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Specific Incorporation of an Artificial Nucleotide Opposite a Mutagenic DNA Adduct by a DNA Polymerase

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Supporting Information

ABSTRACT: The ability to detect DNA modification sites at single base resolution could significantly advance studies regarding DNA adduct levels, which are extremely difficult to determine. Artificial nucleotides that are specifically incorporated opposite a modified DNA site offer a potential strategy for detection of such sites by DNA polymerase-based systems. Here we investigate the action of newly synthesized base-modified benzimidazole-derived 2′-deoxynucleoside-5′-*O*-triphosphates on DNA polymerases when performing translesion DNA synthesis past the pro-mutagenic DNA adduct O^6-benzylguanine (O^6-BnG). We found that a mutated form of *KlenTaq* DNA polymerase, i.e., *KTqM747K*, catalyzed O^6-BnG adduct-specific processing of the artificial **Benzi**TP in favor of the natural dNTPs. Steady-state kinetic parameters revealed that *KTqM747K* catalysis of **Benzi**TP is 25-fold more efficient for template O^6-BnG than G, and 5-fold more efficient than natural dTMP misincorporation in adduct bypass. Furthermore, the nucleotide analogue **Benzi**TP is required for full-length product formation in O^6-BnG bypass, as without **Benzi**TP the polymerase stalls at the adduct site. By combining the *KTqM747K* polymerase and **Benzi**TP, a first round of DNA synthesis enabled subsequent amplification of **Benzi**-containing DNA. These results advance the development of technologies for detecting DNA adducts.

Exposure to alkylating agents present in the environment, food, and tobacco smoke as well as to certain chemotherapeutic drugs[1] can result in the formation of mutagenic O^6-alkylguanine adducts (O^6-alkylG).[2−5] These DNA adducts occur at low physiological levels, making them difficult to detect. Existing analysis methods, such as ^{32}P-postlabeling, electrochemical or fluorescence detection, immunoassay, mass spectrometry, and accelerator mass spectrometry, have various limitations for the prevalent measurement of DNA adducts in biological samples.[6−9] In one recent demonstration, single-molecule real-time sequencing (SMRT) was used to discriminate between adducts.[10] Further advances involving the ability to amplify DNA adducts could significantly advance studies investigating DNA damage levels and cancer etiology. Herein we present the first report of an artificial nucleotide being incorporated opposite a DNA alkylation adduct by a DNA polymerase. Furthermore, we used this process as a basis for single primer amplification, where the artificial nucleotide in amplified product DNA is a marker for the adduct in the original template.

Thermostable DNA polymerase enzymes can mediate the amplification of DNA, even with synthetic base surrogates.[11−13] To date, nucleotide probes that are specifically incorporated opposite a particular damaged site in DNA have been reported for abasic sites and isoguanine.[14,15] While isoguanine could be amplified, abasic site probes acted as chain terminators, and did not result in full-length DNA replication.[15−17] Artificial nucleotide analogues also offer a potential strategy for detection of chemically alkylated DNA with a DNA polymerase (Figure 1A). However, in previous attempts various artificial nucleotides have been shown to be better incorporated opposite G rather than alkylated O^6-methylguanine (O^6-MeG),[18] and to our knowledge, there are no reports of an artificial nucleotide

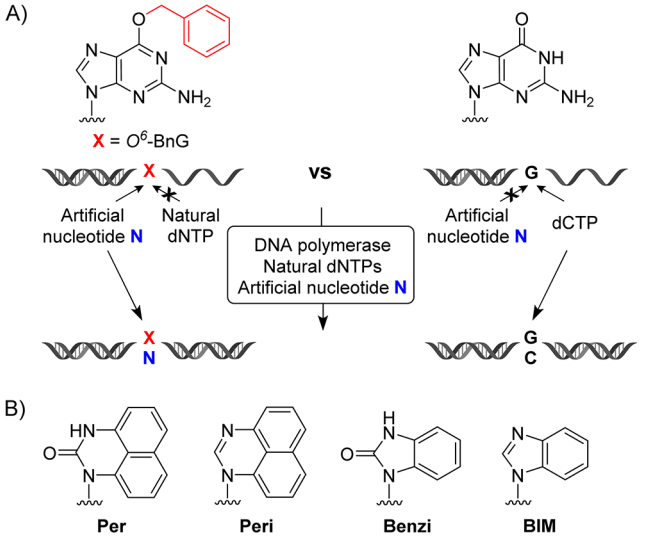

Figure 1. (A) DNA polymerase-mediated specific incorporation of artificial nucleotide N opposite O^6-BnG DNA adduct X vs G in the presence of natural dNTPs. (B) Base analogues for pairing with O^6-alkylG adducts (wavy line indicates attachment in DNA or on 2′-deoxynucleoside-5′-*O*-triphosphate).

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analogue that is preferentially incorporated opposite O^6-alkylG as a substrate in a DNA polymerase-catalyzed DNA synthesis or amplification.

Previously, we developed nucleoside analogues as specific base pairing partners for O^6-BnG adducts within a DNA duplex (Figure 1B).[19,20] Detecting O^6-BnG is relevant for investigating chemicals such as the esophageal carcinogen N-nitrosobenzylmethylamine, and more generally for modeling interactions of mutagenic bulky O^6-alkylguanine adducts with DNA-processing enzymes.[5,21,22] We demonstrated that, when incorporated into DNA, the perimidinone analogue **Per** recognized bulky O^6-BnG by forming more stable DNA duplexes when base paired with O^6-BnG vs G.[19] Structural studies demonstrated that stabilization results from base stacking interactions involving interstrand intercalation of **Per** in the DNA duplex.[23] The nucleoside analogues shown in Figure 1B were further explored as components of primers in DNA polymerase IV-mediated (*Sulfolobus solfataricus*, *Dpo4*) postlesion DNA synthesis, in which extension of **Benzi** from the 3′-primer end was specific when paired with O^6-alkylG vs G.[24,25] However, triphosphate derivatives of these nucleosides have not been available, therefore there is no data regarding their potential incorporation by DNA polymerases.

In this study the two base-modified 2′-deoxynucleoside-5′-*O*-triphosphates **BIM**TP and **Benzi**TP were synthesized by adapting the synthetic strategy of Borch and co-workers (Supporting Information (SI), Scheme S1).[26] A limiting factor in examining the performance of nucleotide probes in DNA replication are traditional difficulties in low-yielding synthesis and purification of nucleoside triphosphates[27] with limited recent advances, and using standard Ludwig conditions[28] for these examples was ineffective. In order to synthesize **BIM**TP and **Benzi**TP, nucleoside analogues **1a**,**b**[20] were reacted with a phosphoramidic dichloride reagent[29,30] to yield benzotriazole intermediates **2a**,**b**, which were subsequently benzylated to yield phosphoramidite intermediates **3a**,**b**.[26] These monophosphates were activated by catalytic hydrogenolysis. Subsequent addition of pyrophosphate resulted in formation of triphosphates **4a**,**b**, which were purified by reverse-phase HPLC and chemically characterized by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, and HR-ESI-MS/MS.

We tested the processing of artificial nucleotides **BIM**TP and **Benzi**TP opposite templates G and O^6-BnG by various DNA polymerases. For our studies we chose A-, B-, and Y-family DNA polymerases *Dpo4*, *Therminator*, *DeepVent*$_R$ *(exo-)*, *KOD*, *KlenTaq*, and a mutant thereof, *KTqM747K*, based on features like lesion bypass ability, acceptance of artificial nucleotides, and thermostability. We performed single-nucleotide incorporation experiments in which a 5′-end radiolabeled 23 nucleotide (nt) primer and a 28-nt template containing either G or O^6-BnG (positioned at nt 24) were incubated with DNA polymerase and corresponding dNTPs (Figure 2A). Products were separated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. The level of primer extension (in %) was calculated as a ratio of the amount of n+1 product formed per original amount of primer (n = 23 nt). The archaeal translesion polymerase *Dpo4*, known to bypass O^6-BnG adducts,[31] catalyzed higher incorporation levels of **Benzi**MP at the adduct site compared to G (SI, Figure S1). This observation is consistent with the hypothesis that the G:**Benzi** base pair may suffer from an unfavorable steric interaction between hydrogen bond donor N−H on G and the N−H donor on **Benzi**, while O^6-BnG presents instead a H-bond acceptor positioned favorably to interact with the N−H donor on **Benzi** (Figure 2C). However, while Dpo4 has been shown to catalyze extension from a terminal O^6-BnG:**Benzi** pair, the enzyme is neither thermostable nor processive.[25] *Therminator* DNA polymerase, which has an established enhanced ability to incorporate modified nucleotide substrates (SI, Figure S2),[32] *KOD* (SI, Figure S3), and *DeepVent*$_R$ *(exo-)* (SI, Figure S4) were tested: in these cases, the polymerases processed natural dNTPs, **BIM**TP, and **Benzi**TP opposite G and O^6-BnG. Due to lack of specificity for probe incorporation opposite the adduct these enzymes were not further investigated.

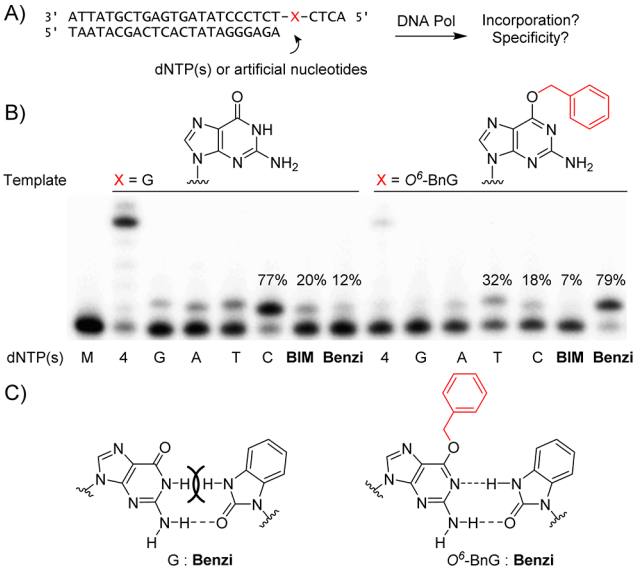

Figure 2. (A) DNA polymerase-mediated primer extension and sequences used in this study. (B) DNA synthesis by *KTqM747K* past template X = G, or O^6-BnG with natural or artificial triphosphates. Abbreviations: M, blank; 4, all four dNTPs; G, dGTP; A, dATP; T, dTTP; C, dCTP; **BIM**, **BIM**TP; **Benzi**, **Benzi**TP. Final dNTP concentrations were 10 μM; polymerase concentrations were 5 nM. (C) Proposed structures of G:**Benzi** and O^6-BnG:**Benzi** base pairs. Curved lines indicate a possible steric clash in G:**Benzi**; wavy lines represents connectivity to DNA strand.[25]

Next, we explored *KlenTaq* polymerase (SI, Figure S5), which is able to incorporate large C7-modified 7-deazapurine nucleotides and can replicate unnatural base pairs,[33,34] as well as a mutant thereof, *KTqM747K* (Figure 2B), that efficiently bypasses various DNA lesions.[35,36] When replicating O^6-BnG, *KlenTaq* incorporated correct dCMP (14%), whereas the mutant *KTqM747K* misincorporated a dTMP (32%). In the presence of all four dNTPs both polymerases were stalled by the O^6-BnG DNA adduct and little full-length product was formed. **BIM**TP and **Benzi**TP were both accepted as substrates, but **BIM**TP was better processed opposite G (*KlenTaq*, 14%; *KTqM747K*, 20%) than O^6-BnG (*KlenTaq*, 5%; *KTqM747K*, 7%). Notably, *KlenTaq* and *KTqM747K* both incorporated **Benzi**MP specifically opposite the O^6-BnG adduct (*KlenTaq*, 39%; *KTqM747K*, 79%) while only little insertion was observed for template G (*KlenTaq*, 3%; *KTqM747K*, 12%). Under these conditions incorporation of artificial nucleotides opposite G might be negligible as incorporation levels are comparable to background levels for formation of non-cognate natural base pairs. Moreover, opposite O^6-BnG, **Benzi**MP was even better incorporated than natural dTMP or dCMP. Because *KTqM747K* mediated more incorporation of **Benzi**MP

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Table 1. Steady-State Kinetic Parameters for Nucleotide Incorporation Mediated by *KTqM747K* DNA Polymerase

dNTP	K_M [μM]	k_{cat} [min^{-1}]	k_{cat}/K_M [μM^{-1} min^{-1}]	efficiency relative to natural dNTPs[a]	efficiency relative to **Benzi**TP[b]
X = G					
dCTP	0.073 ± 0.02	14	190	1	1,600
BenziTP	48 ± 8	0.24	0.005	0.00003	0.04
X = O^6-BnG					
dCTP	88 ± 13	0.64	0.007	0.000037	0.06
dTTP	30 ± 2	0.63	0.021	0.00011	0.2
BenziTP	7.7 ± 0.6	0.90	0.120	0.00062	1

[a]Relative efficiency equals efficiency (k_{cat}/K_M) relative to that of dCTP processing opposite G; [b]relative efficiency equals efficiency (k_{cat}/K_M) relative to that of **Benzi**TP processing opposite O^6-BnG.

opposite O^6-BnG (79%) than did *KlenTaq* (39%), further experiments focused on the *KTqM747K* mutant and artificial nucleotide **Benzi**TP. We also tested **Benzi**TP processing opposite other natural templates A, T, and C (SI, Figure S6). Highest **Benzi**MP incorporation was observed for template A (88−90%); opposite T and C only little **Benzi**TP was processed (15−41% opposite T and 8−31% opposite C).

Steady-state kinetic experiments were performed to quantitatively analyze the differences in *KTqM747K* catalysis of incorporating **Benzi**MP or natural dNTPs opposite adducted O^6-BnG, as well as templating G, A, T, or C (Table 1; SI, Table S3). Kinetic parameters K_M and k_{cat} were determined under steady-state conditions by monitoring n+1 product formation over time.[37] In *KTqM747K*-mediated O^6-BnG bypass, misincorporation of a dTMP was 3 times more efficient than incorporation of dCMP based on comparison of catalytic efficiencies k_{cat}/K_M (Table 1, X = O^6-BnG). However, incorporation of dCMP opposite O^6-BnG was 30 000-fold less efficient than dCMP opposite G. When comparing catalytic efficiencies of **Benzi**MP incorporation opposite O^6-BnG vs G, the polymerase was 25-fold more efficient for O^6-BnG than G (Table 1). Also, *KTqM747K* catalyzed **Benzi**MP incorporation opposite O^6-BnG 5-fold more efficiently than *KlenTaq* (SI, Table S2). Furthermore, *KTqM747K* incorporated **Benzi**MP opposite O^6-BnG more efficiently than natural dNTPs, i.e., 17-fold more than dCMP and 5-fold more than dTMP (Table 1, X = O^6-BnG). **Benzi**TP was most efficiently processed opposite A (SI, Table S3), but at a rate 30-fold less efficiently than dTTP. These data demonstrate the adduct-specific incorporation of **Benzi**MP by *KTqM747K* in favor over natural nucleotides.

With the knowledge that *KTqM747K* is stalled by the bulky O^6-BnG adduct in the presence of all four natural dNTPs, and that **Benzi**MP is selectively incorporated opposite the adduct, we tested whether adding **Benzi**TP to all four dNTPs would promote the formation of full-length products (Figure 3A). Thus, 5′-end radiolabeled 19-nt primer and 28-nt template (O^6-BnG positioned at 24 nt) were incubated with dNTPs plus **Benzi**TP at increasing concentrations. Again, primer elongation was stalled at the adduct site with natural dNTPs only (Figure 3B, dNTPs = 4, band at 24 nt), but when **Benzi**TP was supplemented, full-length products were formed (Figure 3B, dNTPs = 4+N, bands at 28 nt). To examine the generality of this phenomenon with regard to the sequence context defined by the bases flanking the DNA adduct, we altered the template sequence in order to mimic the cancer-relevant mutational hotspot in codon 12 of the *kras* gene,[38] i.e., 5′-GXT-3′, and observed the same results (SI, Figure S7). We further tested the impact of an additional purine flanking base, both up- and downstream of the adduct, and found full-length synthesis again

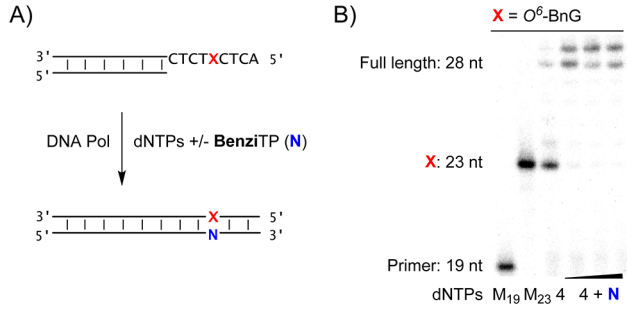

Figure 3. Full extension of O^6-BnG template by *KTqM747K*. (A) Running start primer extension. (B) PAGE analysis of extension products with O^6-BnG template. Abbreviations: 19 nt, primer; 23 nt, X = O^6-BnG; 28 nt, full-length product; M_{19}, marker of 19-nt primer; M_{23}, marker of 23-nt at adduct site X; 4, all four dNTPs (10 μM); 4+N, all four dNTPs (10 μM) plus **Benzi**TP (10, 50, and 100 μM). *KTqM747K* concentrations were 5 nM.

to be promoted when **Benzi**TP was supplemented and dNTP concentration increased (SI, Figure S7). Under these conditions, a prominent 29-nt band was observed, likely from template-independent nucleotide addition.[39,40] These data suggest that **Benzi**MP is incorporated opposite the DNA adduct, promoting further extension with nucleotides, whereas without **Benzi**TP the polymerase is stalled.

With the new information that **Benzi**TP promotes full-length product formation in *KTqM747K* catalysis of O^6-BnG bypass, we further tested whether we could amplify DNA containing the artificial nucleotide, thereby marking the O^6-BnG site in template DNA (Figure 4A). O^6-BnG template DNA (28 nt, X = O^6-BnG positioned at 24 nt) was linearly amplified with *KTqM747K* polymerase by extension of a single primer (19 nt) in the presence of **Benzi**TP (N), where X templated for N. In presence of four natural dNTPs only, we observed a 24-nt

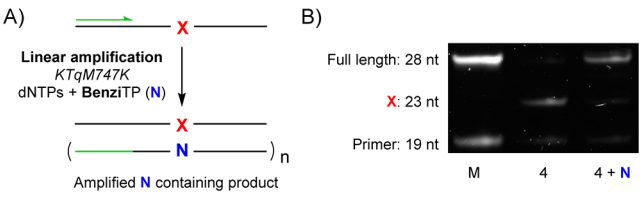

Figure 4. (A) *KTqM747K* DNA polymerase-mediated linear amplification of O^6-BnG template X with **Benzi**TP (N). (B) PAGE analysis of linear amplification reactions. Abbreviations: M, marker of primer (19 nt) and full-length product (28 nt); 4, four natural dNTPs (each at 10 μM); 4+N, four natural dNTPs plus **Benzi**TP (each at 10 μM). *KTqM747K* concentrations were 25 nM.

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product elongated to the adduct site X; however, supplementing with **Benzi**TP resulted in amplification of the full-length 28-nt product (Figure 4B).

In summary, we developed a new process for linear amplification of DNA containing an alkylation adduct. The newly synthesized artificial nucleotide analogue **Benzi**TP is processed opposite the DNA adduct O^6-BnG by *KlenTaq* DNA polymerase mutant *KTqM747K*. Thus, exchanging the non-polar methionine to the cationic lysine fosters the selectivity toward the non-canonical base pair. Methionine 747 is in close proximity to the templating nucleotide and therby might modulate selection and incorporation, but the precise mechanistic basis remains elusive and awaits further studies. Nevertheless, this demonstration is the first example of an artificial nucleotide promoting efficient DNA product formation in replicating an alkylation damaged site in DNA. Furthermore, we performed single-primer amplification, producing increased copy numbers of DNA containing an artificial base marking the adducted site in original template DNA. These findings could advance strategies that combine engineered DNA polymerases with synthetic nucleic acid probes for the amplification-based detection of DNA adducts relevant to understanding cancer etiology and drug toxicity.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, synthesis, chemical characterization, additional figures of primer extension analysis, and steady-state kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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