Sequence-Specific Quantitation of Mutagenic DNA Damage via Polymerase Amplification with an Artificial Nucleotide

Claudia M. N. Aloisi, Arman Nilforoushan, Nathalie Ziegler, and Shana J. Sturla*

ABSTRACT: DNA mutations can result from replication errors due to different forms of DNA damage, including low-abundance DNA adducts induced by reactions with electrophiles. The lack of strategies to measure DNA adducts within genomic loci, however, limits our understanding of chemical mutagenesis. The use of artificial nucleotides incorporated opposite DNA adducts by engineered DNA polymerases offers a potential basis for site-specific detection of DNA adducts, but the availability of effective artificial nucleotides that insert opposite DNA adducts is extremely limited, and furthermore, there has been no report of a quantitative strategy for determining how much DNA alkylation occurs in a sequence of interest. In this work, we synthesized an artificial nucleotide triphosphate that is selectively inserted opposite O€-carboxymethyl-guanine DNA by an engineered polymerase and is required for DNA synthesis past the adduct. We characterized the mechanism of this enzymatic process and demonstrated that the artificial nucleotide is a marker for the presence and location in the genome of O€-carboxymethyl-guanine. Finally, we established a mass spectrometric method for quantifying the incorporated artificial nucleotide and obtained a linear relationship with the amount of O€-carboxymethyl-guanine in the target sequence. In this work, we present a strategy to identify, locate, and quantify a mutagenic DNA adduct, advancing tools for linking DNA alkylation to mutagenesis and for detecting DNA adducts in genes as potential diagnostic biomarkers for cancer prevention.

INTRODUCTION

DNA integrity is continuously threatened by endogenous and exogenous DNA-reactive chemicals. The resulting chemical adducts to DNA can initiate adverse biological consequences including cell death and mutation. O€-Alkyl-guanines (O€-alkylGs) are mutagenic DNA adducts that have been linked to carcinogenesis. They can form from anticancer drugs, antibiotics, and environmental exposures such as cigarette smoke or red and processed meat consumption. In particular, O€-carboxymethyl-guanine (O€-CMG; Figure 1B) was significantly higher in exfoliated colonocytes of people who eat meat vs vegetarians and has been detected upon gastrointestinal digestion of meat, suggesting its formation as a molecular initiating event in colorectal carcinogenesis (CRC) linked to meat consumption. Mutagenic potential of O€-CMG, there is however a lack of data concerning the presence and accumulation of O€-CMG in CRC hotspot regions of the genome.

To establish a cause-effect relationship between O€-CMG and CRC, the detection and quantification of DNA damage in the genome is critically needed. O€-CMG is typically detected by P32 postlabeling, immunoblotting, and mass spectrometry. The most sensitive O€-CMG measurement was by ESI-MS with a LOQ of 73.4 amol, allowing detection of 0.05 O€-CMG per 10^7 nucleotides in human cells. However, for all approaches, only the total level of damage can be determined and information on the genomic location of O€-CMG is lost.

ABSTRACT: DNA mutations can result from replication errors due to different forms of DNA damage, including low-abundance DNA adducts induced by reactions with electrophiles. The lack of strategies to measure DNA adducts within genomic loci, however, limits our understanding of chemical mutagenesis. The use of artificial nucleotides incorporated opposite DNA adducts by engineered DNA polymerases offers a potential basis for site-specific detection of DNA adducts, but the availability of effective artificial nucleotides that insert opposite DNA adducts is extremely limited, and furthermore, there has been no report of a quantitative strategy for determining how much DNA alkylation occurs in a sequence of interest. In this work, we synthesized an artificial nucleotide triphosphate that is selectively inserted opposite O€-carboxymethyl-guanine DNA by an engineered polymerase and is required for DNA synthesis past the adduct. We characterized the mechanism of this enzymatic process and demonstrated that the artificial nucleotide is a marker for the presence and location in the genome of O€-carboxymethyl-guanine. Finally, we established a mass spectrometric method for quantifying the incorporated artificial nucleotide and obtained a linear relationship with the amount of O€-carboxymethyl-guanine in the target sequence. In this work, we present a strategy to identify, locate, and quantify a mutagenic DNA adduct, advancing tools for linking DNA alkylation to mutagenesis and for detecting DNA adducts in genes as potential diagnostic biomarkers for cancer prevention.

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Mutation spectra from colon cells of people with CRC resemble the spectrum induced by the carboxymethylating agent potassium diazoacetate (KDA). Spectra measured in the cancer-relevant gene p53 of CRC tissues and in a KDA-treated p53 gene-containing plasmid were rich in C > T transition mutations, potentially arising from the misincorporation of bases by Y- and B-family TLS Pols. In another study, by transfecting a DNA plasmid containing O€-CMG in human cells, it was concluded that O€-CMG moderately blocks DNA replication and induces mutations at substantial frequencies. While there is evidence regarding the mutagenic potential of O€-CMG, there is however a lack of data concerning the presence and accumulation of O€-CMG in CRC hotspot regions of the genome.

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Polymerase-mediated amplification has revolutionized the life sciences, and the first report of its use to amplify a DNA adduct involved the bypass-proficient Pol mutant KlenTaq M747K\(^{30−32}\) and the heterocyclic imidic nucleotide triphosphate (TP) Benzi. Benzi was selectively incorporated opposite \(O^6\)-alkylG, and DNA complementary to the damaged templates could be amplified by sequential repeats of primer extension reactions, with the presence of Benzi marking the damage location. However, the approach lacked any quantitative analysis capacity, meaning that the amount of \(O^6\)-alkylG in the DNA sample could not be determined.

To fulfill both the requirement of quantification of low-occurrence \(O^6\)-CMG and the need to retain information regarding the DNA sequence, we present herein a chemical—biochemical—analytical combined strategy for the quantitative detection of \(O^6\)-CMG in a sequence-targeted manner. We synthesized (Scheme S1) the triphosphate of the aromatic heterocyclic imide nucleoside ExBenzi and showed for the first time that it is specifically incorporated opposite \(O^6\)-CMG by an engineered Pol. We characterized the incorporation rates of ExBenziTP vs natural bases opposite \(O^6\)-CMG and used molecular modeling based on the KlenTaq M747K crystal structure to identify a structural basis for ExBenzi selectivity. Finally, we developed a mass spectrometric method to quantify the incorporated ExBenzi nucleoside, which reflects the amount of initial \(O^6\)-CMG. With this combination of chemical probe, polymerase-mediated synthesis, and mass spectrometric analysis, we were able to detect and quantify the \(O^6\)-CMG adduct in a specific DNA sequence context. With the capacity to target any DNA sequence, this strategy is anticipated to help elucidate how DNA damage in hotspot regions of the genome impact the mutagenesis and carcinogenesis processes.

### RESULTS AND DISCUSSION

#### Artificial Nucleotide Analogue Is Incorporated Opposite the \(O^6\)-CMG DNA Adduct.

To characterize the efficiency of incorporation of ExBenzi opposite \(O^6\)-CMG, we performed primer extension experiments using the engineered DNA Pol KlenTaq M747K, which has an established capacity to bypass and incorporate chemically modified nucleotides.\(^{30−32}\) Thus, a 5’-end radiolabeled 23-nucleotide (nt) primer was annealed to a 28 nt template (SI, Material and Methods) with either G or \(O^6\)-CMG at nt position 24. The ability of KlenTaq M747K to incorporate ExBenzi opposite G or \(O^6\)-CMG was tested by incubating the annealed primer-template DNA with ExBenziTP and KlenTaq M747K at 55 °C for 10 min (Figure 1A). For comparison, the reaction was conducted with other single nucleotides as controls, i.e. the previously reported artificial nucleotide BenziTP and dTTP.

#### Table 1. Steady-State Kinetic Parameters for Nucleotide Incorporation by KlenTaq M747K DNA Polymerase

| \(X^*\) | dNTP | \(K_M\) [\(\mu\)M] | \(k_{cat}\) [min\(^{-1}\)] | \(k_{cat}/K_M\) [\(\mu\)M\(^{-1}\) min\(^{-1}\)] | relative \(k_{cat}/K_M\) |
|---|---|---|---|---|---|
| \(O^6\)-CMG | C | 689 ± 104 | 4.1 | 0.006 | 0.04 |
| T | 196 ± 29 | 3.8 | 0.019 | 0.13 |
| Benzi | 70 ± 14 | 3.7 | 0.062 | 0.44 |
| ExBenzi | 48 ± 7 | 6.8 | 0.142 | 1 |
| G | C | 1.5 ± 0.2 | 26 | 17.5 | 123 |
| T | 45 ± 7 | 2.2 | 0.048 | 0.34 |
| Benzi | 32 ± 6 | 0.10 | 0.003 | 0.02 |
| ExBenzi | 39 ± 5 | 0.14 | 0.003 | 0.02 |

\(^{\text{Relative } k_{cat}/K_M \text{ equals catalytic efficiency } (k_{cat}/K_M) \text{ relative to that of ExBenziTP incorporation opposite } O^6\text{-CMG}}\)
which is the canonical base most frequently incorporated opposite O6-CMG by KlenTaq M747K.30 The percentage of incorporated product was calculated as the ratio of the amount of n+1 extension product to the initial amount of primer (Figure 1B). We found that opposite O6-CMG, ExBenzi is more efficiently incorporated (59%) than T (12%) and that it is incorporated at a higher level than the previously reported Benzi (41%). Opposite a template containing G, there was almost no evidence for nucleotide incorporation (4% for both Benzi and ExBenzi vs 12% for T).

To quantitatively assess the efficiencies of nucleotide incorporation and characterize how replication over O6-CMG by KlenTaq M747K depends on the presence of ExBenziTP, steady-state kinetic analyses of these primer extension processes were performed. Thus, the amount of incorporation of single nucleotides was measured over time at increasing concentration of each nucleotide (Figure S2). Oligonucleotides resulting from single incorporation of nucleotides were separated on polyacrylamide gel and visualized by autoradiography. The equilibrium constant for binding affinity (K_M) and the catalytic turnover (k_cat) were derived for incorporation by KlenTaq M747K of Benzi, ExBenzi, T, or C, opposite G or O6-CMG (Table 1). For ExBenzi incorporation, K_M values for incorporation templated by either damaged or undamaged DNA were very similar (48 and 39 μM, respectively). Interestingly, a higher binding affinity to the enzyme was calculated for ExBenzi and Benzi compared to T and C opposite O6-CMG. Indeed, the K_M for insertion of C (48, 196, and 689 μM, respectively). Conversely, k_cat for the incorporation of ExBenzi opposite O6-CMG was almost 2-fold higher than for the incorporation of Benzi, T, or C in the same context. As a result, the catalytic efficiency (k_cat/K_M) for the incorporation of ExBenzi opposite O6-CMG was 7- and 24-fold higher than for T or C, respectively, opposite O6-CMG (Table 1). Remarkably, ExBenzi displayed the highest selectivity, almost 50-fold, for incorporation opposite damaged over undamaged DNA (Table 1; k_cat/K_M = 0.142 vs 0.003).

Structural Basis for ExBenziTP Selective Incorporation Opposite O6-CMG by KlenTaq M747K. We were interested in understanding the physical basis for the highly selective incorporation of ExBenzi opposite O6-CMG, despite its large size, both when compared to other nucleotides and in terms of template selectivity. Thus, we performed molecular modeling studies with ExBenziTP, BenziTP, or dTTP opposite O6-CMG or G in DNA bound to KlenTaq M747K. Using a molecular mechanics-based computational modeling approach, we built a model starting from the crystal structure of KlenTaq M747K in a ternary complex with double-stranded DNA and an incoming dCTP (PDB ID: 5O7T). We then replaced incoming dCTP with ExBenziTP, BenziTP, or dTTP. The template strand had either G (original structure) or O6-CMG opposite the incoming base. Upon energy minimization to identify the most stable and high-occupancy conformer of the constructed DNA–enzyme complex (Figure 2A), ExBenzi and O6-CMG interacted by two hydrogen bonds (Figure 2B, top): one between the N1 of O6-CMG and the −NH donor of ExBenzi (1.9 Å) and one between the NH2 donor of O6-CMG and the carbonyl group of ExBenzi (2.1 Å). These interactions

Figure 2. Molecular modeling of interactions in the active site of KlenTaq M747K. (A) KlenTaq M747K (PDB ID: 5O7T) was energy minimized with Molecular Operating Environment software. (B) In its active site (zoomed-in region), ExBenziTP paired opposite O6-CMG (top) via two hydrogen bonds and opposite G (middle) via one hydrogen bond. dTTP paired opposite O6-CMG (bottom) via two hydrogen bonds. (C) Overlay of base pairs O6-CMG:ExBenziTP and O6-CMG:TTP (similarly planarity, bottom). Opposite G, ExBenzi is tilted (top). (D) Relative catalytic efficiency (Table 1) of incorporation of ExBenziTP opposite G (blue bar) and opposite O6-CMG (gray) and of TTP opposite O6-CMG (green). (E) 2D ligand–protein interactions are shown between KlenTaq M747K and ExBenziTP (top) and TTP (bottom).
are consistent with previous models\textsuperscript{29,31} and crystallographic analysis\textsuperscript{33} of a similar construct with Benzi. In contrast, the G:ExBenziTP structure predicted only one hydrogen bond (2.0 Å) and a potential steric clash between the −NH moiety on ExBenzi and the −NH at the N1 position on O\textsuperscript{6}-CMG (Figure 2B, middle), expected to hinder catalysis. As a result, the complex with O\textsuperscript{6}-CMG:ExBenziTP is predicted to be more stable (computed free energy of −8.0 kcal/mol) than that of G:ExBenziTP (+32.0 kcal/mol). Furthermore, by overlapping the above computed base pairs, we observed a different orientation for ExBenzi depending on whether it is paired with O\textsuperscript{6}-CMG or G (Figure 2C). When paired with G, the steric clash imposed a slight rotation to ExBenzi that resulted in reduced planarity. The computationally derived geometry and interactions of ExBenzi with O\textsuperscript{6}-CMG vs G help explain the experimentally derived catalytic parameters (Table 1 and graph in Figure 2D).

Having established a structural basis for template-selective incorporation of ExBenzi, we were interested in understanding the selective incorporation of ExBenziTP over T opposite by KlenTaq M747K. Thus, we modeled O\textsuperscript{6}-CMG:TTP in the active site of the enzyme and found a similar planarity (Figure 2C) and hydrogen bond pattern (Figure 2B, bottom) for O\textsuperscript{6}-CMG:TTP and O\textsuperscript{6}-CMG:ExBenziTP, suggesting that in this instance interactions between the paired bases do not determine the selectivity of ExBenzi incorporation or that differences in the interactions are not evident in these models. We speculated that the interactions of the incoming base, ExBenziTP vs dTTP, with the enzyme might account for the highly favorable incorporation of ExBenzi over T opposite O\textsuperscript{6}-CMG, also based on the value of $K_a$ calculated for ExBenziTP and dTTP incorporation, which suggests a stronger binding affinity for ExBenziTP (Table 1). Indeed, the energy calculated for the interaction of KlenTaq M747K with ExBenziTP is −225.2 kcal/mol vs −204.5 kcal/mol with dTTP. Interactions between the triphosphate groups of the incoming nucleotide and the enzyme made the largest contribution to this difference (Figure 2E). For example, when Arg659, predicted to interact strongly with the phosphate of ExBenziTP, was mutated to Ala, the computed energy difference was +43 kcal/mol, whereas when Phe667, predicted to interact with T base, was mutated to Ala, there was not a significant impact on computed energy (Figure S3). The interaction with the enzyme was predicted to be slightly more favorable for ExBenziTP when compared to BenziTP as well (not shown), in agreement with kinetic measurements (Table 1).

The modeling studies performed herein suggest that a combination of interactions of the incoming nucleotide with the polymerase and between the bases of the nascent pair drives the selective reaction. The interaction of an incoming base (e.g. TTP and ExBenziTP) with the enzyme represents a first energetic barrier in the incorporation process.\textsuperscript{34} We speculate that, once in the active site, the interaction of the incoming TP with the templating base promotes the rate-limiting conformational changes of the enzyme required for insertion. Therefore, the stabilization of the system caused by the lowest-energy base pair is hypothesized to be pivotal in driving the base insertion (e.g. ExBenziTP opposite O\textsuperscript{6}-CMG vs G). Our findings provide a structural and energetic basis for the rational development of artificial nucleotides for DNA adduct detection and imply that the artificial nucleotides should be designed to compensate the altered hydrogen-bonding capacity of the DNA adduct and to favorably interact with the Pol enzyme responsible for DNA synthesis. Overall, modeling studies corroborated results of primer extension (Figure 1) and kinetics experiments (Table 1), supporting the further investigation of ExBenzi as a marker for O\textsuperscript{6}-CMG.

**ExBenzi Is Required for Efficient Extension of a DNA Primer on a Damaged DNA Template.** To test whether the polymerase can extend from ExBenzi once it is incorporated opposite O\textsuperscript{6}-CMG, we performed DNA primer extension studies under conditions suitable for synthesis of a full-length complement of the damaged DNA template. Reaction conditions were the same as for single nucleotide incorporation experiments, except that the reaction mixture was supplemented with all four natural dNTPs, with or without BenziTP or ExBenziTP (Figure 3A). Primer elongation products were analyzed on denaturing polyacrylamide gels, and visualized by autoradiography (M = marker lane with 23 nt primer).

![Figure 3](https://dx.doi.org/10.1021/jacs.9b11746)

**Figure 3.** Primer extension past O\textsuperscript{6}-CMG. (A) Polymerase-mediated DNA synthesis. (B) Full extension of DNA primer complementary to undamaged (X = G) or damaged (X = O\textsuperscript{6}-CMG) DNA template, in the presence of all four natural dNTPs (4), and of BenziTP (4 + BenziTP) or of ExBenziTP (4 + ExBenziTP). Reactions were catalyzed by KlenTaq M747K for 10 min, analyzed on denaturing polyacrylamide gels, and visualized by autoradiography (M = marker lane with 23 nt primer).
Quantification of ExBenzi Nucleoside by Mass Spectrometry Is Diagnostic of O⁶-CMG Levels in DNA. Having optimized the artificial nucleotide-engineered polymerase amplification strategy for O⁶-CMG in a DNA sequence, we aimed to overcome limitations in quantifying O⁶-CMG at low, difficult to detect amounts. Thus, we developed a mass spectrometric method for quantification of ExBenzi nucleoside from the amplicons. Linear amplification reactions in the presence of all four natural nucleotides and ExBenziTP were performed using either O⁶-CMG DNA or unmodified DNA as template, analogous to that described in the previous paragraph. Amplified DNA was enzymatically hydrolyzed to nucleosides and purified for mass spectrometric analysis.

ExBenzi nucleoside was quantified by LC-MS/MS in SRM mode, by monitoring the transition m/z 301 → 185 (Figure S7). To correct for normal instrument variation and account for sample loss during processing, we evaluated artificial nucleosides that are structural analogues of ExBenzi, such as Benzi, the perimidinone Per, and the benzimidazole derivatives BIM and ExBIM, as internal standards (IS) that could avoid the need for synthesizing isotopically modified ExBenzi.28–31 ExBIM nucleoside was optimal in having similar chemical properties to ExBenzi nucleoside, eluting at a similar retention time, and having a unique mass transition of m/z 285 → 169. We optimized the preparation of samples to achieve a similar recovery for ExBenzi nucleoside and for the IS ExBIM nucleoside of 90%.

To quantify the ExBenzi nucleoside, calibration curves were prepared (Figure S7) in the presence of sample matrix, consisting of nucleosides, enzyme, and salts (1xKTO_Si), as a matrix effect was observed for the ionization of both nucleosides. Standard samples in matrix were prepared analogously to what was described above for the experimental samples. Briefly, linear amplification reactions were run using an unmodified DNA template under the usual conditions but in the absence of ExBenzi. Samples were spiked with IS ExBIM (10 nM) and with ExBenzi (5–500 nM). By relating the signal of ExBenzi to that of ExBIM, we could quantify ExBenzi nucleoside in a matrix with a LOQ of 20 fmol (Figure S7). Analysis of ExBenzi released from amplicons that were templated with DNA containing O⁶-CMG showed a linear increase of ExBenzi signal with increasing concentrations of O⁶-CMG in the template. ExBenzi could be detected also when reactions were performed with unmodified DNA, probably due to unspecific incorporation or noncovalent binding of ExBenzi, such as intercalation.35 We could reduce the unspecific signal by disrupting such noncovalent interactions by varying pH or salt concentration; however, these additional sample processing steps significantly reduced recovery rates. Therefore, the background levels of ExBenzi in samples arising from amplification of unmodified DNA was used for background subtraction for the analysis. Thus, we found a linear increase of ExBenzi with increasing initial O⁶-CMG levels in the template (Figure S5).

The approach described here establishes for the first time a chemical basis for amplification and quantitation of DNA damage in a sequence-targeted manner. This approach is useful for the study of isolated oligonucleotides or longer DNA; however, the sensitivity is insufficient for sequence-specific aduct detection of samples from cells. In cultured cells exposed to azaserine (0–450 μM), O⁶-CMG levels were 0.3–9.1 lesions/10⁷ nucleotides.32 The low occurrence of O⁶-CMG is even more relevant in the case of sequence-specific

Alternating cycles (50) of DNA melting (95 °C, 30 s), annealing (42 °C, 30 s), and extension steps (55 °C, 30 s) were performed. Products were separated on a polyacrylamide gel and visualized by fluorescence (Figure 4B). Under these conditions, a signal for the full-length product was recorded only in the presence of ExBenziTP.

To assess whether the ExBenzi-promoted primer extension from damaged DNA could be applied to longer DNAs, we performed primer extension on a modified 4.7 kb plasmid. A 6-FAM-labeled 23 nt primer and O⁶-CMG 28 nt template, analogous to that described in the previous paragraph. Amplified DNA was enzymatically hydrolyzed to nucleosides and purified for mass spectrometric analysis.

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Interest in understanding the genomic location of DNA adducts is growing in medical sciences, due to causative links to mutagenicity and carcinogenesis, as well as drug efficacy. The O6-CMG DNA adduct found in this study is mutagenic and hypothesized to be linked with CRC development. We developed a strategy combining sequence-specific extension, increase in copy number of marker strands, and quantitation of a unique synthetic nucleoside as a reporter of levels of O6-CMG DNA adducts in a DNA oligonucleotide. The artificial synthetic nucleotide ExBenzi is the most specifically and efficiently incorporated opposite O6-CMG reported to date. We elucidated a structural basis for the high selectivity and specificity involving complementarity of H bonds and planarity of the O6-CMG:ExBenziTP pair in the KlenTaq M747K active site. Finally, we developed a mass spectrometric method for quantification of ExBenzi nucleoside and showed that the amount of ExBenzi incorporated during DNA synthesis correlates linearly with the initial O6-CMG DNA, allowing therefore its quantitation. The detection of O6-CMG with retention of DNA sequence information presented in this study lays a foundation to address relationships between DNA damage and mutations in hotspot regions of the genome needed for predictive or prediagnostic purposes.

## CONCLUSION

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## ASSOCIATED CONTENT

+ Supporting Information
  The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.9b11746.
  Material and methods, characterization data for synthetic triphosphate, supplementary Figures S1–S8, and corresponding references (PDF)

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### Notes

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### Figure 5

Quantitation of ExBenzi in amplicons from DNA containing O6-CMG. ExBenzi incorporated by linearly amplified primer reactions from DNA with varying amounts of O6-CMG by mass spectrometry, based on the calibration curve in Figure S8. Data are the average from four independent replicates, each made of two technical replicates. $R^2 = 0.95$.

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**Detection**; for example, there are only 5 guanines per genome (i.e. $6.6 \times 10^9$ nucleotides) belonging to codons 12 and 13 of the $\kappa$-ras gene, which are commonly mutated in CRC. With the combined strategy described here, assuming all $\kappa$-ras Gs are carboxymethylated, an increase of sensitivity of a few orders of magnitude is needed. Nonetheless, for cell-based applications, there are several aspects that can be developed in future studies to address this limitation, including polymerase enzyme engineering/evolution, higher mass spectrometric sensitivity, and sample enrichment.

**Bypass of O6-MeG Occurs Independent of ExBenzi.** In addition to the hurdle of extremely low abundance of DNA adducts in cells, other adducts form, mostly O6-MeG, under related conditions. Since ExBenzi also can be incorporated opposite O6-MeG, and potentially unrelated adducts from different sources, the presence of O6-MeG in biological samples by the approach described here could lead to overestimation of O6-CMG. Incorporation rates opposite O6-MeG are generally higher than O6-CMG (6-fold increase for T and ExBenzi; Table S1). Despite this, an integral selection feature of the O6-CMG-targeting approach is that DNA synthesis past O6-CMG by KlenTaq M747K is low in the presence of natural dNTPs, requiring ExBenzi for extension, whereas O6-MeG is generally readily extended in the absence of ExBenzi (Figure S1B), due to the high incorporation of T (Figure S1A). Therefore, the combination of ExBenziTP and KlenTaq M747K is a poor indicator of O6-MeG levels, and these observations are consistent with those previously reported for Benzi. Nevertheless, further approaches may be envisioned, such as extending preannealed primers first in the absence of ExBenziTP, resulting mainly in the extension of primer annealed to O6-MeG, then adding ExBenziTP to extend past O6-CMG targets (Figure S1C). Future work will be focused on characterizing chemical and biochemical aspects that drive adduct–triphosphate interactions and on targeting structural and enzymatic features to enable complementary strategies that address diverse adducts in heterogeneous biological samples.
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