Published in final edited form as:

Chembiochem. 2016 November 03; 17(21): 2033–2037. doi:10.1002/cbic.201600420.

Mechanism of Error-Free Bypass of the Environmental Carcinogen N-(2'-Deoxyguanosin-8-yl)-3-aminobenzanthrone Adduct by Human DNA Polymerase η **

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The environmental pollutant 3-nitrobenzanthrone produces bulky aminobenzanthrone (ABA) DNA adducts with both guanine and adenine nucleobases. A major product occurs at the C8 position of guanine (C8-dG-ABA). These adducts present a strong block to replicative polymerases but, remarkably, can be bypassed in a largely error-free manner by the human Y-family polymerase η (hPol η). Here we report the crystal structure of a ternary Pol•DNA•dCTP complex between a C8-

**Supported by NIH grants P01 CA-160032 & R01 CA-55678 (M.E., M.P.S.), R01 ES-05509 (M.P.S), R01 ES-009127 & R01 ES-021762 (A.K.B.), and P30 CA-68485 (VICC).

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Supporting Information (experimental procedures, Table S1, Figures S1, S2) for this article is available via the WWW. Atomic coordinates and structure factors have been deposited in the Protein Data Bank (<http://wwpdb.org/>): PDB code 5JUM.

dG-ABA containing template: primer duplex and hPol η . The complex was captured at the insertion stage and provides crucial insight into the mechanism of error-free bypass of this bulky lesion. Specifically, bypass involves accommodation of the ABA moiety inside a hydrophobic cleft to the side of the enzyme active site and formation of an intra-nucleotide H-bond between phosphate and ABA amino moiety, allowing the adducted guanine to form a standard Watson-Crick pair with the incoming dCTP.

Keywords

Human DNA Polymerase η ; X-ray Crystallography; 3-Nitrobenzanthrone; DNA Damage; Lesion Bypass; Environmental Carcinogen

3-Nitrobenzanthrone (3-nitro-7H-benz-[d,e]anthracen-7-one, 3-NBA, Scheme 1) is a product of incomplete combustion^[1,2] and has been identified as a component of diesel exhaust^[2–4] and as an environmental contaminant.^[1,5] The International Association for Research on Cancer (IARC) has classified diesel exhaust as carcinogenic to humans^[6] and 3-NBA is a suspected culprit of this carcinogenicity.^[1,6] 3-NBA has been found to be both highly mutagenic^[7–10] and genotoxic,^[11–15] and is itself classified as a potential human carcinogen by the IARC.^[6]

Enzymatic reduction *in vivo* engenders a conversion of 3-NBA to aminobenzanthrone (ABA, Scheme 1).^[16,17] Intermediates in the reduction pathway can form reactive nitrenium ions that alkylate DNA resulting in ABA adducts.^[18–20] Three different adducts have been identified, and they occur on the C8 (Scheme 1) or N^2 positions of guanine (major adducts), or the N^6 position of adenine (minor adduct).^[21,22] Due to the bulky nature of these adducts, it is believed that they present strong blocks to replicative polymerases and bypass is instead accomplished by lower-fidelity translesion synthesis (TLS) polymerases.^[8,23,24] In humans these include the Y-family Pols η , ι , κ and Rev1.^[23–25]

However, C8-dG-ABA, one of the two major adducts formed,^[8,21] has previously been implicated in significant blocking of bypass by TLS polymerases in a nucleotide excision repair-deficient human cell line.^[8] When bypass did occur, of the two dG and one dA adducts, the C8-dG-ABA adduct triggered the highest mutation frequency.^[8] Nevertheless, the majority of bypass events were non-mutagenic, with nearly 70% correct incorporation of

cytosine reported.^[8] Another study specifically examined the C8-dG-ABA adduct in human embryonic kidney (HEK293T) cells, and also observed correct incorporation of cytosine opposite this lesion in 86% of bypass events.^[26] A series of siRNA knockdown experiments showed that each of the Y-family polymerases contributed to the bypass of this lesion.^[26] However, hPol η played the most significant role in the mutagenic insertion step, with a decrease in mutation frequency of 39% upon knockdown of hPol η .^[26]

Using a physiological concentration of each nucleotide (100 μ M), hPol η incorporated correct dCTP with a probability of 59% opposite the C8-dG-ABA lesion (Figure 1A) after 10 s of incubation (Figure 1B, left). The probability is defined as the percentage of the incorporation of a specific nucleotide among all four possible nucleotide incorporations opposite a template nucleotide. Opposite the lesion, there was also incorporation of incorrect nucleotides following the pattern of dATP (28%) > dTTP (9%) > dGTP (5%). At longer reaction time points (30s and 120 s, Figure 1B), the probability of a misincorporation event was greater because correct dCTP incorporation was almost completed while more misincorporation occurred.

Using pre-steady-state kinetics for measuring the rates of nucleotide incorporation by hPol η onto the 20/26-mer-C8-dG-ABA DNA substrate (Figure 1A),^[27] we calculated the observed rate constants (k_{obs}) for each dNTP at 100 μ M by using the hyperbolic equation ($k_{\text{obs}} = k_p + [\text{dNTP}]/(K_d + [\text{dNTP}])$), where k_p is the maximum dNTP incorporation rate constant, and K_d is the apparent equilibrium dissociation constant of the dNTP. For example, to calculate the estimated probability for correct incorporation of dCTP at 100 μ M, we took that k_{obs} and divided it by the sum of k_{obs} values obtained for all dNTPs on the same substrate ($(k_{\text{obs, dCTP}} / \sum k_{\text{obs, dNTP}}) \times 100\%$). Based on our calculation, the probability for hPol η to correctly insert dCTP was 81%, higher than 59% observed after the 10s reaction time in the single-nucleotide incorporation assay (Figure 1B). This suggests that 10s was still too long to accurately estimate the correct nucleotide incorporation probability opposite the lesion. However, the kinetic probabilities of hPol η incorporating incorrect dNTPs opposite C8-dG-ABA follow the pattern of dATP (13%) > dTTP (4%) > dGTP (2%), which is similar to the one depicted in Figure 1B.

To gain a better understanding of the mechanism of error-free bypass of the C8-dG-ABA adduct by hPol η , we determined the crystal structure of a ternary Pol•DNA•dCTP complex trapped at the insertion stage. The hPol η catalytic core encompassing residues 1–432 was expressed in *E. coli* and purified following published protocols^[28] (see the Supporting Information for materials and methods). The DNA duplex is composed of a 12-mer template with the C8-dG-ABA lesion (X) 5'-CAT XAT GAC GCT-3' and an 8-mer primer 5'-AGC GTC AT-3'. Crystals of the complex with dCTP were grown in the presence of Ca^{2+} instead of Mg^{2+} to prevent incorporation of the incoming nucleoside triphosphate into the primer strand. The structure was phased with molecular replacement using hPol η as the search model and refined to a resolution of 2.6 Å.

A summary of crystal data, data collection and refinement parameters is given in Table 1 and an example of the electron density around the final model of the complex is depicted in Figure 2. The final model allows visualization of 10 of the template nucleotides (the 5'-

terminal C and A outside the active site are disordered) and the entire primer strand. Perhaps surprisingly, the C8-dG-ABA adducted nucleotide adopts the *anti* conformation at the active site of hPol η (Figure 3, Figure S1). The conformation is stabilized by a H-bond between the ABA amino moiety and the phosphate group, with the C8-dG-ABA 2'-deoxyribose adopting a C3'-*endo* pucker.

As a consequence the adducted G forms a standard Watson-Crick base pair with the incoming dCTP. The ABA adduct is tilted relative to the guanine plane by about 80° and rotated outwards into the major groove of the template-primer duplex.

However, rather than exhibiting enhanced conformational flexibility that is often associated with portions of a structure that are extruded from the duplex, the structure offers evidence that the polyaromatic hydrocarbon moiety assumes a configuration that is stabilized by multiple interactions. Thus, ABA forms a sandwich with the amide group of Ser-62 and the T3 template residue that is lodged outside the active site. The carbonyl oxygen of Ser-62 engages in an $n \rightarrow \pi^*$ interaction with the ABA keto group and C β -H and C2'-H from Ser-62 and T3, respectively, engage in C-H $\rightarrow \pi$ interactions with aromatic rings from opposite sides of the ABA plane (Figure 3). Trp-64 seals the backside of this mostly hydrophobic pocket. Both, the keto oxygens of ABA and T3 (O2) are too far removed from Trp-64 N-H (distances of 3.71 and 3.83 Å, respectively; Figure S1) to engage in H-bonds.

The extensive interactions observed for ABA at the hPol η active site extend to the base, sugar and phosphate portions of the adducted dG (Figure 3, Figure S1). On the minor groove side, the NH₂ group of Gln-38 from the finger domain forms H-bonds with O4' of the 2'-deoxyribose and N3 of the base. The C γ 2(H)₃ methyl group of Ile-48 stacks onto the six-membered ring of guanine. In the major groove the hydroxyl group of Ser-61 is positioned somewhat far to establish H-bonds with the O δ and N7 acceptor functions of guanine (distances of 3.80 and 3.75 Å, respectively). Finally, the OH group of Tyr-39 stabilizes the template strand conformation with a H-bond to the non-bridging OP1 phosphate oxygen of C8-dG-ABA. On the opposite side of the active site, the guanidino moiety of Arg-61 from the finger domain stacks on top of the nucleobase of dCTP. The NH₂ moiety of the arginine forms salt bridges with the α - and β -phosphate groups and helps align the former for attack by the 3'-OH of the terminal primer nucleotide (distance O3'...P α = 3.85 Å) together with the two divalent metal ions.

The tight fit between the nascent C8-dG-ABA:dCTP base pair and hPol η is further illustrated in Figure 4 which depicts a space filling model of the active site of the polymerase. Finger residues Ser-62 and Arg-61 (foreground, center) protrude from the ceiling along with Gln-38, and bear down on the G:C base-pair plane at the split between the primer and template strands.

Two metal ions are accommodated in a pocket on one side of the active site, by conserved Glu and Asp residues. On the other side, where the template strand enters the active site, the ABA adduct fits snugly into a cleft formed by Ser-62, Met-63 (main chain) and Trp-64 and extended by T3 from the template strand. The structure thus offers insight into how hPol η

stabilizes a configuration of the template strand that allows error-free bypass of the bulky C8-dG-ABA lesion.

We recently reported the NMR solution structure of a DNA 12mer duplex containing a C8-dG-ABA nucleotide opposite dC.^[29] In this structure, ABA was intercalated in the duplex, with the adducted G in the *syn* conformation and extruded into the major groove. The intercalated adduct thus takes the place of a base pair and thereby forces the opposing cytosine into the major groove (Figure S2). We superimposed this DNA duplex model with base pairs on the 5'-side of the adducted dG deleted onto the template-primer duplex of the ternary hPol η complex, such that the intercalated ABA assumes the position of the T:A pair at the -1 position at the Pol active site (Figure 5A). This configuration illustrates a potential basis for error-prone bypass of C8-dG-ABA, in that incoming dATP or dGTP might be preferred due to favorable stacking. Insertion could then proceed in a non-templated fashion, causing a mutation, or opposite a residue located 5'-adjacent to C8-dG-ABA, thus causing a frameshift.

Drastically different conformations and interactions of aromatic moieties depending on the environment, i.e. inside a DNA duplex studied alone or contained in a template-primer strand at the active site of a DNA polymerase, have previously been observed. For example, the dNaM and d5SICS components of an unnatural base pair that lack the ability to establish H-bonds are stacked in the structure of an isolated DNA duplex. However, at the active site of KlenTaq DNA polymerase, dNaM in the template strand and incoming d(5SICS)TP form a side-by-side Watson-Crick like base pair.^[31]

hPol η inserts dCTP ca. 3.5-fold more efficiently than dATP opposite another C8 adduct of dG, 8-oxoG.^[32] Incorporation of dATP occurs with the adducted dG in the *syn* orientation and stacked inside the template strand. However, in comparison to the C₈=O₈ moiety of 8-oxoG, the ABA adduct is far too bulky to be accommodated within the minor groove.

The enzyme from yeast (scPol η) is known to bypass bulky acetylaminofluorene DNA adducts, such as *N*-2'-(deoxyguanosin-8-yl)-2-acetylaminofluorene (AAF-dG). But the presence of the acetyl moiety precludes formation of a H-bond between amino group and 5'-phosphate and thus the *anti* conformation. Hence bypass of the lesion is error-prone, with insertion of all four dNTPs opposite AAF-dG.^[33] Consistent with these observations at the functional level, crystal structures of the complex between scPol η and a DNA duplex that contains AAF-dG show aminofluorene stacked on the adjacent template-primer duplex in one case, with dG rotated into the major groove, and, dG rotated toward the templating position and partially freeing the active site in a second case (Figure 6A).^[33] Neither structure contained an incoming dNTP. Crystal structures of ternary complexes between the Y-family TLS DNA polymerase Dpo4 from *Sulfolobus solfataricus* and AF-dG at the -1-position (post-replicative, extension state) revealed both *syn*- and *anti*-orientations of the adducted nucleotide.^[34] Thus, in the former structure, the AF moiety is stacked between the base pair at the -2 position and the nascent dC:dGTP pair, with the primer residue opposite AF-dG disordered in the model (Figure 6B). In the structure with the *anti*-orientation of AF-dG, the adducted nucleotide forms a Watson-Crick pair with primer dC, followed by the nascent dC:dGTP pair (Figure 6B).^[34]

Incorporation of AF-dG (but not of AAF-dG), albeit at a much reduced rate compared to that of native nucleotides, has also been observed for a replicative, high-fidelity DNA polymerase, the thermophilic *Bacillus* DNA polymerase I fragment (BF). The crystal structure of the BF:DNA complex at the pre-insertion state shows the adducted residue in a *syn* conformation, and in the *anti* conformation in the post-insertion state and forming a Watson-Crick pair with dC, with the AF moiety protruding into the major groove.^[35]

In summary, we present the first crystal structure of hPol η in complex with a bulky C8-dG aromatic amine lesion. The structure provides a model for the correct (Figure 1) but slow bypass of the C8-dG-ABA adduct.^[26] Finally, the C8-dG-ABA:hPol η complex constitutes the second example of an insertion-stage bypass structure with an adduct lodged in the hydrophobic cleft to the side of the active site. A somewhat similar orientation was found for *O*⁴MedT (Figure 5B) that is replicated by hPol η in an error-prone fashion.^[30] However, unlike in the case of C8-dG-ABA where only the ABA portion is inserted into the cleft, the entire *O*⁴MedT nucleobase can be accommodated inside it, with the lesion reaching all the way to the back of the pocket.

Experimental Section

See supporting information for further details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Single-nucleotide incorporation opposite a C8-dG-ABA lesion catalyzed by hPol η at 37 °C. (A) 20/26-mer-C8-dG-ABA; the adducted dG is marked by an X. (B) A pre-incubated solution of hPol η (130 nM) and 32 P-labeled 20/26-mer-C8-dG-ABA DNA substrate (30 nM) was mixed with an indicated dNTP (100 μ M) for 10 s, 30 s, or 120 s, before the reaction was quenched with 0.37 M EDTA. Product concentration ([P]) was quantified by calculating the intensity ratio of the P band(s), over the sum of the P band(s), and the band of remaining 20-mer, and multiplying by the total DNA concentration (30 nM). The probability for dNTP incorporation opposite C8-dG-ABA is calculated as $([P] \text{ from dNTP incorporation} / \Sigma[P] \text{ from each dNTP incorporation}) \times 100\%$. “Blank” indicates no addition of dNTPs.

Figure 2.

Quality of the final Fourier $2F_o - F_c$ sum electron density (1σ threshold) around the active site region of the ternary complex between hPol η , C8-dG-ABA adducted template-primer duplex and dCTP. Carbon atoms of hPol η , C8-dG-ABA and dCTP are colored in light green, magenta and yellow, respectively, Ca^{2+} ions are pink spheres, and the remainder of the duplex is colored in olive.

Figure 3.

Active site configuration in the ternary hPol η -insertion-step complex with dCTP opposite C8-dG-ABA. (A) View into the DNA major groove, and (B) rotated by $\sim 90^\circ$ around the horizontal axis and looking roughly along the normal to the nucleobase plane of the incoming dCTP. Carbon atoms of C8-dG-ABA, incoming dCTP, and selected hPol η side chains are colored in magenta, yellow, and light green, respectively, and Ca^{2+} ions are pink spheres.

Figure 4. Space filling model of the hPol η active site with C8-dG-ABA (magenta carbon atoms) accommodated inside a cleft to the side of the active site that allows for extensive hydrophobic interactions between ABA moiety and surrounding residues from the polymerase finger domain. Carbon atoms of the incoming dCTP are colored in yellow.

Figure 5.

Model of error-prone bypass of C8-dG-ABA and comparison of hPol η complexes with the C8-dG-ABA and *O*⁴MedT adducts. (A) Superimposition of the crystal structure of the hPol η (Pol ribbon in cyan) complex with C8-dG-ABA (magenta/olive) and dCTP (yellow) and the NMR structure of DNA (lilac) with the C8-dG-ABA (light green) lesion.^[29] (B) Superimposition of the hPol η complexes with C8-dG-ABA (magenta/olive)/dCTP (yellow; Pol ribbon in cyan) and *O*⁴MedT (light green/lilac)/dATP (brown; Pol ribbon in pink).^[30]

Figure 6.

Conformations of dG C8-aromatic amine adducts at the active sites of TLS DNA polymerases. (A) Superimposition of the template-primer duplexes with *anti* C8-dG-ABA (hPol η , this work) and *syn* AAF-dG in complex with scPol η (PDB ID 2XGP, complex B).^[33] The DNA conformations deviate considerably (r.m.s.d. sugar-phosphate backbone atoms $> 2\text{\AA}$) and the AAF moiety in the scPol η complex is lodged in the minor groove which is impossible with the human enzyme. (B) Superimposition of post-insertion complexes of Dpo4 with *syn* and *anti* AF-dG (PDB IDs 3KHG and 3KHH, resp.).^[34] The

AF moiety in the latter is lodged in the major groove. Neither the complexes of AAF-dG with scPol η nor those of Dpo4 with AF-dG constitute good models for the bypass of C8-dG-ABA by hPol η .

Scheme 1.

Chemical structures of 3-NBA, N-OH-ABA and one of the major *in vivo* ABA adducts: C8-dG-ABA.

Table 1

Selected crystal data, data collection parameters, and structure refinement statistics

<i>Data Collection</i>	
Space group	$P6_1$
Resolution [$\text{\AA}$]	50.0-2.60 (2.64-2.60)
Unit cell $a=b$, c [$\text{\AA}$]	99.11, 81.69
Completeness [%]	100 (100)
$I/\sigma(I)$	15.3 (2.0)
R-merge [%]	13.3 (88.8)
Redundancy	7.6 (7.5)
<i>Refinement</i>	
R-work [%]	16.6 (21.4)
R-free [%] ^b	23.8 (28.1)
Avg. B [$\text{\AA}^2$]	39.0
R.m.s.d. bonds [$\text{\AA}$]	0.009
R.m.s.d. angles [deg.]	1.1

^aStatistics for the highest-resolution shell are shown in parentheses.^bBased on 5% of the reflections.