemical Synthesis

2.1.1. Starting material for synthesis of a DNA oligomer containing

dG^{C8-N-ABA}—All starting materials, reagents and solvents were of commercial grade and used as such unless otherwise specified. Anhydrous solvents were purchased from Aldrich (St. Louis, MO). THF was dried by standard methods. NMR (¹H and ¹³C) spectra were recorded on a Bruker (Coventry, UK) AC-400 spectrometer. Samples prepared for NMR analysis were dissolved in either CDCl₃ or DMSO-*d*₆. Chemical shifts are reported in δ ppm relative to TMS in the proton spectra and to the deuterated solvent in the carbon spectra. LCMS (electrospray ionization) was recorded on a Micromass Quattro II (Manchester, UK) triple quadrupole mass spectrometer in acetonitrile mobile phase and the cone voltage set to 30 V. Mass spectra of the modified oligonucleotide were acquired on a QStar Elite hybrid QTOF MS mass spectrometer (AB Sciex, USA).

2.1.2. Synthesis and characterization of (*O*⁶-Benzyl-3',5'—bis-*O*-(*tert*-butyldimethylsilyl)-*N*²-dimethoxytrityl-2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (**4**, Scheme 2)—

*O*⁶-Benzyl-8-bromo-3',5'-*O*-bis(*tert*-butyldimethylsilyl)-*N*²-dimethoxytrityl-2'-deoxyguanosine [25, 26] (**3**) (5 g, 5.08 mmol), 3-aminobenzanthrone (1.02 g, 4.14 mmol), tris (dibenzylidenacetone)dipalladium (Pd₂(dba)₃) (380 mg) and *rac*-2, 2'- bis(diphenylphosphino)-1,1'-binaphthyl (64 mg) were preheated in degassed toluene at 90 °C. After 40 min, sodium *tert*-butoxide (621 mg, 6.5 mmol) was added and the reaction mixture was stirred for an additional hour at 100 °C. The reaction was cooled and the filtrate was concentrated and purified by chromatography on aluminum oxide using 5–20% ethyl acetate in hexane to provide compound **4** (4.97g, 85%) in Scheme 2. ¹H NMR (400 MHz, DMSO-*d*₆) δ -0.27(3H, s), -0.15(3H, s), 0.14 (6H, s), 0.66 (9H, s), 0.95(9H, s), 1.64(1H, br s), 2.22 (1H, br s), 3.80 (7H, br s), 4.14 (3H, m), 4.50 (1H, br s), 5.08 (2H, br s), 6.14 (1H, br s), 6.80 (4H, m), 7.28–7.72 (19H, m), 8.24 (2H, m), 8.38 (2H, m), 8.49 (1H, d, *J* = 7.1 Hz), 8.76 (1H, d, *J* = 7.1 Hz). ¹³C NMR (100 MHz, DMSO-*d*₆) δ -5.7, -4.7, 1.1, 14.2, 18.0, 18.2, 25.7, 25.8, 25.9, 36.7, 39.9, 39.9, 55.2, 60.4, 63.1, 67.5, 70.2, 72.6, 86.2, 88.5, 112.0, 112.8, 112.9, 112.9, 117.1, 121.0, 122.8, 125.9, 125.9, 126.5,

128.6129.1, 129.1, 133.4, 136.7, 137.1, 137.7, 146.3, 147.2, 156.2, 158.1, 184.1. MS (ESI): m/z : calculated for $[M+H]^+$ $C_{67}H_{75}N_6O_7Si_2$ 1131.5236, observed 1131.5.

2.1.3. DMT deprotection of the dG adduct—To the protected dG adduct **4** (100 mg, 0.088 mmol) dissolved in 2 mL dichloromethane was added 1M zinc bromide solution in isopropanol: dichloromethane (1:1) (2 mL). The reaction was stirred for 15 min, quenched with aqueous sodium bicarbonate, and extracted with dichloromethane. The organic layer was combined, dried and purified by using column chromatography on aluminum oxide using 5–30% ethyl acetate in hexane to afford (*O*⁶-benzyl-3',5'-bis-*O*-(*tert*-butyldimethylsilyl)-2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (44 mg, 60%). ¹H NMR (300 MHz, CDCl₃) δ -0.33 (3H, s), -0.19 (3H, s), 0.16 (3H, s), 0.18 (3H, s), 0.60 (9H, s), 0.97 (9H, s), 2.43 (1H, m), 3.08 (1H, m), 3.82 (2H, m), 3.96–3.98 (1H, m), 4.21 (1H, s), 4.59 (1H, s), 4.71 (2H, br s), 5.59 (2H, s), 6.42 (1H, t, *J* = 7.1 Hz), 7.20 (1H, m), 7.34 – 7.36 (4H, m), 7.50 – 7.55 (3H, m), 7.76–7.81 (2H, m), 8.34 – 8.37 (2H, m), 8.50 (3H, m), 8.71 (1H, s), 8.82 (1H, m). ¹³C NMR (100 MHz, CDCl₃) δ -5.65, -4.66, -4.51, 14.2, 18.1, 18.2, 24.7, 36.8, 39.2, 53.5, 60.4, 63.1, 67.8, 72.5, 85.4, 85.4, 88.1, 117.7, 122.8, 125.5, 125.6, 127.8, 127.9, 128.2, 128.3, 130.1, 133.2, 136.5, 136.9, 147.5, 153.6, 157.5, 183.9. MS (ESI): m/z : calculated for $[M+H]^+$ $C_{49}H_{57}N_6O_5Si_2$ 829.3929, observed 829.0.

2.1.4. TBDMS deprotection of the dG Adduct—To the detritylated compound, (*O*⁶-benzyl-3',5'-bis-*O*-(*tert*-butyldimethylsilyl)-2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (200 mg, 0.242 mmol) in THF (5 mL), triethylamine trihydrogenfluoride (100 μL) was added and stirred overnight at room temperature. Subsequently, triethylamine (88 μL) was added, the solvent was evaporated, and the compound was purified by column chromatography on silica gel using methylene chloride: methanol as an eluent to afford (*O*⁶-benzyl-2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (122 mg, 84%). ¹H NMR (300 MHz, DMSO-*d*₆) δ 2.22 (1H, m), 2.89 (1H, m), 3.68 (1H, m), 3.76 (1H, m), 3.99 (1H, br s), 4.46 (1H, br s), 5.35 (1H, s), 5.44 (2H, br s), 5.53 (1H, m), 6.30 (2H, s), 6.50 (1H, m), 7.46–7.30 (5H, s), 7.58 (1H, m), 7.83 (1H, m), 7.90 (1H, m), 7.93 (1H, m), 8.52 (1H, m), 8.60 (1H, m), 8.68 (1H, m), 8.70 (1H, d, *J* = 8.0 Hz), 9.33 (1H, s). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 9.0, 47.0, 62.0, 67.1, 71.8, 84.1, 84.5, 88.1, 118.9, 124.0, 126.40, 126.42, 126.7, 126.8, 127.7, 128.0, 128.1, 128.4, 128.4, 128.8, 129.0, 129.9, 130.1, 130.9, 134.3, 135.8, 136.6, 136.8, 137.3, 147.0, 158.7, 183.2. MS (ESI): m/z : calculated for M^+ $C_{34}H_{28}N_6O_5$ 600.2121, observed 600.7.

2.1.5. Benzyl deprotection of the dG adduct—In a round bottom flask (*O*⁶-benzyl-2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (200 mg, 0.330 mmol) was dissolved in 20 mL THF/MeOH (1:1) under inert (N₂) atmosphere. The reaction mixture was purged with N₂ for 10 min. Pd-black (15 mg) was added and the reaction mixture was purged with H₂ for 2 h. It was left overnight with continued stirring under H₂. A solid was separated out and filtered. The filtrate was dried and purified by reversed-phase chromatography on a C18 column using 1–50% acetonitrile in water to give 153 mg of (2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (**5**, Scheme 2) in 90% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 2.09–2.16 (1H, m), 2.86 (1H, br s), 3.61–3.72 (2H, m), 3.90 (1H, br s), 4.39 (1H, s), 5.27 (1H, br s), 5.36 (1H, br s), 6.38 (3H, br s), 7.59 (1H, s), 7.75 (1H, s), 7.84 – 7.91 (2H, m), 8.34 (1H, s),

8.55 (1H, br s), 8.72 (3H, br s), 9.13 (1H, s), 10.63 (1H, s). ^{13}C NMR (75 MHz, DMSO- d_6) δ 9.6, 46.1, 62.0, 71.6, 84.0, 88.1, 116.4, 119.8, 123.8, 125.8, 126.2, 127.0, 127.6, 127.8, 128.3, 128.4, 129.7, 130.1, 130.7, 134.2, 136.7, 144.0, 150.5, 153.5, 156.2, 183.2. MS (ESI): m/z : calculated for M^+ $\text{C}_{27}\text{H}_{22}\text{N}_6\text{O}_5$ 510.1652, observed 510.8.

2.1.6. DMF protection of (2'-deoxyguanosin-8-yl)-3-aminobenzanthrone—

Compound **5** (150 mg, 0.294 mmol) in Scheme 2 was suspended in dry MeOH (10 mL) under argon. *N,N*-Dimethylformamide dimethyl acetal (1 mL) was added and the mixture was stirred for 3 days. The solid precipitate was filtered to afford the pure N^2 -DMF protected (2'-deoxyguanosin-8-yl)-3-aminobenzanthrone. The filtrate was dried and purified by reversed-phase chromatography on a C18 column using 1–50% acetonitrile in water to afford an additional crop of the compound (15 mg). Combined yield (157 mg) of the compound was 95 %. ^1H NMR (400 MHz, CDCl_3) δ 2.20–2.23 (1H, m), 2.87–2.9 (1H, m), 2.93 (3H, s), 3.05 (3H, s), 3.64 (1H, d, J = 12 Hz), 3.75 (1H, d, J = 12 Hz), 3.91 (1H, br s), 4.46 (1H, br s), 5.33 (2H, s), 6.27 (1H, s), 6.44 (1H, s), 7.57–7.60 (1H, m), 7.76–7.78 (1H, m), 7.85 (1H, t, J = 8 Hz), 7.92 (1H, t, J = 8 Hz), 8.34 (1H, d, J = 8 Hz), 8.55 (2H, s), 8.70–8.72 (3H, m), 9.24 (1H, s). ^{13}C NMR (75 MHz, CDCl_3) δ 38.60, 41.6, 44.64, 65.44, 74.97, 87.4, 91.5, 120.27, 120.28, 123.6, 127.3, 129.42, 129.83, 130.44, 131.25, 131.67, 131.87, 133.25, 133.67, 134.24, 137.84, 140.17, 144.97, 148.57, 152.83, 160.30, 160.72, 161.74, 186.76. HRMS (ESI) m/z calculated for $[\text{M}+\text{H}]$, $\text{C}_{30}\text{H}_{28}\text{N}_7\text{O}_5$ 566.2152, observed 566.2183.

2.1.7. DMT protection—In a 50 mL round-bottom flask N^2 -DMF protected (2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (100 mg, 0.177 mmol) was dissolved in dry pyridine (5 mL) under argon., to which DMAP (1 mg) and DMT-Cl (71 mg) were added. The reaction mixture was heated at 50 °C for 2 h. Then solvent was evaporated and purified by column chromatography on silica gel (using 1% Et_3N , 3–5% MeOH- 95% CH_2Cl_2) to afford (2-dimethylformamidine-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (107 mg, 70%). ^1H NMR (400 MHz, CDCl_3) δ 3.0 (3H, s), 3.04 (3H, s), 3.10–3.17 (2H, m), 3.38–3.42 (1H, m), 3.48–3.50 (1H, m), 3.56 (3H, s), 3.57 (3H, s), 3.62–3.76 (1H, m), 4.38 (1H, s), 4.59 (1H, s), 6.46 (1H, br s), 6.52–6.54 (4H, m), 7.06–7.12 (8H, m), 7.18–7.26 (2H, m), 7.42–7.54 (3H, m), 7.71 (1H, t, J = 8 Hz), 8.07 (^1H , d, J = 8 Hz), 8.23 (1H, d, J = 8 Hz), 8.33 (1H, d, J = 8 Hz), 8.38 (1H, s), 8.46 (1H, d, J = 8 Hz), 8.63 (1H, s), 8.71 (1H, d, J = 8 Hz). ^{13}C NMR (100 MHz, CDCl_3) δ 39.44, 40.29, 55.16, 64.02, 72.02, 86.28, 86.62, 86.92, 107.31, 113.06, 114.12, 116.98, 121.06, 122.95, 125.86, 125.99, 126.18, 126.93, 127.54, 127.85, 128.06, 128.16, 128.25, 128.54, 128.99, 129.74, 130.05, 130.17, 133.71, 135.37, 135.44, 136.64, 128.26, 144.3, 146.87, 149.48, 151.69, 154.76, 155.33, 157.23, 158.20, 158.46, 184.10. HRMS (ESI) m/z calculated for $[\text{M}+\text{H}]$, $\text{C}_{57}\text{H}_{46}\text{N}_7\text{O}_7$ 868.3459 and observed 868.3507.

2.1.8. (2-Dimethylformamidine-5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-[*N,N'*-diisopropylamino(2-cyanoethoxy)phosphinyl]-2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (6**)—**DMF and DMT protected adduct (100 mg, 0.115 mmol) was dissolved in dry CH_2Cl_2 (10 mL) under argon and DIEA (0.1 mL) was added. 2-Cyanoethoxy diisopropyl chlorophosphoramidite (50 μL) was added and the mixture was stirred for 30 min. Subsequently, the solvent was evaporated and the product was purified by

column chromatography on silica gel (using 1% Et₃N, 0.5–2% MeOH, 96% CH₂Cl₂) to afford the phosphoramidite **6** (74 mg, 60%) as a mixture of two diastereomers. ¹H NMR (400 MHz, CDCl₃) (mixture of two diastereomers) δ 1.07–1.34 (2H, m), 1.99 (1H, br s), 2.42–2.45 (1H, m), 2.59–2.65 (2H, m), 2.69–2.76 (2H, m), 3.09 (3H, br s), 3.15–3.16 (3H, m), 3.29–3.34 (1H, m), 3.39–3.40 (1H, m), 3.44–3.50 (2H, m), 3.56–3.57 (6H, br s), 3.57–3.91 (4H, m), 4.44–4.45 (1H, m), 4.63–4.76 (1H, m), 6.40–6.45 (1H, m), 6.50–6.55 (3H, m), 7.07–7.13 (6H, m), 7.23–7.24 (2H, m), 7.49–7.53 (2H, m), 7.72–7.76 (1H, m), 8.07–8.17 (1H, m), 8.28 (1H, br s), 8.35–8.39 (1H, m), 8.48–8.54 (3H, m), 8.73–8.74 (1H, m). ¹³C NMR (100 MHz, CDCl₃) (mixture of two diastereomers) δ 20.42, 20.49, 20.66, 20.73, 21.72, 24.80, 35.4, 38.75, 41.47, 43.52, 43.64, 46.28, 53.05, 55.23, 58.13, 58.5, 58.7, 63.26, 63.64, 73.68, 73.85, 74.19, 74.37, 85.9, 86.14, 86.79, 86.82, 113.18, 115.52, 115.77, 117.68, 117.88, 122.96, 120.59, 122.96, 124.97, 126.00, 126.35, 127.09, 127.13, 127.37, 127.96, 128.13, 128.22, 128.25, 128.64, 129.18, 129.77, 130.07, 130.16, 133.64, 135.18, 135.25, 135.37, 136.91, 138.24, 144.3, 145.88, 146.01, 148.84, 148.89, 155.39, 155.45, 156.99, 157.67, 157.79, 158.62, 183.0. ³¹P NMR (300 MHz, CDCl₃): 148.9, 148.8. HRMS (ESI) *m/z* calculated for [M+H], C₆₀H₆₃N₉O₈P 1068.4537, observed 1068.4524.

2.2. Kinetic Assays

2.2.1. Buffers—All pre-steady-state kinetic assays were performed in optimized reaction buffer R (50 mM HEPES, pH 7.5, 5 mM MgCl₂, 50 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 10% glycerol (v/v), and 0.1 mg/ml bovine serum albumin)⁵. All electrophoresis mobility shift assays (EMSA) were performed in buffer S (50 mM Tris-Cl, pH 7.5 at 23 °C, 5 mM MgCl₂, 50 mM NaCl, 5 mM DTT, 10% glycerol (v/v), and 0.1 mg/ml bovine serum albumin). The EMSAs were conducted using running buffer A (50 mM Tris acetate, pH 7.5, at 25 °C, 0.5 mM EDTA, and 5.5 mM magnesium acetate). All concentrations are final after mixing. Unless otherwise noted, all reactions were performed at 37 °C.

2.2.2. Enzymes and DNA Substrates—Full length Dpo4 was expressed in *E. coli* and purified as previously described.[6, 7] All primers and templates except 26-mer-dG^{C8-N}-ABA in Table 1 were purchased from the Integrated DNA Technologies, and purified by denaturing polyacrylamide gel electrophoresis (PAGE). The DNA primers were 5'-³²P-labeled by incubation with [γ-³²P] ATP (Perkin Elmer) and OptiKinase (United States Biochemical) for 3 h at 37 °C. The 5'-³²P-labeled primers were annealed to the unlabeled control 26-mer or 26-mer-dG^{C8-N}-ABA at a molar ratio of 1.00:1.35. The solution was first

denatured at 95 °C (for undamaged DNA) or 75 °C (for 26-mer-dG^{C8-N-ABA}) for 5 minutes and then slowly cooled to room temperature.

2.2.3. Running Start Assays—The running start assays were performed as previously described.[8, 9, 27] Briefly, 5'-³²P-labeled DNA (100 nM) and Dpo4 (100 nM) were preincubated in buffer R and subsequently mixed rapidly with a solution containing all four dNTPs (200 μM each) at 37 °C via rapid chemical-quench flow apparatus (KinTek). The reactions were quenched by the addition of EDTA to 0.37 M at specific time points. The reaction products were separated by denaturing PAGE (17% polyacrylamide, 8 M urea) and quantitated using a Typhoon Trio (GE Healthcare).

Quantitative analysis of the running start assays was performed by determining the relative lesion bypass efficiencies (dG^{C8-N-ABA} bypass %) as a function of reaction time. For each time point t , dG^{C8-N-ABA} bypass% = (B/E) × 100, where the total dG^{C8-N-ABA} bypass events (B) was calculated from the concentration of all intermediate products with sizes greater than or equal to the 21mer, and the total dG^{C8-N-ABA} “encounter” events (E) equaled the summation of the 20-mer concentration and the total dG^{C8-N-ABA} bypass events (B). To quantitatively define the dG^{C8-N-ABA} bypass efficiency, t_{50}^{bypass} was defined as the time required to bypass 50% of the total dG^{C8-N-ABA} lesions encountered.

2.2.4. Electrophoretic Mobility Shift Assays—Buffer S containing 5'-³²P-labeled DNA (5 nM) was titrated with Dpo4 (0 – 220 nM). Binary complexes were then separated from free DNA by native PAGE at a constant voltage of 80 V for 30 min at 25 °C using running buffer A. Complex formation was quantitated by using a Typhoon Trio (GE Healthcare). The plot of the binary complex (Dpo4•DNA) concentrations versus Dpo4 concentrations was fit to Equation 1 to yield $K_{d, \text{DNA}}$, the equilibrium dissociation constant for the binary complex (Dpo4•DNA) at 25 °C.

$$[\text{Dpo4} \bullet \text{DNA}] = 0.5(K_{d, \text{DNA}} + E_0 + D_0) - 0.5[(K_{d, \text{DNA}} + E_0 + D_0)^2 - 4E_0D_0]^{1/2} \quad (\text{Eq. 1})$$

In Equation 1, E_0 is the active Dpo4 concentration while D_0 is the DNA concentration.

2.2.5. Substrate Specificity Assays—A previously described single-turnover dNTP incorporation assay was employed to determine the maximum dNTP incorporation rate constant (k_p), and equilibrium dissociation constant ($K_{d, \text{dNTP}}$) of an incoming dNTP.[6–8, 27] These two kinetic parameters yield the dNTP incorporation efficiency ($k_p/K_{d, \text{dNTP}}$). Briefly, Dpo4 (120 nM) and 5'-³²P-labeled DNA substrate (30 nM) were preincubated in Buffer R and then mixed rapidly with a solution containing increasing concentrations of dNTP. The reactions were quenched at specific time points by the addition of EDTA to 0.37 M. Reaction samples were analyzed by denaturing PAGE (17% acrylamide, 8 M urea) and quantitated by using a Typhoon Trio (GE Healthcare). The plot of product formation versus time for each dNTP concentration was fit to a single exponential equation (Equation 2),

$$[\text{Product}] = A(1 - \exp(-k_{\text{obs}}t)) \quad (\text{Eq. 2})$$

where k_{obs} is the observed reaction rate constant and A is the reaction amplitude. Next, the k_{obs} values were plotted against the dNTP concentrations and the plot was then fit to a hyperbolic equation (Equation 3),

$$k_{obs} = k_p [\text{dNTP}] / \{ [\text{dNTP}] + K_{d,\text{dNTP}} \} \quad (\text{Eq. 3})$$

where k_p is the maximum dNTP incorporation rate constant and $K_{d,\text{dNTP}}$ is the equilibrium dissociation constant for the binding of dNTP to the binary complex (Dpo4•DNA) to form the ternary complex (Dpo4•DNA•dNTP).

2.2.6. Biphasic Kinetic Assays—Dpo4 (120 nM) and 5'-³²P-labeled DNA (30 nM) were first preincubated in buffer R and the solution was then mixed rapidly with a solution containing both 5 μ M DNA trap D-1 (Table 1) and 1.2 mM correct dNTP in buffer R by using a rapid chemical-quench flow apparatus. The reactions were quenched at specific times by the addition of EDTA to 0.37 M. The samples were resolved and quantitated as described above. The product formation was plotted versus time and the plot was fit to a double-exponential equation (Equation 4),

$$[\text{Product}] = A_f [1 - \exp(-k_f t)] + A_s [1 - \exp(-k_s t)] \quad (\text{Eq. 4})$$

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

3. Results

3.1. Synthesis of 26-mer-dG^{C8-N-ABA}

Synthesis of C8-dG-ABA was reported by Takamura-Enya and coworkers by Pd-catalyzed cross-coupling of 8-amino-dG with 3-bromobenzanthrone in the presence of XANTPHOS as the ligand.[28] Since conversion of the protected 8-bromo-dG to 8-amino-dG adds two steps and gives low yield of the amine, which drastically reduces the overall yield of the desired adduct, we attempted the reverse coupling of appropriately protected 8-bromo-dG, **3**, with 3-aminobenzanthrone (Scheme 2). Using Pd₂dba₃ as the coupling agent with the ligand BINAP and NaOtBu base at 100 °C for 2 h, we were able to achieve the coupling yield (85%) of adduct **4** (Scheme 2) comparable to the reported reverse approach (80%).[28] Deprotection of **4** and subsequent conversion to the phosphoramidite monomer **6** (Scheme 2) for DNA synthesis also proceeded with a good yield. The protected monomer **6** was used to synthesize a 26-mer-dG^{C8-N-ABA} template (Table 1), by standard protocol in an automated DNA synthesizer, except that the coupling time for this ABA-adducted monomer was increased to 20 min. Deprotection of the oligonucleotides was carried out with concentrated H₄OH in the presence of β -mercaptoethanol. The 26-mer-dG^{C8-N-ABA} template was purified by reverse-phase HPLC followed by denaturing PAGE and was analyzed by ESI-MS (Figs. S1 and S2 in Supporting Information). The m/z gave 8142 Da, which is 243 Da higher than the calculated mass of the unmodified 26-mer (7899.1 Da, Table 1) indicating the presence of dG^{C8-N-ABA} in the 26-mer-dG^{C8-N-ABA} template.

3.2. Bypass of a Site-Specifically Placed dG^{C8-N-ABA} Lesion by Dpo4

Running start assays were employed to examine the response of Dpo4 to a site specifically placed dG^{C8-N-ABA} lesion in the 26-mer-dG^{C8-N-ABA} template relative to the corresponding undamaged 26-mer template (Table 1). Dpo4 gradually elongated the 17-mer primer and synthesized the full-length product 26-mer in about 8 s with the control 17/26-mer DNA substrate, whereas it took about 900 s to achieve the same result with the damaged 17/26-mer-dG^{C8-N-ABA} DNA substrate (Fig. 1). Thus, a single dG^{C8-N-ABA} lesion slowed Dpo4-catalyzed DNA polymerization by more than 100-fold. To quantitatively study the polymerase pause sites, the t_{50}^{bypass} , defined as the time required to bypass 50% of the total dG^{C8-N-ABA} lesions encountered by Dpo4, was estimated in Fig. 2 (see Experimental Procedures). Consistent with the observations in Fig. 1, the t_{50}^{bypass} value was 30 s which is 270-fold greater than the t_{50} value of 0.11 s for the time to traverse 50% of undamaged dG encountered by Dpo4 at the same template position. Furthermore, the time required to extend 50% of the 21-mer lesion bypass product encountered by Dpo4 ($t_{50}^{\text{extension}}$) was estimated to be 600 s (Fig. 2), which is 320-fold greater than the $t_{50}^{\text{extension}}$ value of 1.85 s for the time to traverse 50% of the same position in the undamaged template. As a result, relative to replication of the undamaged DNA substrate, Fig. 1B shows the strong accumulation of intermediate products of 20-mer and 21-mer for replication of the damaged template, indicating that Dpo4 paused strongly during the bypass of the lesion and also at the subsequent extension step. In summary, the 20-fold greater $t_{50}^{\text{extension}}$ versus t_{50}^{bypass} (Fig. 2) indicates that Dpo4 pauses significantly more while extending the bypass product than while bypassing the lesion. A similar Dpo4 pausing pattern has been observed previously with another bulky lesion dG^{AP}. [10] Besides the accumulation of the 20-mer and 21-mer products in Fig. 1B, the 25-mer intermediate product also accumulated in the presence of both control and damaged DNA substrates (Fig. 1). The accumulation was caused by “polymerase slippage” via primer realignment against the 26-mer template which contains three consecutive dCs at its 5'-terminus (Table 1). [10] Lastly, a small amount of 27-mer was synthesized by Dpo4 with the control DNA substrate 17/26-mer, resulting from a Dpo4-catalyzed blunt-end addition to the full-length 26-mer product. [29]

3.3. Moderate Effect of a dG^{C8-N-ABA} Lesion on DNA Binding by Dpo4

The significant impact of dG^{C8-N-ABA} on the rate of DNA synthesis (Fig. 1B) may be attributed to an adverse effect on DNA binding by Dpo4. To examine this possibility, EMSAs were employed to measure the binding affinities of Dpo4 to several undamaged and damaged DNA substrates. As an example, Fig. 3 shows how the binding affinity between Dpo4 and [³²P]-labeled 21/26-mer-dG^{C8-N-ABA} was measured. First, 5'-³²P-labeled 21/26-mer-dG^{C8-N-ABA} (5 nM) was titrated with varying concentrations of Dpo4 and the resulting binary complex (Dpo4•21/26-mer-dG^{C8-N-ABA}) was separated from free 21/26-mer-dG^{C8-N-ABA} via native PAGE (Fig. 3A). The titration was repeated four times. After quantitation, the concentration of Dpo4•21/26-mer-dG^{C8-N-ABA} was plotted against the total concentration of Dpo4 and the plot was then fit to Eq. 1 (“Experimental Procedures”) to obtain a $K_{d, \text{DNA}}$ of 29 ± 7 nM (Fig. 3B). Based on measured $K_{d, \text{DNA}}$ values in Table 2, Dpo4 bound to the undamaged DNA substrates with $K_{d, \text{DNA}}$ values ranging from 3.1 to 4.0 nM. In comparison, the single dG^{C8-N-ABA} lesion weakened the binding of the damaged

DNA substrates to Dpo4 with affinities in the range of 5.0 to 29 nM. Notably, the affinity ratio (Table 2) is greater with 20/26-mer-dG^{C8-N-ABA} (3-fold) and 21/26-mer-dG^{C8-N-ABA} (7.8-fold). However, we concluded that the impact of the lesion on the DNA binding affinity of Dpo4 contributed but was not sufficient to explain the strong accumulation of the 20-mer and 21-mer in Fig. 1B.

3.4. Kinetics of dNTP Incorporation in the presence of a dG^{C8-N-ABA} lesion

Other than the DNA binding affinity, the dG^{C8-N-ABA} lesion may affect the kinetics of nucleotide incorporation opposite the lesion and/or at adjacent template positions. To test this scenario, we determined the maximum dNTP incorporation rate constant k_p , the equilibrium dissociation constant of dNTP binding $K_{d,dNTP}$, and the substrate specificity (or efficiency) $k_p/K_{d,dNTP}$ for a single dNTP incorporation into undamaged or damaged DNA substrates with primers 18-mer to 22-mer under single turnover reaction conditions. As a representative example, Fig. 4 shows the measurement of the k_p and $K_{d,dNTP}$ values for dATP incorporation into 18/26-mer-dG^{C8-N-ABA}. First, a preincubated solution of Dpo4 (120 nM) and 5'-³²P-labeled 18/26-mer-dG^{C8-N-ABA} (30 nM) was rapidly mixed and reacted with varying concentrations of dATP and quenched at various time points. The plot of product concentrations versus reaction times at each dATP concentration was fit to Eq. 2 ("Experimental Procedures") to yield the observed rate constant, k_{obs} . The k_{obs} values were then plotted against dATP concentrations and the plot was fit to Eq. 3 ("Experimental Procedures") to produce a k_p of $2.0 \pm 0.4 \text{ s}^{-1}$ and a $K_{d,dATP}$ of $2,200 \pm 600 \text{ }\mu\text{M}$ (Table 3). With varying sizes of the primer, we similarly measured the kinetic parameters for correct or incorrect nucleotide incorporation opposite dG^{C8-N-ABA} and at the template positions upstream and downstream from the lesion (Table 3). The $k_p/K_{d,dNTP}$ values for correct dNTP incorporations into 18/26-mer-dG^{C8-N-ABA}, 19/26-mer-dG^{C8-N-ABA}, and 22/26-mer-dG^{C8-N-ABA} are either similar to or less than 10-fold lower than the efficiency values with the corresponding control DNA substrates. The efficiency values are 120- and 170-fold lower for correct dNTP incorporation into 20/26-mer-dG^{C8-N-ABA} and 21/26-mer-dG^{C8-N-ABA} than into the control 20/26-mer and 21/26-mer substrates, respectively (Table 3). Thus, relative to undamaged DNA, Dpo4 had little difficulty elongating damaged DNA at non-pause sites (18/26-mer-dG^{C8-N-ABA}, 19/26-mer-dG^{C8-N-ABA}, and 22/26-mer-dG^{C8-N-ABA}) but struggled at the two strong pause sites (20/26-mer-dG^{C8-N-ABA} and 21/26-mer-dG^{C8-N-ABA}), leading to the accumulation of 20-mer and 21-mer observed in Fig. 1B. Furthermore, with damaged DNA substrates containing a single site specifically placed dG^{C8-N-ABA} lesion, we found that Dpo4 incorporated correct over incorrect nucleotides with 10- to 10,000-fold greater efficiency, resulting in polymerase fidelity in the range of 10^{-1} to 10^{-6} (Table 3). At the non-pause sites, the polymerase fidelity (10^{-3} to 10^{-6}) is similar to the fidelity measured with control DNA.[7] In comparison, the polymerase fidelity (10^{-1} to 10^{-3}) at the pause sites is 100-fold lower on average. Consequently, the probability of correct dNTP incorporation was above 98% at all sites tested in the damaged template, except with 21/26-mer-dG^{C8-N-ABA}, where the probability of correct nucleotide incorporation fell to 84.3% (Table 3).

3.5. Biphasic Kinetics of dNTP Incorporation at Polymerase Pause Sites

Our previous kinetic studies have shown that correct dNTP incorporation follows biphasic kinetics at each polymerase pause site induced by various DNA lesions.[8, 10, 27, 30] To examine if the kinetic trend is consistent at the two Dpo4 pause sites caused by dG^{C8-N-ABA}, we performed a DNA trap experiment. A preincubated solution of Dpo4 (120 nM) and 5'-³²P-labeled 20/26-mer-dG^{C8-N-ABA} or 21/26-mer-dG^{C8-N-ABA} (30 nM) was rapidly mixed with a solution containing correct dNTP (1.2 mM) and unlabeled D-1 DNA (5 μM, Table 1). The reaction was quenched at various times by addition of EDTA to 0.37 M. Previously, we have shown that a 167-fold molar excess of undamaged D-1 over 5'-³²P-labeled damaged DNA was sufficient to trap any free Dpo4 before its rebinding to the damaged DNA substrate.[10, 27] As expected, biphasic kinetics of dNTP incorporation was observed with both damaged DNA substrates. Each plot of product concentration versus reaction time (Fig. 5) was fit to a double exponential equation (Eq. 4), yielding the biphasic kinetic parameters listed in Table 4. In comparison, the data fit poorly to a single exponential equation (Data not shown). This can be attributed to the fact that with either 20/26-mer-dG^{C8-N-ABA} or 21/26-mer-dG^{C8-N-ABA}, the first phase rate constant (k_f) is substantially greater than the second phase rate constant (k_s) while the reaction amplitudes in the first phase (A_f) and the second phase (A_s) are comparable. The total reaction amplitudes ($A_f + A_s$) were 44% with 20/26-mer-dG^{C8-N-ABA} and 14.3% with 21/26-mer-dG^{C8-N-ABA}, indicating that a substantial amount of damaged DNA was not converted into products during the DNA trap assays. This low overall reaction amplitude was possibly caused by the lesion and/or the reaction conditions. To exclude the latter possibility, similar DNA trap assays were previously performed with the undamaged 20/26-mer and 21/26-mer substrates [10]. Only a single fast phase of nucleotide incorporation was observed with the control substrates [10]. The reaction amplitudes with both 20/26-mer and 21/26-mer (Table 4) are ~90% [10], demonstrating that the presence of dG^{C8-N-ABA} significantly lowered overall reaction amplitudes at the polymerase pause sites. Furthermore, to examine if the biphasic kinetics of nucleotide incorporation onto damaged DNA were affected by dNTP concentration, the same DNA trap assay with 20/26-mer-dG^{C8-N-ABA} was performed under 450 or 1,000 μM dCTP (Fig. 5A) and the kinetic data were listed in Table 4. Notably, the biphasic data with 450, 1,000, and 1,200 μM dCTP (Table 4) reveal that the reaction amplitudes of the fast and slow phases were unchanged while their rate constants increased with higher dCTP concentrations.

4. Discussion

4.1. Polymerase Pause Sites

During genomic replication, a bulky lesion like dG^{C8-N-ABA} is expected to act as a road-block for high-fidelity, replicative DNA polymerases, which are known to have a tight active site and select against DNA base pairs with non-canonical or distorted geometry.[31] For low-fidelity, Y-family DNA polymerases like Dpo4, their loose and flexible active site tolerates damaged DNA which is geometrically distorted by various lesions as demonstrated by the ternary crystal structures of Dpo4, an incoming nucleotide, and DNA containing various lesions.[32–40] Our running start assays show that Dpo4 was indeed capable of bypassing the site-specifically placed dG^{C8-N-ABA} lesion and synthesized full-length

products albeit at a significantly slower rate than copying a corresponding undamaged DNA template (Fig. 1). The slower full-length product formation rate is primarily due to two consecutive strong polymerase pause sites evidenced by the accumulation of the 20-mer and 21-mer intermediate products (Fig. 1B). A similar polymerase pausing pattern was observed in our published kinetic studies of the bypass of dG^{AP}. [10] During polymerization, Dpo4 paused when incorporating a nucleotide opposite the dG^{C8-N-ABA} lesion, and even more so while extending the bypass product. These conclusions are consistent with the 270- and 320-fold longer t_{50} values for Dpo4 to traverse the 21st and 22nd positions, respectively, from the 3'-terminus of the damaged template 26-mer-dG^{C8-N-ABA} than the corresponding positions on the control template 26-mer.

4.2. Kinetic Basis for Polymerase Pausing as a Result of dG^{C8-N-ABA}

Our DNA-binding assays revealed that the dG^{C8-N-ABA} lesion only reduced the binding affinity of Dpo4 to the damaged DNA substrates by up to 7.8-fold when compared to the corresponding undamaged DNA substrates (Fig. 6D), and Dpo4 binds to the damaged DNA substrates at non-pause sites with 2–6 fold higher affinities than at pause sites (Table 2). Although these affinity differences contribute to the strong pausing of Dpo4 at the two template positions (Fig. 1B) based on the large t_{50} differences, a reduction in DNA-binding affinity alone is not sufficient to induce such significant pausing by Dpo4. To further investigate the kinetic basis behind the bypass of dG^{C8-N-ABA} catalyzed by Dpo4, individual nucleotide incorporation efficiencies at five template positions were measured with either damaged or undamaged DNA substrates (Table 1). Our kinetic data (Table 3) demonstrated that the dG^{C8-N-ABA} lesion did not alter the kinetic trend that correct nucleotides were incorporated with significantly higher rate constants (k_p) and efficiencies ($k_p/K_{d,dNTP}$) than incorrect nucleotides at all template positions. For the three series reactions during polymerization including 18-mer→19-mer→20-mer, 19-mer→20-mer→21-mer, and 20-mer→21-mer→22-mer, they share the following kinetic pattern based on the kinetic data in Table 3: the first conversion step has higher k_p and $k_p/K_{d,dNTP}$ values for correct nucleotide incorporation than the 2nd conversion step, leading to the accumulation of the intermediates 19-mer, 20-mer, and 21-mer, respectively. The reason why the 19-mer accumulated only at early time points is because the fast and efficient conversion of 19-mer→20-mer (0.48 s^{-1} , $9.6 \times 10^{-3} \mu\text{M}^{-1}\text{s}^{-1}$) was completed at later time points (Figure 1B). In contrast, the strong accumulation of 21-mer product is due to large differences (22–28 fold) in both the rate constants and efficiencies in the conversion of 21-mer→22-mer (0.0089 s^{-1} , $2.2 \times 10^{-5} \mu\text{M}^{-1}\text{s}^{-1}$) relative to the conversion of 20-mer→21-mer (0.25 s^{-1} , $4.9 \times 10^{-4} \mu\text{M}^{-1}\text{s}^{-1}$). Similarly, a strong accumulation of 20-mer was contributed by the slower and less efficient conversion of 20-mer→21-mer (0.25 s^{-1} , $4.9 \times 10^{-4} \mu\text{M}^{-1}\text{s}^{-1}$) than the conversion of 19-mer→20-mer (0.48 s^{-1} , $9.6 \times 10^{-3} \mu\text{M}^{-1}\text{s}^{-1}$).

Notably, the 22-mer intermediate product did not accumulate (Fig. 1B), despite that the conversion of 22-mer→23-mer ($3.2 \times 10^{-3} \mu\text{M}^{-1}\text{s}^{-1}$) has 3-fold lower efficiency than the conversion of 19-mer→20-mer ($9.6 \times 10^{-3} \mu\text{M}^{-1}\text{s}^{-1}$) and the correct nucleotide incorporation efficiency ratio is 11-fold higher at this template position (Fig. 6A). The lack of 22-mer accumulation was due to the kinetic pattern in the reaction series 21-mer→22-mer→23-mer, whereas the 2nd step (4.5 s^{-1}) is much faster than the first step (8.9×10^{-3}

s^{-1}). This kinetic pattern is opposite from the pattern deduced from the kinetic series reactions discussed above. Notably, these kinetic patterns for intermediate product formation have been previously revealed in the Dpo4 catalyzed bypass of an abasic site,[8] a cisplatin-d(GpG) adduct,[27] and a dG^{AP} [10].

4.3. Large Effect of dG^{C8-N-ABA} on the Kinetic Parameters of dNTP Incorporation

When comparing the kinetic parameters obtained with control and damaged templates, the correct nucleotide incorporation efficiency ratio, $(k_p/K_{d,dNTP})_{\text{control}}/(k_p/K_{d,dNTP})_{\text{damaged}}$, increases dramatically from non-pause to pause sites (Fig. 6C). For example, the ratios (Table 3) are less than 10-fold at non-pause sites while the ratios are 120 and 170 with primers 20-mer and 21-mer, respectively. Furthermore, the single dG^{C8-N-ABA} lesion in 20/26-mer-dG^{C8-N-ABA} and 21/26-mer-dG^{C8-N-ABA} weakened their binding to Dpo4 by 3.0- and 7.8-fold, respectively (Fig. 6D and Table 2). Thus, the impact of dG^{C8-N-ABA} on Dpo4•DNA binding is significantly smaller than on nucleotide incorporation at and near the lesion site. Since the overall efficiency of a polymerase at a template position is a function of both its binding affinity to DNA and nucleotide incorporation efficiency during processive polymerization, Dpo4 was 360- and 1,330-fold less efficient while elongating primers 20-mer and 21-mer, respectively, with the damaged template 26-mer-dG^{C8-N-ABA} than with the control template 26-mer. Notably, the k_p was 46- and 182-fold slower with 20/26-mer-dG^{C8-N-ABA} and 21/26-mer-dG^{C8-N-ABA}, respectively, than with the corresponding control substrates (Fig. 6A), while the equilibrium dissociation constant ratio $(K_{d,dNTP})_{\text{damaged}}/(K_{d,dNTP})_{\text{control}}$ was less than 2-fold (Fig. 6B). Thus, the ratio of $(k_p/K_{d,dNTP})_{\text{control}}/(k_p/K_{d,dNTP})_{\text{damaged}}$ at each Dpo4 pause site (Fig. 6C) is dictated by the ratio of $(k_p)_{\text{control}}/(k_p)_{\text{damaged}}$. Oddly, correct dCTP was incorporated with a 10-fold greater $K_{d,dNTP}$ in the presence of 22/26-mer-dG^{C8-N-ABA} than in the presence of control 22/26-mer while the k_p value was not altered by the lesion, leading to a nucleotide incorporation efficiency ratio of 10 at this non-pause site (Fig. 6). It is unclear how an embedded template nucleotide dG^{C8-N-ABA} weakened the ground-state binding of an incoming nucleotide to Dpo4•22/26-mer-dG^{C8-N-ABA}.

4.4. Kinetic Mechanism for dG^{C8-N-ABA} Bypass by Dpo4

Since the presence of dG^{C8-N-ABA} did not significantly affect the binding of Dpo4 to both 20/26-mer-dG^{C8-N-ABA} and 21/26-mer-dG^{C8-N-ABA}, in comparison to the respective control DNA substrates, it is likely that Dpo4 holds onto the damaged DNA substrates when incorporating a correct incoming dNTP with low k_p values. This possibility was confirmed by the DNA trap assays which demonstrated that some of the Dpo4•DNA complex did not dissociate even in the slow reaction phase (Fig. 5). The biphasic kinetics of nucleotide incorporation at each pause site show that a fast phase (A_f, k_f) preceded a slow phase (A_s, k_s) (Fig. 5 and Table 4). In both cases, the sum of the contribution from the fast ($A_f k_f$) and slow ($A_s k_s$) phases yielded rate constants with 20/26-mer-dG^{C8-N-ABA} ($0.18 s^{-1}$) and 21/26-mer-dG^{C8-N-ABA} ($0.009 s^{-1}$) which were relatively close to the respective observed k_{obs} values of 0.18 and $0.007 s^{-1}$, estimated using Equation 3, 1.2 mM dCTP in Fig. 5, and corresponding measured $K_{d,dNTP}$ and k_p values (Table 3). In comparison, similar DNA trap assays with the control 20/26-mer and 21/26-mer substrates only exhibited single, fast phase kinetics with greater reaction amplitudes of 87% and 90%, respectively (Table 4). The less than 100%

reaction amplitudes with the control substrates can be attributed to experimental errors, inaccurate measurements of oligomer concentrations, incomplete or imperfect annealing of DNA duplexes, or/and Dpo4 binding at the blunt end rather than the staggered end of DNA. [29] Together, the kinetic data in the presence of a DNA trap suggest that the slow phase observed with the damaged DNA substrates was due to the formation of nonproductive complexes between Dpo4 and DNA ($E \cdot DNA_n^N$) while the fast phase exhibited by each control or damaged DNA substrate indicates the formation of a productive complex ($E \cdot DNA_n^P$), which was turned over rapidly once a nucleotide was bound. Notably, the k_f values are smaller with the damaged DNA substrates than with the control DNA substrates, suggesting that even in the fast phase, Dpo4 did not bind to damaged DNA as productively as it interacted with undamaged DNA. Furthermore, since the slow elongation of $E \cdot DNA_n^N$ occurred in the presence of a large molar excess of unlabeled DNA trap, $E \cdot DNA_n^N$ was first transformed into $E \cdot DNA_n^P$ without dissociation with a first order rate of k_e , and subsequently elongated (Scheme 3A). Interestingly, the mechanism in Scheme 3A has been implicated into the bypass of an abasic site,[8] a cisplatin-d(GpG) adduct,[27] and a dG^{AP} lesion by Dpo4.[10] If the mechanism in Scheme 3A is correct at each of the two polymerase pause sites in Fig. 1B, the slow phase rate constant (k_s) which is dominated by k_e , should not be altered when the dNTP concentration is changed. To test this possibility, we performed the same DNA trap assays with 20/26-mer-dG^{C8-N-ABA} in the presence of different dCTP concentrations. We did not perform such DNA trap experiments with 21/26-mer-dG^{C8-N-ABA} because the overall reaction amplitude (4.3 nM) with this DNA substrate (Table 4) is small and the limited accuracy of the kinetic data may not allow us to draw a definite conclusion. With 20/26-mer-dG^{C8-N-ABA}, we performed the DNA trap assays with dCTP concentration of 450, 1,000, and 1,200 μ M (Figure 5A). The biphasic kinetic data in Table 4 indicate that the reaction amplitudes (A_f and A_s) do not change with the variation of correct dCTP concentration, which is expected since dNTP concentration should not affect the binding of DNA and Dpo4. In contrast, both the fast and slow phase rate constants are 2- to 3-fold higher in the presence of 1,200 than 450 μ M of dCTP while the rate constants with 1,000 μ M dCTP are in the middle. These biphasic kinetic data suggest that the kinetic mechanism in Scheme 3A is inaccurate for the bypass of dG^{C8-N-ABA} by Dpo4 and prompted us to propose a new one shown in Scheme 3B. In this mechanism, while the fast phase k_f is still governed by the process of nucleotide incorporation onto $E \cdot DNA_n^P$ as in Scheme 3A, the slow phase rate constant (k_s) is a function of both the process of slow nucleotide incorporation onto $E \cdot DNA_n^N$ (k_2) and the interconversion between the non-productive and productive binary complexes (k_e). Additionally, our kinetic data in Table 4 also suggest that k_e cannot be much larger than k_2 . Otherwise, Scheme 3B will be simplified as Scheme 3A.

Lastly, the sum of A_f and A_s with either 20/26-mer-dG^{C8-N-ABA} (44%) or 21/26-mer-dG^{C8-N-ABA} (14.3%) is significantly smaller than the reaction amplitude (~90%) observed with either 20/26-mer or 21/26-mer (Table 4). Therefore, significant percentages of 20/26-mer-dG^{C8-N-ABA} (90% – 44% = 46%) and 21/26-mer-dG^{C8-N-ABA} (90% – 14% = 76%) bound by Dpo4 were either catalytically incompetent ($E \cdot DNA_n^D$) or dissociated during the trap assays if the maximum reaction amplitudes with the two damaged substrates can be as high as their corresponding control substrates (~90%). As the formation of the ternary

complexes $E \cdot DNA_n^N \cdot dNTP$ and $E \cdot DNA_n^P \cdot dNTP$, $dNTP$ binds to $E \cdot DNA_n^D$ and yields $E \cdot DNA_n^D \cdot dNTP$. Previously, such ternary complexes ($E \cdot DNA_n^D \cdot dNTP$) have been proposed as Dpo4, HIV-1 reverse transcriptase, and T7 DNA polymerase bind to damaged DNA containing dG^{AP} , [10] N^2 -methylguanine, [41] O^6 -benzylguanine, [42] and O^6 -methylguanine. [42] Taken together, the above analysis suggests the kinetic mechanism in Scheme 3B, rather than in Scheme 3A, is accurate for the bypass of $dG^{C8-N-ABA}$ by Dpo4.

Although there are no reported crystal structures of Dpo4 in complex with DNA containing $dG^{C8-N-ABA}$, several published structures of the Y-family polymerases and DNA containing similarly bulky lesions support the lesion bypass mechanisms (Scheme 3). [35, 36, 43] For example, the binary crystal structures of yeast Pol η and DNA containing a damaged templating nucleotide N -2-acetylaminofluorene- dG (AAF- dG) [43] show that the AAF moiety either stacks above the junction base pair, or partially rotates out of the DNA helix but still blocks incoming $dCTP$. The first conformation of AAF completely blocks an incoming $dNTP$ and thereby represents $E \cdot DNA_n^D$. The second conformation of AAF likely represents $E \cdot DNA_n^N$, which requires subtle to mild structural changes in order for AAF to be completely rotated out of the DNA helix. If the AAF moiety is completely out of the DNA duplex, the binary complex is considered to be in the form of $E \cdot DNA_n^P$, which productively binds an incoming nucleotide and rapidly incorporates it. Interestingly, such a productive conformation ($E \cdot DNA_n^P \cdot dNTP$) has been displayed by the ternary structures of Dpo4, $dNTP$, and DNA containing either benzo[a]pyrene diol epoxide (BPDE)- dG or BPDE- dA , [35, 36] illustrating that the BPDE moiety is flipped out of the DNA helix into a structural gap between the Little Finger and Palm domains of Dpo4 and the distance between the primer 3'-OH and the α -phosphate of the incoming $dNTP$ (3.9 Å) is close to the optimum catalytic distance (3.4 Å). [36, 44] Although these binary and ternary structures with AAF- dG [43] and BPDE [35, 36] are instructive, it remains to see how DNA containing $dG^{C8-N-ABA}$ binds to Dpo4 and forms the three conformations of $E \cdot DNA_n^D$, $E \cdot DNA_n^N$, and $E \cdot DNA_n^P$.

4.5. Biological relevance of the kinetic studies of $dG^{C8-N-ABA}$

Fig. 1B shows that a single $dG^{C8-N-ABA}$ considerably stalls DNA replication catalyzed by Dpo4 *in vitro*. Consistently, $dG^{C8-N-ABA}$ is recently shown to be the strongest replication block of the three major 3-NBA derived DNA adducts in an assay with nucleotide excision repair-deficient human XPA cells. [45] Furthermore, the fidelity values in Table 3 indicate that Dpo4 is prone to misincorporate nucleotides at the two pause sites (Fig. 1B). The error-prone manner is especially severe during the extension of the lesion bypass product, where Dpo4 misincorporates $dCTP$, $dATP$, and $dTTP$ opposite dC with high frequencies of 10.3, 3.8, and 1.6%, respectively. Notably, $dG^{C8-N-ABA}$, inducing primarily G-to-A transitions followed by G-to-T transversions, is found to be the most mutagenic 3-NBA adduct with a mutational frequency of 30.6% in the nucleotide excision repair-deficient human XPA cells. [45] The cellular results agree with our *in vitro* fidelity values although it remains to determine the identity of the polymerase which bypasses $dG^{C8-N-ABA}$ in the XPA cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABA	Aminobenzanthrone
dG^{AP}	<i>N</i> -(deoxyguanosin-8-yl)-1-aminopyrene
dG^{C8-N-ABA}	<i>N</i> -(deoxyguanosin-8-yl)-3-aminobenzanthrone
dNTP	3'-deoxynucleotide 5'-triphosphate
Dpo4	<i>Sulfolobus Solfataricus</i> DNA polymerase IV
EMSA	electrophoresis mobility shift assay
E•DNA_n^D	catalytically incompetent or dead-end binary complex
E•DNA_n^N	non-productive binary complex
E•DNA_n^P	productive binary complex
E•DNA_n^D•dNTP	catalytically incompetent ternary complex
E•DNA_n^N•dNTP	non-productive ternary complex
E•DNA_n^P•dNTP	productive ternary complex
3-NBA	3-Nitrobenzanthrone
NitroPAH	nitro polyaromatic hydrocarbon
PAGE	polyacrylamide gel electrophoresis

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Highlights

3-Nitrobenzanthrone is one of the most mutagenic compounds from diesel exhaust

Pre-steady-state kinetic analysis of DNA damage bypass

A mechanism of lesion bypass by a model Y-family DNA polymerase is proposed

Figure 1. Running Start Assay

A preincubated solution of 100 nM Dpo4 and 100 nM 5'-³²P-labeled (A) 17/26-mer, or (B) 17/26-mer-dG^{C8-N-ABA} was rapidly mixed with all four dNTPs (200 μM each) for various times before being quenched by addition of EDTA to 0.37 M. Sizes of important products are indicated, and the 21st position marks the position of the dG^{C8-N-ABA} lesion from the 3'-terminus of the DNA template.

Figure 2.

Determination of t_{50}^{bypass} and $t_{50}^{extension}$. The percentages of the extension of the intermediate products 20-mer/26-mer-dG^{C8-N}-ABA (●) and 21-mer/26-mer-dG^{C8-N}-ABA (■) out of all polymerase encounter events were determined by quantitation of the gels in Figure 1 (see 2.2.3.) and then plotted against reaction time.

Figure 3. Determination of $K_{d, \text{DNA}}$

(A) A gel image shown the formation of the binary complex Dpo4•DNA during titration. Dpo4 (0 – 220 nM) was titrated into a solution containing 5'-³²P-labeled 21/26-mer-dG^{C8-N}-ABA (5 nM). The binary complex of Dpo4•DNA was separated from free DNA by native PAGE. The titration was repeated four times. (B) Plot of the binary complex concentration versus the total concentration of Dpo4. Each error bar represents the standard deviation of a complex concentration based on four independent experiments. The data were fit to Equation 1 (see 2.2.4.) which yielded a $K_{d, \text{DNA}}$ of 29 ± 7 nM.

Figure 4. Kinetics of dATP incorporation onto 18/26-mer-dG^{C8}-N-ABA

(A) Dpo4 (120 nM) was first preincubated with 5'-³²P-labeled 18/26-mer-dG^{C8}-N-ABA (30 nM), and was then rapidly mixed with increasing concentrations of dATP (50 μM , ●; 150 μM , ■; 300 μM , ◆; 500 μM , ▲; 700 μM , ●; 1000 μM , ■; 1600 μM , ◆) for the indicated times before being quenched. Each time course was fitted to Equation 2 to yield a k_{obs} . (B) the k_{obs} values were plotted against corresponding dATP concentrations and the plot was fit to Equation 3 to produce a k_p of $2.0 \pm 0.4 s^{-1}$ and a $K_{d, dATP}$ of $2,200 \pm 600 \mu M$.

Figure 5. Biphasic kinetics of correct dNTP incorporation in the presence of a DNA trap
 A preincubated solution of 120 nM Dpo4 and 30 nM 5'-³²P-labeled 20/26-mer-dG^{C8-N-ABA} (A) or 21/26-mer-dG^{C8-N-ABA} (B) was mixed rapidly with 5 μM unlabeled 21/41-mer D-1 (DNA trap) and correct dNTP for various times before being quenched by EDTA. The plots of product versus time were fit to Eq. 4 and the data were listed in Table 4. (A) Dpo4•20/26-mer-dG^{C8-N-ABA} was mixed with the DNA trap and 450 (Blue), 1,000 (Red), or 1,200 μM (Black) dCTP. The inset is a magnification of the time points from 0 to 20 seconds, showing the first phase of product formation. (B) Dpo4•21/26-mer-dG^{C8-N-ABA} was mixed with the DNA trap and 1,200 μM dGTP.

Figure 6. Quantitative effects of a dG^{C8-N-ABA} lesion on DNA binding and correct dNTP incorporation catalyzed by Dpo4

The ratios of the kinetic parameters including k_p (**A**), $K_{d, \text{dNTP}}$ (**B**), $k_p/K_{d, \text{dNTP}}$ (**C**), and $K_{d, \text{DNA}}$ (**D**) between damaged and corresponding control DNA substrates were plotted against the primer sizes.

Scheme 1.

Bioactivation of 3-Nitrobenzanthrone. The nitro group of 3-NBA is first activated by a nitroreduction step followed by the formation of an intermediate group (N-AcO-ABA or N-OSO₃H-ABA), which is subsequently converted to a nitrenium ion and reacts with adenosine or guanosine to form a DNA adduct, e.g. dGC8-N-ABA.

Scheme 2.

Chemical Synthesis of (2-Dimethylformamidino-5'-O-(4,4'-dimethoxytrityl)-3'-O-[N,N'-diisopropylamino(2-cyanoethoxy)phosphinyl]-2'-deoxyguanosin-8-yl)-3-aminobenzanthrone.

Scheme 3.
Proposed Kinetic Mechanisms (A) and (B) of Lesion Bypass.

Table 1

Sequences of DNA oligonucleotides

Primers (position ^a)	Sequences
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'
^b 26-mer-dG ^{C8-N-ABA}	3'-TTGCTGCCGGTCACTTAAGCGCGCCC-5'
DNA trap	
D-1 (21/41-mer)	5'-CGCAGCCGTCCAACCAACTCA-3'
	3'-GCGTCGGCAGGTTGGTTGAGTAGCAGCTAGGTTACGGCAGG-5'

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

^bG designates dG^{C8-N-ABA}.

Table 2

Binding Affinity of Dpo4 to Damaged and Control DNA Substrates at 23 °C

DNA Substrate	Damaged DNA ^a (nM)	Control DNA ^{b,c} (nM)	Affinity Ratio ^d
19/26-mer	7.4 ± 0.8 ^e	3.1 ± 0.5	2.4
20/26-mer	12 ± 3	4.0 ± 0.2	3.0
21/26-mer	29 ± 7	3.7 ± 0.2	7.8
22/26-mer	5.0 ± 0.9	3.8 ± 0.6	1.3

^a Refer to those DNA substrates with the template 26-mer-dG^{C8-N}-ABA^b Refer to those undamaged DNA substrates with the control template 26-mer^c Values are from Table 2 of reference [10]^d Calculated as $(K_d, \text{DNA})_{\text{damaged}} / (K_d, \text{DNA})_{\text{control}}$ ^e Error is a data fitting error.

Table 3
Kinetic parameters for dNTP incorporation into damaged DNA substrates containing dG^{C8-N}-ABA

dNTP	$K_{d,dNTP}$ (μ M)	k_p (s^{-1})	$(k_p/K_{d,dNTP})_{damaged}$ (μ M ⁻¹ s ⁻¹)	Efficiency Ratio ^{a,b}	Fidelity ^c	Probability ^d
18/26-mer-dG^{C8-N}-ABA						
dCTP	32 ± 5	2.83 ± 0.08	0.088	1.25	-	99.95
dATP	2200 ± 600	(2.0 ± 0.4) × 10 ⁻²	9.1 × 10 ⁻⁶	0.85	7.3 × 10 ⁻⁶	0.01
dGTP	350 ± 40	(6.2 ± 0.3) × 10 ⁻⁴	1.8 × 10 ⁻⁵	1.6	1.4 × 10 ⁻⁵	0.02
dTTP	-	-	1.6 × 10 ⁻⁵	5.4	1.3 × 10 ⁻⁵	0.02
19/26-mer-dG^{C8-N}-ABA						
dGTP	50 ± 9	0.48 ± 0.02	9.6 × 10 ⁻³	2.6	-	99.82
dATP	430 ± 60	(2.9 ± 0.2) × 10 ⁻³	6.7 × 10 ⁻⁶	2.1	7.0 × 10 ⁻⁴	0.07
dCTP	220 ± 80	(1.4 ± 0.2) × 10 ⁻³	6.4 × 10 ⁻⁶	22	6.7 × 10 ⁻⁴	0.06
dTTP	-	-	5.5 × 10 ⁻⁶	1.6	5.7 × 10 ⁻⁴	0.05
*20/26-mer-dG^{C8-N}-ABA						
dCTP	510 ± 60	0.25 ± 0.02	4.9 × 10 ⁻⁴	120	-	98.93
dATP	640 ± 170	(1.9 ± 0.3) × 10 ⁻⁴	3.0 × 10 ⁻⁶	6.3	6.1 × 10 ⁻⁴	0.61
dGTP	1200 ± 400	(8.1 ± 0.1) × 10 ⁻⁴	6.8 × 10 ⁻⁷	66	1.4 × 10 ⁻⁴	0.14
dTTP	970 ± 390	(1.6 ± 0.4) × 10 ⁻⁴	1.6 × 10 ⁻⁶	52	3.3 × 10 ⁻⁴	0.32
*21/26-mer-dG^{C8-N}-ABA						
dGTP	400 ± 80	(8.9 ± 0.7) × 10 ⁻⁴	2.2 × 10 ⁻⁵	170	-	84.26
dATP	450 ± 50	(4.5 ± 0.2) × 10 ⁻⁴	1.0 × 10 ⁻⁶	2.4	4.3 × 10 ⁻²	3.83
dCTP	160 ± 20	(4.3 ± 0.1) × 10 ⁻⁴	2.7 × 10 ⁻⁶	12	1.1 × 10 ⁻¹	10.34
dTTP	420 ± 80	(1.7 ± 0.1) × 10 ⁻⁴	4.0 × 10 ⁻⁷	4.5	1.8 × 10 ⁻²	1.57
22/26-mer-dG^{C8-N}-ABA						
dCTP	1400 ± 300	4.5 ± 0.7	3.2 × 10 ⁻⁴	11	-	99.55
dATP	470 ± 70	(5.4 ± 0.4) × 10 ⁻⁴	1.1 × 10 ⁻⁵	0.38	3.4 × 10 ⁻⁴	0.34
dGTP	1100 ± 200	(1.4 ± 0.2) × 10 ⁻⁴	1.3 × 10 ⁻⁶	6.5	4.1 × 10 ⁻⁴	0.04

dNTP	K_d dNTP (μ M)	k_p (s^{-1})	$(k_p/K_d, \text{dNTP})_{\text{damaged}}$ ($\mu\text{M}^{-1}s^{-1}$)	Efficiency Ratio ^{a,b}	Fidelity ^c	Probability ^d
dTTP	770 ± 140	$(1.7 \pm 0.2) \times 10^{-4}$	2.2×10^{-6}	5.0	6.9×10^{-4}	0.07

* Denote pause sites.

^a Calculated as $(k_p/K_d, \text{dNTP})_{\text{control}}/(k_p/K_d, \text{dNTP})_{\text{damaged}}$.

^b Values for $(k_p/K_d, \text{dNTP})_{\text{control}}$ 19/26-mer are from supplemental Table 1 of reference [10]. Kinetic parameters for 18/26-mer are listed in SI Table 1.

^c Calculated as $(k_p/K_d, \text{incorrect dNTP})_{\text{damaged}}/((k_p/K_d, \text{correct dNTP})_{\text{damaged}} + (k_p/K_d, \text{incorrect dNTP})_{\text{damaged}})$.

^d Calculated as $((k_p/K_d, \text{dNTP})_{\text{damaged}}/(\sum (k_p/K_d, \text{dNTP})_{\text{damaged}})) \times 100$.

Biphasic kinetic parameters for correct dNTP incorporation into a 5'-³²P-labeled DNA substrate (30 nM) in the presence of a DNA trap (5 μM) at 37 °C

Table 4

DNA substrate	Correct dNTP	A_f^a (nM)	k_f (s ⁻¹)	A_s^a (nM)	k_s (s ⁻¹)
Nucleotide concentration = 1,200 μM					
<i>b</i> ₂₀ /26-mer	dCTP	26 ± 1 (86.7%)	4.6 ± 0.5	-	-
<i>b</i> ₂₁ /26-mer	dGTP	27.0 ± 0.4 (90%)	2.3 ± 0.1	-	-
20/26-mer-dG ^{CS-N} -ABA	dCTP	8.6 ± 0.7 (29%)	0.60 ± 0.07	4.5 ± 0.7 (15%)	0.05 ± 0.01
21/26-mer-dG ^{CS-N} -ABA	dGTP	2.2 ± 0.4 (7.3%)	0.12 ± 0.06	2.1 ± 0.5 (7%)	0.0028 ± 0.0025
Nucleotide concentration = 1,000 μM					
20/26-mer-dG ^{CS-N} -ABA	dCTP	8.6 ± 1.7 (29%)	0.32 ± 0.09	4.7 ± 1.7 (16%)	0.04 ± 0.01
Nucleotide concentration = 450 μM					
20/26-mer-dG ^{CS-N} -ABA	dCTP	8.7 ± 0.2 (29%)	0.23 ± 0.03	4.7 ± 0.6 (16%)	0.021 ± 0.005

^aPercentage after each reaction amplitude is calculated as (reaction amplitude/30 nM) × 100%.

^bReaction amplitude (*A*) and rate constant (*k*) values are from Table 4 of reference [10].