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Translesion synthesis of 6-nitrochrysene-derived 2′-deoxyadenosine adduct in human cells

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Abstract

6-Nitrochrysene (6-NC) is a potent mutagen in bacteria and carcinogenic in animals. It is the most potent carcinogen ever tested in newborn mouse assay. DNA lesions resulting from 6-NC modification are likely to induce mutations if they are not removed by cellular defense pathways prior to DNA replication. Earlier studies showed that 6-NC-derived C8–2'-deoxyadenosine adduct, *N*-(dA-8-yl)-6-AC, is very slowly repaired in human cells. In this study, we have investigated replication of *N*-(dA-8-yl)-6-AC in human embryonic kidney (HEK 293T) cells and the roles of translesion synthesis (TLS) DNA polymerases in bypassing it. Replication of a plasmid containing a single site-specific *N*-(dA-8-yl)-6-AC adduct in HEK 293T cells showed that human DNA polymerase (hPol) η and hPol κ played important roles in bypassing the adduct, since TLS efficiency was reduced to 26% in the absence of these two polymerases compared to 83% in polymerase-competent HEK 293T cells. The progeny from HEK 293T cells provided 12.7% mutants predominantly containing A→T transversions. Mutation frequency (MF) was increased to 17.8% in hPol η-deficient cells, whereas it was decreased to 3.3% and 3.9% when the adduct containing plasmid was replicated in hPol κ- and hPol ζ-deficient cells, respectively. The greatest reduction in MF by more than 90% (to MF 1.2%) was observed in hPol ζ-knockout cells in which hPol κ was knocked down. Taken together, these results suggest that hPol κ and hPol ζ are involved in the error-prone TLS of *N*-(dA-8-yl)-6-AC, while hPol η performs error-free bypass.

Graphical Abstract

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AUTHOR STATEMENT

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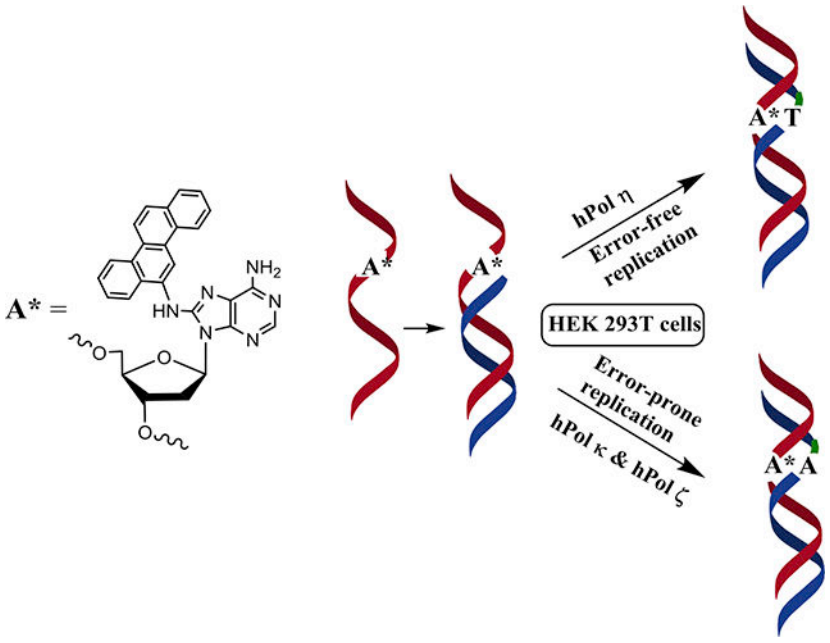
Conflict of interest statement

The authors declare no conflict of interest.



Keywords

nitroaromatic compounds; 6-aminochrysene; carcinogen-DNA adduct; translesion synthesis; mutagenicity; cellular replication

1. INTRODUCTION

Nitropolycyclic aromatic hydrocarbons (NO_2-PAHs) are common environmental pollutants as they are formed during combustion of diesel and other fossil fuels [1–4]. They are also present in certain foods and beverages. The International Agency for Research on Cancer (IARC) labeled diesel exhaust as “carcinogenic to humans” (Group 1) and one of its NO_2-PAH contaminant 6-nitrochrysene (6-NC) as “probably carcinogenic to humans” (Group 2A) [5]. 6-NC is mutagenic in bacteria and carcinogenic in experimental animals [6–9]. It is the most potent carcinogen ever tested in the newborn mouse assay and its carcinogenic potency in rat mammary gland is higher than that of the potent carcinogens benzo[*a*]pyrene and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) [9–11].

6-NC is metabolically activated by two different pathways: the first involves nitroreduction to form 6-hydroxyaminochrysene (*N*-OH-6-AC) and the second involves both nitroreduction and ring oxidation. *N*-OH-6-AC, generated in the first pathway forms three DNA adducts, *N*-(dG-8-yl)-6-AC (**1**), 5-(dG-N^2-yl)-6-AC (**2**), and *N*-(dI-8-yl)-6-AC (**4**), the latter being produced by deamination of the 2′-deoxyadenosine adduct, *N*-(dA-8-yl)-6-AC (**3**) (Scheme 1) [12–15]. The second pathway generates *trans*-1,2-dihydroxy-1,2-dihydro-6-hydroxyaminochrysene (1,2-DHD-6-NHOH-C), which forms 5-(dG-N^2-yl)-1,2-DHD-6-AC, *N*-(dG-8-yl)-1,2-DHD-6-AC, and *N*-(dA-8-yl)1,2-DHD-6-AC [14]. All these DNA adducts have been detected in several organs of rats treated with 6-NC orally or by intraperitoneal injection [12, 14].

When the nucleotide excision repair (NER) efficiency of *N*-(dG-8-yl)-1,2-DHD-6-AC, *N*-(dG-8-yl)-6-AC, and *N*-(dA-8-yl)-6-AC were compared in HeLa cell extracts, *N*-(dG-8-yl)-6-AC repair was most facile [16], even though *N*-(dG-8-yl)-6-AC was estimated to be 15-fold more resistant to NER than the *cis*-Pt adduct [17]. NER of *N*-(dG-8-yl)-1,2-DHD-6-AC is ~2-fold more resistant than *N*-(dG-8-yl)-6-AC whereas *N*-(dA-8-yl)-6-AC is repaired 8-fold more slowly than *N*-(dG-8-yl)-6-AC [16]. It was speculated that slow repair of the 6-NC adducts and thus their persistence in mammalian tissues play a part in the carcinogenicity of 6-NC [16]. However, mutagenicity of these adducts in mammalian cells have never been reported.

We have recently developed a total synthesis method to prepare site-specifically incorporated *N*-(dA-8-yl)-6-AC in any desired sequence [18]. We have also shown that this adduct is mutagenic in *Escherichia coli*. In this article, we report the mutagenicity of this adduct in human embryonic kidney (HEK) 293T cells. We also determined the roles of the translesion synthesis (TLS) DNA polymerases on the bypass and mutagenicity of the DNA adduct.

2. MATERIALS AND METHODS

2.1. Materials

All materials, including reagents and solvents, were of commercial grade. [$\gamma-^{32}$P] ATP was purchased from Perkin Elmer Health Sciences Inc. (Shelton, CT). The enzymes were purchased from New England Biolabs (Beverly, MA). All unmodified oligodeoxynucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Synthetic siRNA duplex against hPol η (SI02663619), hPol κ (SI04930884), and negative control siRNA (1027280) were purchased from Qiagen (Valencia, CA). HEK 293T cells with knockout of a single TLS polymerase hPol η, hPol κ, hPol ι, and hPol ζ, and simultaneous knockout of hPol η/hPol ζ and hPol η/hPol κ were a gift from Professor Yinsheng Wang (University of California, Riverside, CA), which were produced by using the CRISPR-Cas9 genome editing method [19, 20].

2.2. Methods

2.2.1. Construction and characterization of a pMS2 vector containing a single *N*-(dA-8-yl)-6-AC adduct and its replication in HEK 293T cells.—We have constructed a single-stranded pMS2 vector containing a single *N*-(dA-8-yl)-6-AC adducted site. This vector contained neomycin and ampicillin resistance genes and was prepared as previously reported [21, 22]. Briefly, the pMS2 DNA (100 pmols) was digested with an excess of *EcoR*V (500 pmol, 8.1 μg) for 4 h at 37 °C, followed by room temperature overnight. A mixture of a 4-fold excess of a 60-mer scaffold oligonucleotide and the digested plasmid was incubated in a water bath heated to 75 °C, which was allowed to cool to room temperature and then left overnight at 4 °C to form a gapped DNA template. The control and *N*-(dA-8-yl)-6-AC containing oligonucleotides were phosphorylated with T4 polynucleotide kinase, hybridized to the gapped pMS2 DNA, and ligated overnight at 16 °C. Unligated oligonucleotides were removed by passing them through a Centricon-100, and the DNA was precipitated with ethanol. The scaffold was removed by T4 DNA polymerase (16

units, 16 °C, 1 h), and the construct was purified by phenol extraction followed by Sephadex G25 filtration. The HEK 293T or TLS polymerase knockout HEK 293T cells were grown to 60–80% confluency and transfected with 150 ng of construct in 6 μL of Lipofectamine cationic lipid reagent (Invitrogen, Carlsbad, CA). Following transfection with modified or unmodified pMS2 vector, the cells were allowed to grow at 37○C in 5% CO_2 for 24 h and the plasmid DNA was collected and purified. It was used to transform *E. coli* (DH10B) and transformants were analyzed by oligonucleotide hybridization followed by DNA sequence analysis [22, 23].

2.2.2. TLS assay in human cells.—*N*-(dA-8-yl)-6-AC-containing or control pMS2 vector was mixed with a single-stranded pMS2 DNA construct with a 15-mer oligonucleotide sequence at the ligation site different from the *N*-(dA-8-yl)-6-AC containing (or control) DNA sequence, which was used as an internal control. The molar ratio of internal control to unmodified control vector was 1:1, and those for the internal control to *N*-(dA-8-yl)-6-AC containing vector was 1:3. As expected, this construct gave equal number of progeny as the control vector. Transfection was carried out as described above. TLS efficiency was determined as the percentages of the colonies originating from the *N*-(dA-8-yl)-6-A containing plasmid relative to the internal control vector.

2.2.3. Mutational analyses of TLS products from human cells.—For the siRNA knockdown experiments, prior to transfection of the control and *N*-(dA-8-yl)-6-AC containing vectors, synthetic siRNA duplex was transfected into HEK 293T cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA). HEK 293T cells were plated in 6-well plates at 50% confluence. After 24 h incubation, they were transfected with 100 pmoles of siRNA duplex mixed with Lipofectamine, diluted in Opti-MEM (Gibco), per well. One day before transfection of the plasmid, cells were seeded in 24-well plates at 70% confluence. Cells were then co-transfected with another aliquot of siRNA and either control plasmid or lesion-containing plasmid. After 24 h incubation, progeny plasmids were isolated as described earlier. The efficiency of knockdown, determined by RT-PCR, was at least 70% [22].

3. RESULTS AND DISCUSSION

3.1. Experimental system.

Our experimental system involves construction of an *N*-(dA-8-yl)-6-AC containing single-stranded plasmid pMS2 that carries f1 and SV40 origins of replication, neomycin and ampicillin resistance genes, the SV40 early promoter, SV40 small tumor antigen splice sites, and SV40 early poly-adenylation site, which enable it to be replicated in both mammalian cells and *E. coli* [24]. The lesion containing pMS2 construct was mixed with an unmodified plasmid that contained a different DNA sequence at the site of ligation and the mixed DNA was transfected in HEK 293T cells. The latter was used as an internal control to calculate the TLS efficiency. DNA recovered from the transfected HEK 293T cells was used to transform *E. coli* cells for amplification and analysis (see Figure S1 in SI).

The mechanism of replication of circular ssDNA is well understood in bacteria, but it has not been clearly defined in mammalian cells. However, based on several studies, a model has

been proposed. The studies on porcine circovirus, the genome of which contains circular ssDNA, showed that DNA polymerase α-primase synthesizes primers at several sites on ssDNA [25]. Méchali and Harland suggested that the mechanism is similar to lagging strand synthesis [26]. PCNA, the processivity factor for the eukaryotic DNA polymerase, is needed for the DNA synthesis on ssDNA [26]. This suggests that the replicative DNA polymerases Pol α and δ catalyze DNA synthesis in mammalian cells. However, lesion bypass may require participation of TLS polymerases.

3.2. Construction of a plasmid containing a single *N*-(dA-8-yl)-6-AC adduct and its replication in HEK 293T cells.

A 15-mer 5′-GCCCTCAA*CAAGATG where A* denotes *N*-(dA-8-yl)-6-AC was synthesized as reported [18]. The DNA sequence of this oligonucleotide was selected from the *TP53* gene codon 129–133, since codon 131 is a hotspot for A→T transversions in patients with urothelial cancers by exposure to another nitroaromatic carcinogen, aristolochic acid [27–29]. The 15-mer containing *N*-(dA-8-yl)-6-AC and a control were ligated to a gapped plasmid DNA to prepare site-specifically modified pMS2 plasmid constructs, according to a method reported earlier [18]. The lesion containing pMS2 construct and an unmodified plasmid DNA (containing a 20-mer sequence 5′-AGGGTTTACCCAGTCACGTT-3′ at the ligation site different from the DNA sequence of either the lesion containing or control DNA) in 3:1 ratio were co-transfected into HEK 293T cells with or without deficiency in TLS polymerases. As indicated earlier, the unmodified construct was used as an internal control, and equal number of progeny was obtained when it was replicated with an equal amount of control plasmid DNA. Cells were incubated for 24 h to allow for one round of replication, following which the DNA of the replicates were isolated and used to transform *E. coli* DH10B cells. The percentages of the colonies originating from the lesion-containing plasmid relative to the unmodified plasmid, indicating the percentage of TLS, were determined by oligonucleotide hybridization followed by DNA sequencing. The experimental procedure for construction of the DNA adduct containing plasmid, its replication in HEK 293T cells, and TLS efficiency and mutational analyses of the progeny are schematically displayed in Figure S1 in the SI. The DNA sequences of the hybridization probes are provided in Figure S2 in the SI.

3.3. TLS efficiency in human cells.

In HEK 293T cells, the TLS efficiency for the *N*-(dG-8-yl)-6-AC containing plasmid was 83±3%, as compared to 100% progeny generated from the undamaged DNA (Figure 1). Next, we determined the TLS efficiency of the adduct containing construct in cells deficient in various TLS polymerases (hPol η, hPol κ, hPol ι, and hPol ζ). While cells with hPol ι knockout had little effect on replication, TLS efficiency were reduced to 45±3%, 60±2%, and 72±2% in hPol η-, hPol κ-, and hPol ζ-deficient cells, respectively. TLS efficiency was further reduced in deficiency in two TLS polymerases. For example, TLS efficiency was 34±2% in cells with simultaneous knockout of hPol η and hPol ζ. Likewise, when hPol κ was knocked down in hPol ζ knockout cells, TLS efficiency of 54±2% was lower than either polymerase-deficient cell lines. The largest reduction in TLS efficiency to 26±1% was observed in cells with simultaneous knockout of hPol η and hPol κ. This suggests that

primarily hPol η and hPol κ but also hPol ζ play a role in bypassing this DNA adduct in HEK 293T cells.

3.4. Error-free and error-prone TLS of *N*-(dA-8-yl)-6-AC in human cells.

DNA sequence analysis indicated that 12.7±2.7% of the progeny from the HEK 293T cells were mutants, which comprised of ~11% targeted and 1.7% semi-targeted mutations (Figure 2). Semi-targeted mutations are defined as base substitutions and deletions detected near *N*-(dG-8-yl)-6-AC even though the lesion might have been replicated correctly as a template adenine. Targeted mutations included ~7% A*→T, 3% A*→G, and 0.5% A*→C (Figure 3). Mutation frequency (MF) was increased to 17.8±0.2% in hPol η-deficient cells, whereas it decreased to 3.3±0.5%, 10.1±0.6%, and 3.9±0.1% in hPol κ-, hPol ι-, and hPol ζ-deficient cells, respectively. This suggests that hPol η is involved in error-free bypass of *N*-(dG-8-yl)-6-AC, whereas hPol κ and hPol ζ play critical roles in error-prone bypass. hPol ι also has a role in error-prone bypass of the DNA adduct, though its effect was modest. In cells carrying simultaneous knockouts of hPol η and hPol ζ, the MF (4.4±0.3%) was only slightly higher than that in hPol ζ-deficient cells. Likewise, in cells with simultaneous knockout of hPol η and hPol κ, the MF (4.9±0.2%) was higher than that in hPol κ-deficient cells but much lower than that in hPol η-deficient cells. The most pronounced effect on MF was noted in hPol ζ-deficient cells in which hPol κ was knocked down, when the MF dropped to 1.2±0.2%.

In each of these cases the major type of targeted mutation was A*→T, except in hPol κ-deficient cells, in which the major type of targeted mutation was A*→G (Figure 3). This suggests that hPol κ is involved in A*→T mutations. However, in cells deficient in both hPol κ and hPol ζ, A*→T mutation was the foremost targeted mutations. We hypothesize that the error-prone base insertion of A and to a lesser extent C opposite *N*-(dG-8-yl)-6-AC is carried out by hPol κ, but extension for both is executed by hPol ζ. In contrast, hPol η performs a significant fraction of error-free bypass of *N*-(dG-8-yl)-6-AC. Semi-targeted mutations at low level were detected in all cases, which frequently occurred at the 5′ A or 3′ C adjacent to *N*-(dG-8-yl)-6-AC. We believe that the semi-targeted mutations were the result of helix destabilization by the DNA adduct, which have been detected at a low level with other bulky adducts [22, 30] and at a high frequency with certain intra-strand DNA-DNA (e.g., G[8,5-Me]T and T[5- Me,8]G) and DNA-peptide cross-links [31, 32].

Based on these results we propose Scheme 2 for error-free and error-prone TLS of *N*-(dA-8-yl)-6-AC.

It is remarkable that *N*-(dA-8-yl)-6-AC induces predominantly A*→G mutations in both uninduced and SOS-induced *E. coli* cells, and A*→T mutations occur at a much lower frequency [18]. But in human cells the trend is reversed with A*→T occurring at a much higher frequency than A*→G. This suggests a different role of the leading TLS polymerases in TLS of the adduct in these two organisms. It is also interesting that in cells with deficiency in both hPol κ and hPol ζ MF was very low (Figure 2 & 3), but the efficiency of TLS was significant and much higher than hPol η/hPol ζ or hPol η/hPol κ double knockout cells (Figure 1). This further implies a critical role in error-free bypass of *N*-(dA-8-yl)-6-AC by hPol η.

4. Conclusions

While mutagenicity of many bulky dG adducts in mammalian cells have been published [33], there is a paucity of reports on the mutagenicity of bulky dA adducts. Of the limited number of studies on the mutagenic outcome of bulky dA adducts [34–40], none has evaluated a bulky C8-dA adduct. To our knowledge, this is the first study on replication of a bulky NO_2-PAH-derived C8-dA adduct in human cells, which showed that its bypass relies heavily on the TLS polymerases and that it is mutagenic inducing A*→T and A*→G mutations. The mutations are induced when bypass is conducted by a coordination of hPol κ and hPol ζ, whereas error-free TLS is carried out by hPol η.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

NO_2-PAH	nitropolycyclic aromatic hydrocarbons
6-NC	6-nitrochrysene
***N*-(dG-8-yl)-6-AC**	*N*-(deoxyguanosine-8-yl)-6-aminochrysene
5-(dG-N^2-yl)-6-AC	5-(deoxyguanosine-N^2-yl)-6-aminochrysenene
***N*-(dA-8-yl)-6-AC**	*N*-(deoxyadenosine-8-yl)-6-aminochrysene
NER	nucleotide excision repair
PAH	polynuclear aromatic hydrocarbon
MF	mutation frequency
TLS	translesion synthesis

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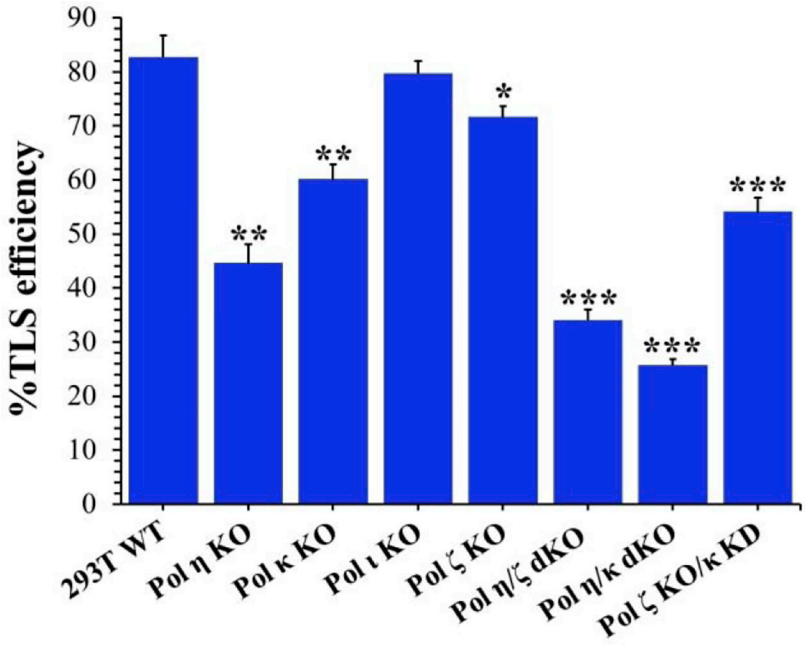


Figure 1.

The TLS efficiencies of the *N*-(dA-8-yl)-6-AC containing construct in HEK 293T cells and various TLS polymerase knockout (KO) HEK 293T cells. The data represent the means and standard errors of the mean from 2–4 independent replication experiments. The statistical significance of the difference in % TLS efficiency between HEK 293T and TLS pols knockouts was calculated using two-tailed, unpaired Student's t test. (*P < 0.05; **P < 0.01; ***P < 0.001).

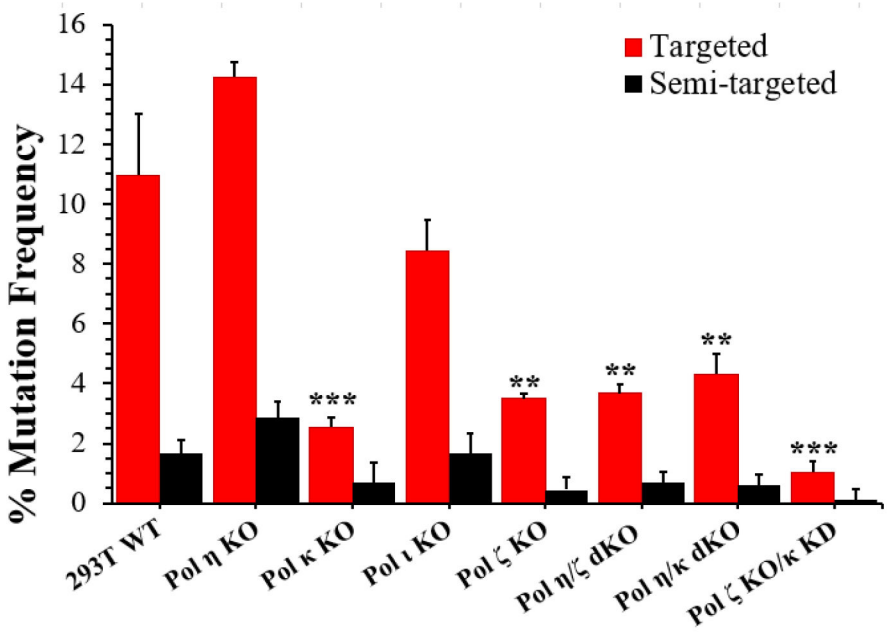


Figure 2.

Targeted and semi-targeted mutations induced in the progeny from the *N*-(dA-8-yl)-6-AC construct in HEK 293T and various polymerase knockout (KO) cells. The data represent the mean and the standard deviation (of the total MF) from 2–4 independent experiments. The statistical significance of the difference in MFs between HEK 293T and TLS pols knockouts was calculated using two-tailed, unpaired Student's t test. (*P < 0.05; **P < 0.01; ***P < 0.001).

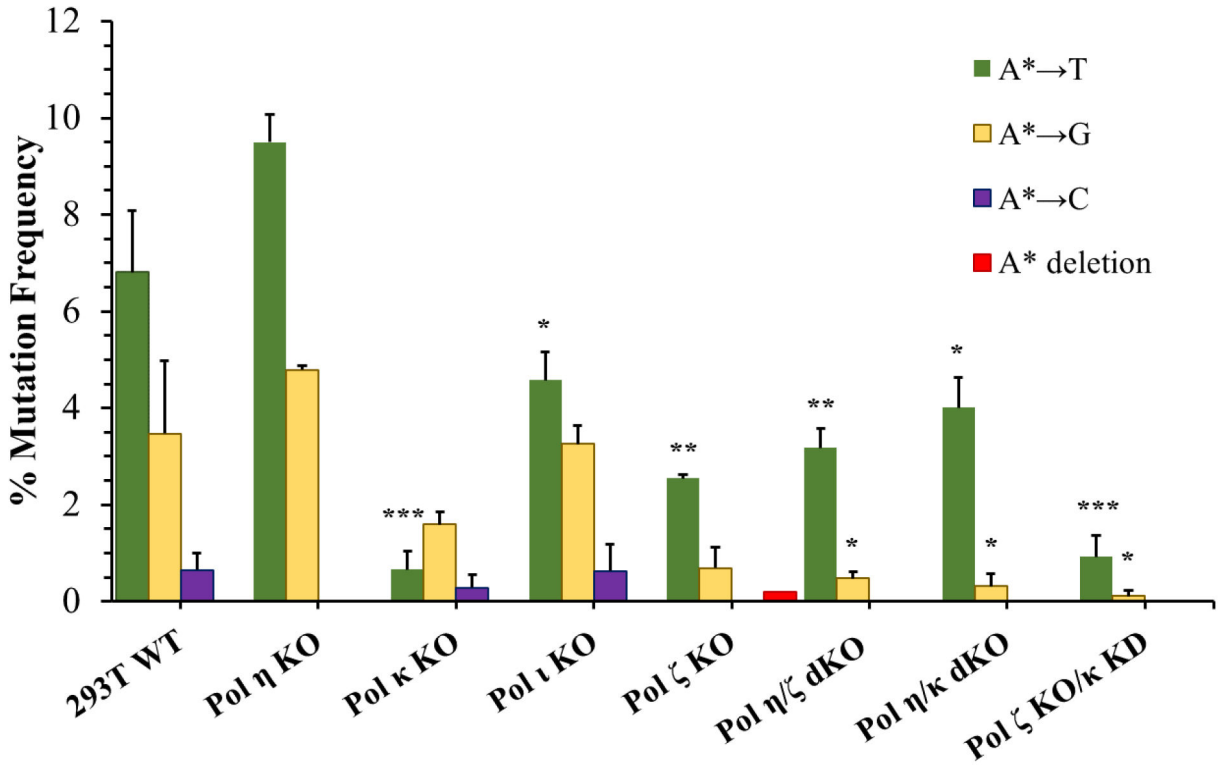
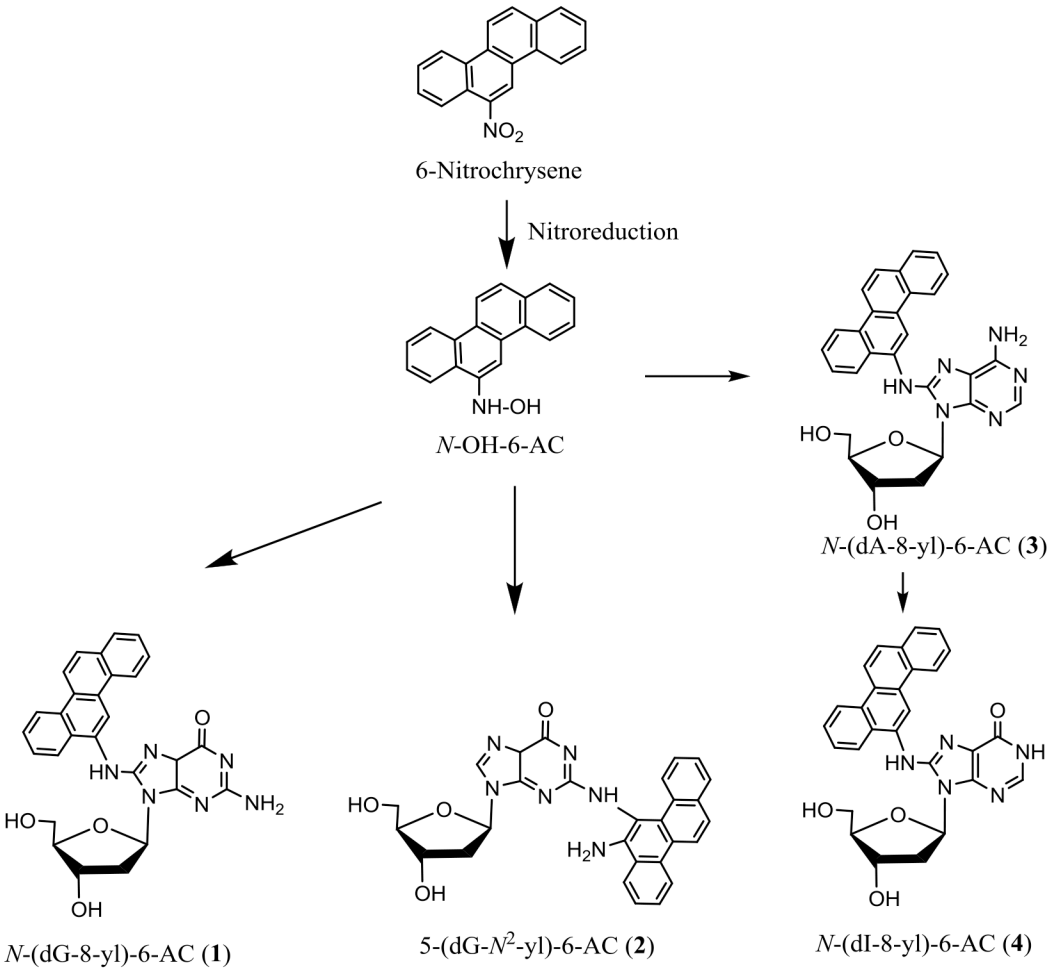
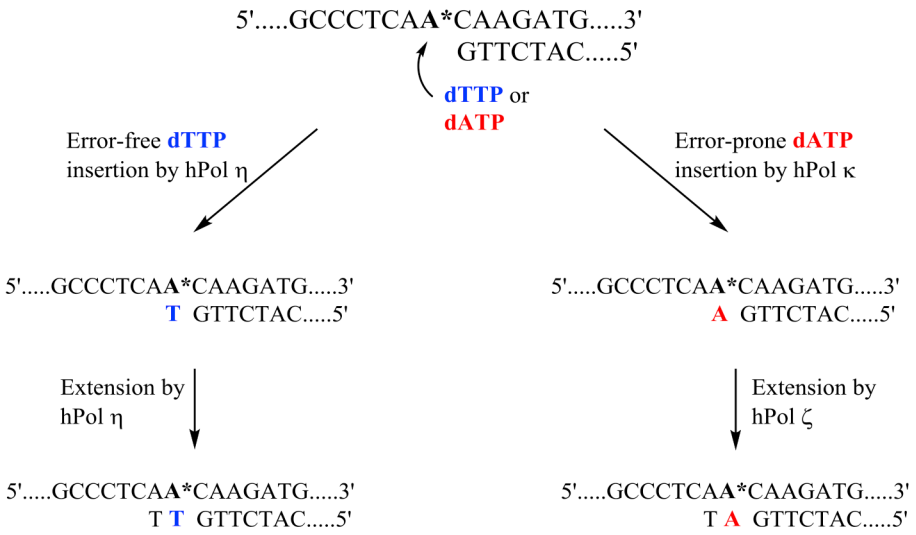


Figure 3.

The types and frequencies of targeted mutations induced in the progeny from the *N*-(dA-8-yl)-6-AC construct in HEK 293T cells and various polymerase knockout (KO) HEK 293T cells. The data represent the mean and the standard deviation (of the total targeted MF) from 2–4 independent experiments. The statistical significance of the difference in targeted MFs between HEK 293T and TLS pols knockouts was calculated using two-tailed, unpaired Student's t test. (*P < 0.05; **P < 0.01; ***P < 0.001).



Scheme 1.
Metabolic activation of 6-NC and the DNA adducts formed by the nitroreduction pathway.



Scheme 2.

Postulated pathways of error-free and error-prone TLS of *N*-(dA-8-yl)-6-AC construct in HEK 293T cells. A similar error-prone pathway can be envisioned for dCTP insertion by hPol κ opposite the adduct and its extension by hPol ζ causing A*→G mutations.