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Bypass of mutagenic O^6-Carboxymethylguanine DNA Adducts by Human Y- and B-Family Polymerases

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ABSTRACT: The generation of chemical alkylating agents from nitrosation of glycine and bile acid conjugates in the gastrointestinal tract is hypothesized to initiate carcinogenesis. O^6-carboxymethylguanine (O^6-CMG) is a product of DNA alkylation derived from nitrosated glycine. Although the tendency of the structurally related adduct O^6-methylguanine to code for the misincoporation of TTP during DNA replication is well-established, the impact of the presence of the O^6-CMG adduct in a DNA template on the efficiency and fidelity of translesion DNA synthesis (TLS) by human DNA polymerases (Pols) have hitherto not been described. Herein, we characterize the ability of the four human TLS Pols η, ι, κ and ζ and the replicative Pol δ to bypass O^6-CMG in a prevalent mutational hot-spot for cancer. The results indicate that Pol η replicates past O^6-CMG, incorporating dCMP or dAMP, whereas Pol κ exclusively performs error-free insertion and Pol ι displays the lowest fidelity. Additionally, we found that the subsequent extension step was carried out with high efficiency by TLS Pols η, κ and ζ, while Pol ι was unable to extend from a terminal mismatch. These results provide a first basis of O^6-CMG-promoted base misincorporation by Y- and B-family polymerases potentially leading to mutational signatures associated with cancer.

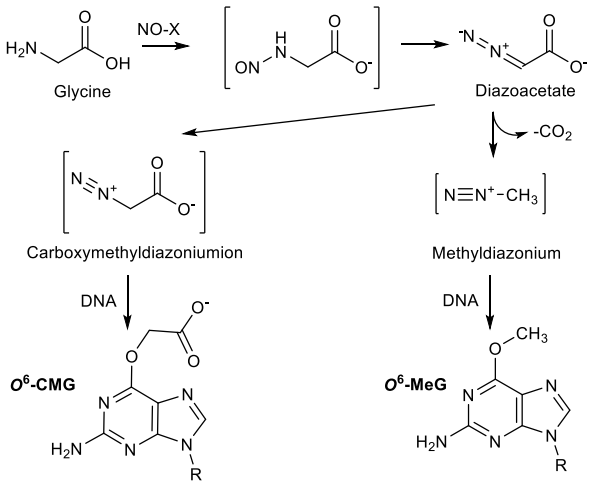
INTRODUCTION

Consuming red and processed meat increases the risk of developing colorectal cancer (CRC), one of the leading causes of cancer death worldwide.[1,2] Most frequent mutations associated with CRC have been identified in adenomatous polyposis coli (*APC)*, Kirsten-ras (*KRAS)* and *TP53* genes.[3,4] The onset of colorectal cancer is special in the progression from normal epithelium to carcinoma involving sequential alterations in *APC*, *KRAS* and *TP53*.[4] For *KRAS,* a majority of G:C→A:T transition mutations are observed specifically at codon 12 and 13, and these hotspot mutations appear in approximately 40% of all colon cancers.[3,5,6] These point mutations may arise from endogenous or exogenous genotoxic chemicals, such as *N*-nitroso compounds (NOC), for which regular exposure through eating meat may alter DNA bases and cause *KRAS* mutations.[7] Nitrosamines are found in food, for example in cured meats and even vegetables, but their endogenous formation in the large intestine by nitrosation of amines and amides is of particular concern.[8,9] NOCs are potent carcinogens that, upon metabolic activation by P450s, alkylate DNA and give rise to DNA adducts. The formation of DNA adducts is a key initiating step in a genotoxic mechanism of carcinogenesis if the adducts miscode during DNA synthesis.

Elucidating chemical and biochemical aspects of the events linking nitrosamine formation and DNA alkylation with mutations occurring in colon cancers is a critical basis of understanding how early biomarker profiles could inform future cancer risk. Nitrosation can arise from the reaction of amines or amides with N_2O_3 at neutral or alkaline pH. N_2O_3 is an oxidation product of NO from foodborne nitrate or after exposure to ionizing radiation.[10] Nitrosation of glycine (Scheme 1), its bile acid conjugate glycocholic acid, or in *N*-glycyl-peptides can occur in the gastrointestinal tract yielding *N*-nitrosoglycine. This reactive intermediate can be converted into the potent DNA alkylating agent diazoacetate, delivering a carboxymethyl group to DNA bases. Amongst different nucleobase sites, alkylation at the *O*6-position of guanine is often a low abundance adduct, but can be strongly miscoding, and O^6-CMG, together with O^6-methylguanine (O^6-MeG), has been detected in DNA from exfoliated human colon cells and blood. The highest adduct levels were measured in the sigmoid colon and rectum.[11-13] Moreover, levels of O^6-CMG from exfoliated colon cells have been shown to increase with increased meat consumption.[12-14] O^6-alkylguanine (O^6-alkylG) adducts, especially O^6-MeG, are primarily repaired by O^6-methylguanine DNA methyltransferase (MGMT) via direct damage reversal.[15] Recent-

ly, O^6-CMG also was found to be an MGMT substrate, leading to inactivation of the protein MGMT.[16]

Scheme 1. Proposed formation of O^6-CMG and O^6-MeG by the nitrosation of glycine. R indicates connection to 2-deoxyribose in a DNA strand.



DNA damage occurs frequently and many adducts can be repaired, yet adducts prone to evade repair and/or induce replication errors can effectively initiate carcinogenesis. If high fidelity DNA replication machinery does not accommodate the DNA adduct, it potentially stalls, resulting in replication fork collapse, double strand breaks and genomic instability.[17] DNA damage tolerance can rescue DNA replication and overcome blocking adducts by allowing replication past DNA adducts in a process called translesion DNA synthesis (TLS).[17,18] In humans, the Y-family polymerases (Pols) η, ι, κ and Rev1 as well as the B family Pol ζ, have been identified to perform TLS.[18] These enzymes have open active sites allowing the accommodation of bulky adducts, but lack 3'-5' proofreading activity and have low fidelity and processivity. Indeed, they act in a potentially error-prone manner and might be accountable for mutagenic events, therefore understanding how human pols process particular DNA adducts is an important aspect of elucidating carcinogenesis mechanisms for genotoxins.[19]

TLS has been characterized for O^6-MeG, O^6-[4-(3-pyridyl)-4-oxobutyl]guanine (O^6-pobG) and O^6-benzylguanine (O^6-BnG) using the archeal TLS Pol *Sulfolobus solfataricus* Dpo4[20-23] and with eukaryotic TLS Pols η, ι, κ and ζ.[24-26] These O^6-alkylguanine adducts are relevant in terms of endogenous methylation, tobacco nitrosamine exposure, and the model hepatocarcinogen methylbenzylnitrosamine. In comparing these previously characterized O^6-alkylG TLS substrates, the larger the alkyl group on the O^6-position of guanine, the greater the reduction in bypass capacity of the human enzymes.[24] Moreover O^6-alkylG adducts are strongly miscoding, O^6-MeG templates the incorporation of T, resulting in G to A transition mutations.[24,27,28] For O^6-CMG, despite the growing concern about adverse effects of high meat intake,[1] so far no studies have addressed their propensity to miscode during bypass.

In vitro toxicity data available for O^6-CMG suggest it to be mutagenic.[29] In a functional yeast assay a plasmid containing the human *p53* cDNA sequence was treated with potassium diazoacetate (KDA), a stable form of nitrosated glycine, yielding detectable levels of O^6-CMG. Accordingly the treated plasmids were used to transform yeast cells and the resulting colonies, harboring a mutated *p53* sequence, were recovered. Subsequent DNA sequencing revealed a mutation pattern dominated by GC→AT transition mutations and GC→TA and AT→TA transversion mutations, which were attributed to the occurrence of O^6-CMG.[29] This observation suggests a potential for O^6-CMG to promote the misincorporation of thymine. Furthermore, recent crystal structures of DNA containing O^6-CMG suggest a chemical basis for the mispairing involving a Watson-Crick-type or high-wobble-type O^6-CMG:T pair.[30,31] Nevertheless, to our knowledge, no data is available regarding O^6-CMG as a substrate for TLS catalyzed by human DNA polymerases, therefore its miscoding properties are not known.

In this study we report the preparation of a novel phosphoramidite reagent for the synthesis of DNA containing the O^6-carboxymethylguanine adduct. We describe the synthesis and characterization of a 48mer oligodeoxyribonucleotide corresponding to the human *KRAS* sequence that incorporates the O^6-CMG adduct at the position of codon 12. Subsequently we assessed the ability of four different human TLS Pols of the Y (Pols η, ι and κ) and B (Pol ζ) family to perform TLS and post-lesion DNA synthesis (PLS) with O^6-CMG-modified DNA as a template. We characterized the four different TLS Pols and a replicative Pol in performing running- and standing-start primer extension and compared the proficiency of the TLS Pols on the basis of steady-state kinetics experiments. This study provides first evidence that the O^6-CMG adduct can be bypassed by human TLS Pols and to a certain extent by replicative Pol δ, but that this is at the cost of potential base misincorporation associated with GC→AT and GC→TA mutations. This suggests a chemical basis for the generation of mutations frequently observed in colorectal cancer.

EXPERIMENTAL DETAILS

Materials. [γ-^{32}P]ATP was obtained from PerkinElmer Life Sciences (Waltham, MA). The natural dNTPs and T4 polynucleotide kinase were purchased from Bioconcept (Switzerland). TRIS-HCl (pH 7.0 at 25°C), TRIS-HCl (pH 8.0 at 25°C), NaCl, $MgCl_2$, dithiothreitol (DTT) and glycerol were all purchased from Invitrogen (Switzerland). Bovine serum albumin (BSA) was obtained from New England Biolabs (Ipswich, MA). All other reagents were purchased from Sigma Aldrich (Switzerland) and used without further purification. The human Pols ι and κ were purchased from Enzymax (Lexington, KY). The human DNA Pol ζ four-subunit-complex (Rev3, Rev7, PolD2 and PolD3) was expressed and purified by Trenzyme GmbH, Konstanz, Germany. Human recombinant Pols η and δ were expressed and purified as described previously.[32,33] Synthetic details and characterization of nucleoside in-

termediates 1-6 are described in Supplementary Information.

Preparation of DNA substrates. The sequences for the unmodified oligonucleotides (ODNs) were as follows: T_{48G} (48mer), 5'-GAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAGTGCC-3'; P_{16} (16mer) 5'-GGCACTCTTGCCTACG-3'; P_{19} (19mer) 5'-GGCACTCTTGCCTACGCCA-3'; P_{20N} (20mer) 5'-GGCACTCTTGCCTACGCCAN-3' where N indicates the four natural nucleotides (G, A, T or C) at the 3' terminus. All sequences were purchased from Eurofins Genomics (Germany). The modified sequence T_{48CMG} (48mer) 5'-GAATATAAACTTGTGGTAGTTGGAGCTGXTGGCGTAGGCAAGAGTGCC-3' contains the O^6-CMG at the position indicated by X. For the incorporation of O^6-CMG in T_{48CMG}, the sequence was synthesized on a Mermade 4 DNA synthesizer from Bio Automation Corporation (Plano, TX) using standard conditions and base-labile phosphoramidites (iPr-Pac-dG-CE, Ac-dC-CE and dA-CE). The modified T_{48CMG} oligonucleotide was deprotected as described previously by Millington *et al.* [34] and purified by reversed phase high-performance liquid chromatography (RP-HPLC) with a linear gradient from 11-16 % (v/v) acetonitrile in 50 mM triethylammonium acetate over 25min. The presence of the desired product was confirmed by mass spectrometry performed on an Agilent MSD ion trap mass spectrometer with electrospray ionization, operated in negative ion mode. The concentration was determined by UV spectroscopy at 260 nm on a *NanoDrop* 1000.

Running-start primer extension assays. T4 polynucleotide kinase and [γ-^{32}P] ATP were used to label primers strands at the 5' end. Labeled primer P_{16} was annealed to templates by combining with either T_{48G} or T_{48CMG} for five minutes at 95 °C and allowing to cool to room temperature for 3 hours. The primer extension reactions for all investigated polymerases were carried out in a reaction buffer containing 40 mM Tris-HCl (pH 8.0, 25 °C), 1 mM $MgCl_2$, 10 mM DTT, 250 μg/mL BSA and 3 % glycerol. For each reaction, 5 nM 5'-[^{32}P]primer:template DNA and 5, 10 or 15 nM of Pols ι, κ, ζ, η or δ were used. In the case of Pol δ, 5 mM $MgCl_2$ and 100 nM proliferating cell nuclear antigen (PCNA) were present in the reaction. The reaction was started by adding 5 μL dNTP mix (10 μM of each dNTP) to the sample with the buffer, 5'-[^{32}P]primer:template DNA and Pol. The reaction mixture was incubated for 15 minutes at 37 °C and the reaction was terminated by adding 10 μL of quenching solution (95 % formamide, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.05 % xylene cyanol and bromophenol blue). Subsequently elongated primers were separated on a 15 % polyacrylamide 7 M urea gel. The gels were run for three hours at 1500 V and were visualized with a phosphorimager (BioRad, Hercules, CA).

Standing-start primer extension assays. The primer P_{19} was 5'-labeled with ^{32}P and annealed to T_{48G} or T_{48CMG} as described for the running-start experiments. The reactions were carried out in 40 mM Tris-HCl (pH 8.0 at 25 °C), 1 mM $MgCl_2$, 10 mM DTT, 250 μg/mL BSA and 3% glycerol. For each reaction 5 nM 5'-[^{32}P]primer:template DNA and 5 nM of each human TLS Pol were used. The reactions with Pol δ contained 5 mM $MgCl_2$ and 100 nM PCNA. To start the reaction 5 μL of a solution containing 10 μM dCTP, dGTP, dATP, or dTTP, or a mixture of all four dNTPs was added to the sample with the buffer, 5'-[^{32}P]primer:template DNA and Pol. The reaction mixture was incubated for 15 minutes at 37 °C, and the reaction was terminated and visualized as described for the running-start primer extension assays.

Primer extension assays. P_{20N} (N= G, A, T or C) was 5'-labeled with ^{32}P and annealed to T_{48G} or T_{48CMG} as described for the running-start primer extension assays. The reactions were carried out in 40 mM Tris-HCl (pH 8.0 at 25°C), 1 mM $MgCl_2$, 10 mM DTT, 250 μg/mL BSA and 3 % glycerol. For each reaction 5 nM 5'-[^{32}P]primer:template DNA and varying concentrations (indicated in Figure 3) of each TLS Pol were used. To initiate the reaction, 5 μL dNTP mix (5 μM of each dNTP) was added to the sample with the buffer, 5'-[^{32}P]primer:template DNA and Pol. The reaction mixture was incubated for 15 minutes at 37 °C, and terminated and visualized as described for the running-start primer extension assays.

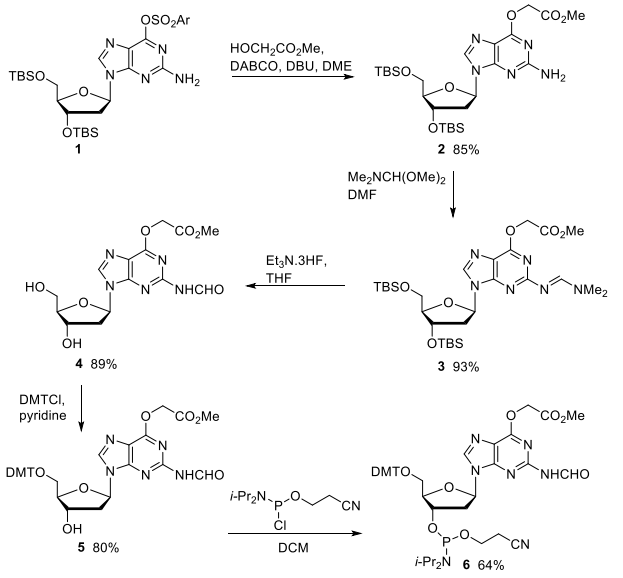
Steady-state kinetic analysis. To measure single nucleotide incorporation, labeled primer strands P_{19} or P_{20N} were annealed to T_{48G} and T_{48CMG} as described in the previous section. Pols (5 nM) were allowed to react with 5'-[^{32}P]primer:template DNA (50 nM) at 37 °C, in a reaction buffer containing 40 mM Tris-HCl (pH 8.0 at 25°C), 1 mM $MgCl_2$, 5 mM DTT, 250 μg/mL BSA, 3 % glycerol and increasing dCTP concentrations in a total volume of 10 μL. The reactions were performed in replicates of three and allowed to proceed for defined time intervals. At varying time, aliquots were removed and quenched with quenching solution containing (95 % formamide, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.05 % xylene cyanol and bromophenol blue). After terminating the reaction, aliquots were applied to a 15 % polyacrylamide 7 M urea gel and separated by electrophoresis. Subsequently the gel was visualized from the phosphorimager screen by a phosphorimager (BioRad, Hercules, CA) and the substrate and product/primer bands were quantified using Quantity One Software (BioRad). The nucleotide incorporation rate was plotted as a function of dCTP concentration and fit to Michaelis-Menten equation using GraphPad Prism 6 software. From the hyperbolic plots parameters k_{cat} and K_m were obtained and used to calculate the frequency of mispair extension (f_{ext}) for each primer:template pair and the dNTP incorporation frequency (f_{inc}) by using the following equation: f_{ext} or $f_{inc} = (k_{cat}/K_M)_{mispaired}/(k_{cat}/K_M)_{paired}$.

RESULTS

Bypass of O^6-CMG catalyzed by human TLS polymerases. In order to compare the ability of human TLS Pols (Pol η, ι, κ and ζ) and the replicative Pol δ to bypass O^6-CMG, we performed running-start DNA synthesis experiments with templates containing synthetically incorporated O^6-CMG which was introduced by an alternative synthetic approach. Previously, we synthesized ODNs

containing O^6-CMG by reaction of methylglycolate with ODN precursors containing the convertible base 2-amino-6-methylsulfonylpurine.[34] In order to synthesize a variety of different O^6-CMG-containing ODNs, we chose instead to prepare a specific phosphoramidite to incorporate the CMG modification directly during DNA synthesis. Xu[35] has described an O^6-CMG phosphoramidite monomer before, but the previous synthesis involved use of the less labile phenylacetyl protecting group for the base. Furthermore the synthesis employed the expensive base quinuclidine during the reaction of mesitylene sulfonate ester **1** with methyl glycolate. As an alternative, we adapted conditions described by Lakshman *et al.*[36] and converted the sulfonate ester **1** into compound **2** by reaction with methyl glycolate, DABCO and DBU in DME (Scheme 2). The amidine protecting group was then introduced by reaction with dimethylformamide dimethylacetal to provide compound **3**. Treatment of compound **3** with triethylamine trihydrofluoride in THF followed by silica column chromatography provided a mixture of products determined to be, following analysis by ^{1}H NMR and MS, a mixture of the amidine and formyl protected product **4**.

Scheme 2. Synthesis of O^6-carboxymethylguanine-phosphoramidite



In a subsequent reaction, when the crude product was purified by column chromatography in the absence of Et_3N, the *N*2-formyl compound **4** was isolated in 89 % yield as the sole product. The instability of the formamidine derivatives of O^6-alkyl-2'-deoxyguanosines, albeit 7-deaza- analogues, has also been noted previously.[37,38] Dimethoxytrityl (DMT) protection of the 5'-OH of compound **4** followed by phosphitylation gave the phosphoramidite **6**. The phosphoramidite was used, in standard solid phase DNA synthesis using base-labile protecting groups. Cleavage of the DNA from the solid support and removal of all protecting groups was achieved by hydrolysis with 0.5 M aq. NaOH for 1 day at room temperature and subsequent addition of conc. aq. ammonia solution (33%) for three days at room temperature to provide the 48mer template containing O^6-CMG (T_{48CMG}). The adduct containing ODN was purified by RP-HPLC and characterized by mass spectrometry. The 48mer template (T_{48CMG}) was annealed with a 16mer primer (P_{16}) and a template containing G (T_{48G}) was used as a control. These substrates were used as templates for primer extension reactions catalyzed by replicative and each of the TLS Pols.

Replicative Pol δ could efficiently elongate the primer on the control (T_{48G}) template at all tested Pol concentrations and the full-length product was observed (Figure 1, Lanes 2-4). However, elongation of the primer paired with the CMG-containing template was impaired (Figure 1, Lanes 5-7). Stronger elongation bands before and at the adduct site (n+4) were observed but only weak extension up to the full-length product was detected. Overall, the presence of O^6-CMG seemed to affect the activity of Pol δ but did not completely inhibit the protein.

Pol ι was unable to achieve efficient extension of the primer paired with either the unmodified or the adduct-containing template (Figure 1, Lanes 9-14). The primer annealed with the CMG-containing template could not be elongated and it seemed that Pol ι was stalled at the site of the adduct (Figure 1, Lanes 12-14). Overall Pol ι was inefficient at all concentrations tested and no full-length product was observed. Overall Pol ι incorporated only a few bases with low efficiency and displayed little processivity.

Pol κ, on the other hand, very efficiently elongated the primer. However it was a hindered by the O^6-CMG adduct compared with the natural template. There was a weak pause at the site of the damage observed in the experiment with 5 nM Pol (Figure 1, Lane 19). However, for all Pol concentrations in the experiments with the natural and modified template an elongation band that was one or two bases shorter than the expected full-length product was observed (Figure 1, Lanes 16-21). A potential explanation for this observation might be the known property of Pol κ to create -1 frameshift mutations.[24,39] The visualized elongation band might therefore still resemble the correct full-length extension but after slippage of Pol κ during the synthesis.

Pol ζ extended the primer paired with the unmodified template to full-length product but no base incorporation opposite O^6-CMG was observed (Figure 1, Lanes 26-28). Indeed, the observation of a strong elongation band at the n+3 position, immediately preceding the adduct suggested that O^6-CMG blocks DNA synthesis by Pol ζ.

From all four TLS Pols tested, Pol η most efficiently bypassed O^6-CMG. It elongated primers paired with the damaged as well as with the unmodified template to the full-length product. Nevertheless, a slight reduction in extension past the CMG adduct was observed (Figure 1, Lanes 33-35), indicating a moderate pausing of Pol η by O^6-CMG. In summary, Pols η and κ efficiently bypassed O^6-CMG, but Pols ι and ζ were blocked.

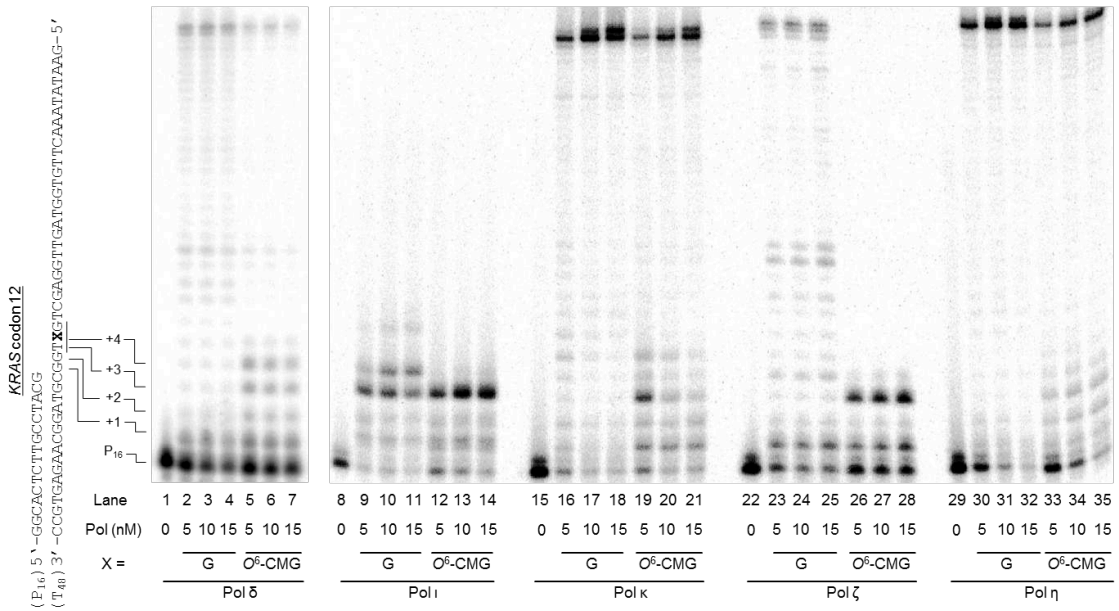


Figure 1. DNA synthesis past O^6-CMG under running start conditions in the presence of increasing concentrations of Pol δ, ι, κ, ζ or η (5, 10 or 15 nM) and 4 dNTPs (10 nM of each dNTP).

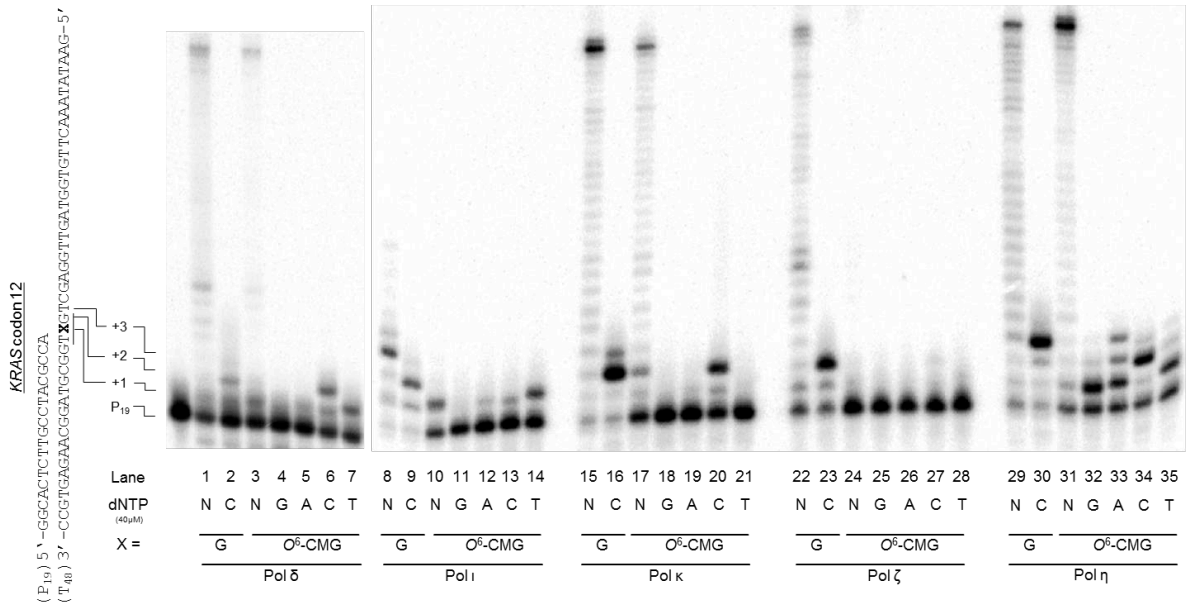


Figure 2. DNA synthesis past O^6-CMG by Pols δ, ι, κ, ζ and η under standing-start conditions from codon 12 of the *KRAS* sequence. N, all four dNTPs; G, dGTP; A, dATP; C, dCTP; T, dTTP.

Impact of O^6-CMG on polymerase fidelity. To assess the accuracy of O^6-CMG bypass by human TLS Pols η, ι, κ, ζ and the human replicative Pol δ, we performed standing-start primer extension experiments. Thus, the primer (P_{19}) was annealed to the 48mer template T_{48CMG} or T_{48G}, representing the O^6-CMG- or G-containing oligonucleotide. For the O^6-CMG bypass by Pol δ, incorporation of dCMP and dTMP opposite the adduct was observed (Figure 2, Lanes 6+7) as well as correct incorporation of dCMP opposite the undamaged G containing template (Figure 2, Lane 2). However, the extension after the bypass on the modified template seemed difficult to be accomplished by Pol δ as there was a stronger n+1 band and only a faint full-length band observed in the experiment with all four dNTPs present (Figure 2, Lane 3).

Pol ι preferentially incorporated dTMP opposite O^6-CMG (Figure 2, Lane 14); minor incorporation of dCMP and dAMP was also apparent from the observation of weak n+1 bands. Additionally, Pol ι incorporated dCMP opposite G on the unmodified template, but the overall efficiency seemed lower than for other polymerases tested.

For Pol κ, a weak n+1 and a strong n+2 elongation band was observed for incorporation of dCMP, but not dGTP, dATP or dTTP, opposite O^6-CMG (Figure 2, Lane 20). This

observation suggests mostly error free bypass of O^6-CMG by Pol κ. For Pol ζ, no elongation bands were observed for the incorporation of dGTP, dATP, dCTP or dTTP opposite O^6-CMG (Figure 2, Lanes 24-28), but a strong n+2 primer elongation band was present for the incorporation of dCMP on the natural template.

Of the polymerases tested, Pol η was most proficient in bypassing O^6-CMG, but did so with low fidelity. Thus, a strong n+2 elongation band was observed for the correct incorporation of dCMP (Figure 2, Lane 34), however n+1 elongation bands were also observed for incorporation of dGMP as well as dTMP (Figure 2, Lanes 32 and 35 respectively), and furthermore, n+1, n+2 and n+3 elongation bands were observed for dATP (Figure 2, Lane 33). The single nucleotide incorporation data suggest that amongst the Pols tested, Pol κ bypasses O^6-CMG most accurately whereas Pol δ, ι, η have a high potential to bypass the adduct in an error prone manner, and that the B-Family Pol ζ is not active in O^6-CMG bypass.

Steady-state kinetics of O^6-CMG bypass by Pol η, κ and ι. Since we observed in qualitative bypass studies that TLS DNA Pol η, κ and ι could process O^6-CMG, we further characterized the steady-state rates for bypass of this adduct. Because of limited availability for replicative Pol δ we did not perform steady-state kinetics and used it only for qualitative comparison with the TLS Pols in the running- and standing-start experiments. Kinetic parameters K_M and k_{cat} were determined under enzyme-limiting conditions with 10-fold excess DNA and varying dCTP concentrations. To compare the frequency of mismatch extension (f^{∞}_{inc}), values were normalized to the k_{cat}/K_M data for the incorporation of dCMP opposite G.

Pol η preferentially incorporated dCMP and dAMP opposite O^6-CMG with a k_{cat}/K_M of 0.36 $\mu M^{-1}min^{-1}$ and 0.09 $\mu M^{-1}min^{-1}$ respectively (Table 1, Entries 2 and 5). Incorporation of dTMP and dGMP were drastically reduced, mostly manifest from unfavorable K_M values of 252 μM and 180 μM, respectively. The incorporation of dCMP opposite G was very efficient (k_{cat}/K_M of 43 $\mu M^{-1}min^{-1}$). Comparing these data with the incorporation efficiencies from the template having O^6-CMG reveals that the presence of O^6-CMG seemed to reduce Pol η bypass between 120- to 17,000-fold.

In accordance with the observations from the standing-start experiments with Pol κ, kinetic parameters were obtained only for incorporation of dCMP opposite O^6-CMG (k_{cat}/K_M of 0.0072 $\mu M^{-1}min^{-1}$, Table 1, Entry 7). No values were accessible for incorporation of dTTP, dGTP or dATP, underscoring a potential error-free bypass by Pol κ. Regardless of the specific and preferred incorporation of dCMP opposite O^6-CMG, Pol-κ-bypass was 265-fold reduced compared to the dCMP incorporation on the unmodified template (k_{cat}/K_M of 1.93 $\mu M^{-1}min^{-1}$, Table 1, Entry 6).

Pol ι most efficiently bypassed O^6-CMG by incorporating dTMP opposite the adduct with a k_{cat}/K_M of 0.0011 $\mu M^{-1}min^{-1}$ (Table 1, Entry 10) whereas dCMP was incorporated with a k_{cat}/K_M of 0.0002 $\mu M^{-1}min^{-1}$ (Table 1, Entry 9). This results in a 6-fold preference for dTTP over dCTP. In summary, these findings indicated that Pol η is able to bypass O^6-CMG most efficiently followed by Pol κ and ι and that Pol η and κ preferentially incorporates dCMP whereas Pol ι favors incorporation of dTMP over dCMP opposite O^6-CMG.

Extension from O^6-CMG by human TLS Pols. TLS can be considered as a two-step process[40] in which one Pol incorporates a nucleotide opposite the adduct and the same or a specialized extender Pol subsequently elongates from the damage. Therefore, we next examined the ability of the four TLS Pols to extend from terminal primer-template mismatches comprised of O^6-CMG or G paired opposite canonical bases.

Pol ι extended unmodified DNA but did not extend any of the mismatched primer ends paired with O^6-CMG (Figure 3, Lanes 6-9). The damage might form a block for efficient extension carried out by Pol ι. Only the correctly paired C:G primer:template was extended to form a strong n+2 elongation band. Extension from the mismatched primers ending with G, A and T paired with the templating G resulted in each case in a weak n+2 elongation band, with T:G appearing to be the most efficiently extended mismatch (Figure 3, Lanes 2-5).

Pol κ efficiently elongated all four primers to full-length product when they were paired with the natural template, favoring extension from C:G and G:G (Figure 3, Lanes 11-14). In the presence of the adduct, Pol κ only elongated C:O^6-CMG but none of the other canonical bases paired with O^6-CMG (Figure 3, Lanes 15-18), demonstrating an exclusive error-free elongation of O^6-CMG which is in accordance with the error-free bypass observed in the standing-start bypass studies.

Pol ζ extended most efficiently from the C:O^6-CMG primer:template construct followed by T:O^6-CMG and A:O^6-CMG (Figure 3, Lane 25-27) and for all three configurations, full-length extension was observed. The G-terminated primer paired with the O^6-CMG template resulted in an n+1 elongation band and no full-length product was visible (Figure 3, Lane 24). In contrast, all four primers paired with the G-containing template could be successfully elongated and full-length products were detected (Figure 3, Lane 20-23).

Pol η could extend all four primers paired with the unmodified template very efficiently with a slight preference for the C:G primer:template construct. For all canonical primers paired with G, full-length products were observed. Furthermore, primers paired with the O^6-CMG template were elongated and full-length elongation bands were observed. However, the C:O^6-CMG and T:O^6-CMG constructs were preferentially extended from the G:O^6-CMG and A:O^6-CMG termini. Taken together, these findings indicated that Pol η and Pol ζ might be best able to extend from a terminal base-pair consisting of O^6-CMG and a canonical nucleobase.

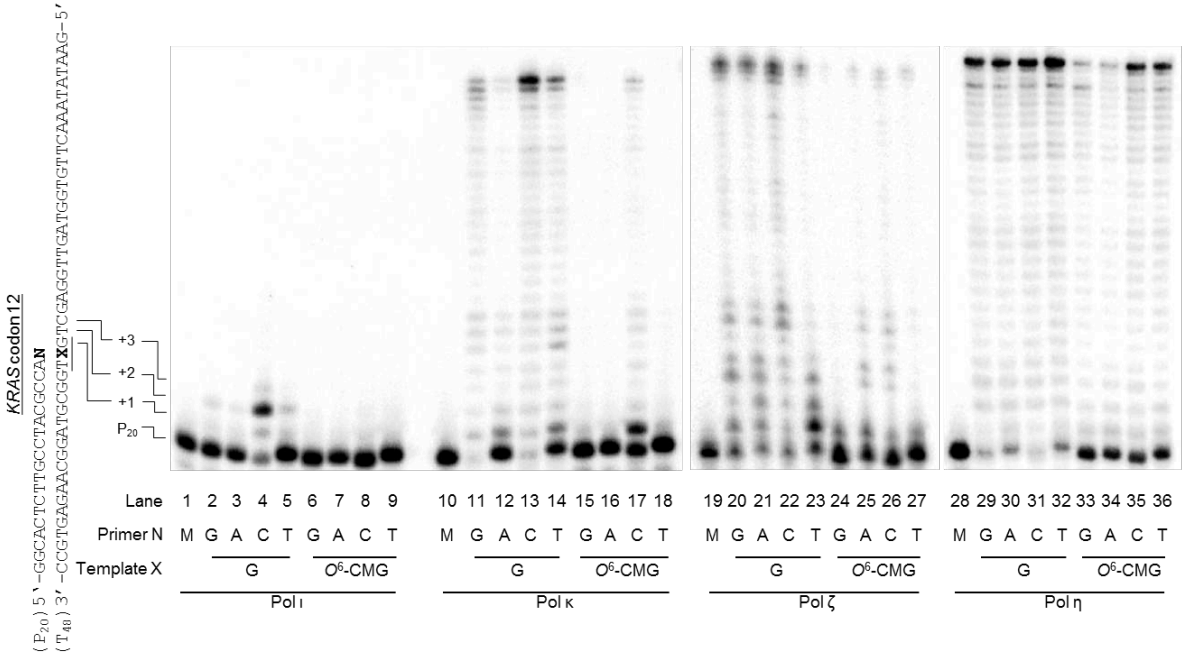


Figure 3. Postlesion DNA synthesis evaluated using primers terminated with G, A, C or T placed opposite G or O^6-CMG in the template. Reactions were carried out in presence of 30 nM Pol ι, 5 nM Pol κ, 20 nM Pol ζ or 20 nM Pol η. M, γ-^{32}P labeled P_{19}; G, A, C and T refers to the terminal base opposite either G or O^6-CMG.

Steady-state kinetics of O^6-CMG PLS by Pol η, κ and ζ. Since we observed that Pols η, κ, and ζ extended the primer:template constructs containing O^6-CMG, we further characterized competing extension pathways by steady-state kinetic analysis of extension from O^6-CMG. Kinetic parameters K_M and k_{cat} were determined under enzyme-limiting conditions with 10-fold excess of DNA and varying dCTP concentrations. To compare the frequency of mismatch extension (f^c_{ext}), values were normalized to the k_{cat}/K_M data for the canonical C:G base pair.

For the primers paired with the modified template, Pol η extended most efficiently from C:O^6-CMG (k_{cat}/K_M 0.7 $\mu M^{-1}min^{-1}$, Table 2, Entry 2) followed by T:O^6-CMG and A:O^6-CMG (k_{cat}/K_M 0.077 $\mu M^{-1}min^{-1}$ and 0.064 $\mu M^{-1}min^{-1}$ Table 2, Entries 3 and 5 respectively). The loss in efficiency for elongation from the G-terminated primer (k_{cat}/K_M 0.033 $\mu M^{-1}min^{-1}$, Table 2, Entry 4) was mainly attributed to the less favored K_M 16.6 μM. However, compared to the extension from the canonical C:G pair (k_{cat}/K_M 4.25 $\mu M^{-1}min^{-1}$, Table 2, Entry 1), the efficiency of primer extension from the modified template was reduced between 6- and 130-fold.

Consistent with the previously observed error-free bypass of CMG, Pol κ was also unable to extend from T-, G- and A-terminated primers paired opposite O^6-CMG. Thus, only kinetic parameters for the extension from C:O^6-CMG could be determined, displaying a k_{cat}/K_M of 0.032 $\mu M^{-1}min^{-1}$ (Table 2, Entry 7). Comparing this value to the C:G extension reveals a 48-fold decreased efficiency for extension from the templated CMG by Pol κ.

The kinetic parameters for Pol ζ demonstrated that C-, T- and A-terminated primers paired with the O^6-CMG template were extended with equal efficiency (Table 2, Entries 9, 10 and 12) and the G:O^6-CMG pairing was the least favored for extension (Table 2, Entries 11). As observed for Pol η, the reduced efficiency for elongation from the G-terminated primer could be attributed to it having the least favorable K_M. Again, these observations related to the canonical C:G construct display reduced efficiencies between 16- and 40-fold for adduct specific extension, compared to the unmodified template. Taken together, Pol η was the most efficient in extending from a terminal mismatch, followed by Pol κ and ζ and all three preferred the extension from a C:O^6-CMG pair.

DISCUSSION

A working model for the concerted action of the multiple human polymerases invoked for DNA synthesis in the presence of modified bases involves polymerase switching. In this process, TLS Pols are deployed to overcome blockages, protecting cells from acute toxicity however at the possible cost of base misincorporation and genomic instability.[40-43] The potential for base misincorporation during DNA replication by a variety of human Pols in bypassing O^6-alkylG adducts has been described;[24] however the presence of O^6-CMG in the template has never been examined for human Y- and B-family polymerases. Herein, we examined the chemical basis of this process by characterizing the propensity of four human TLS Pols, Pol η, κ, ι and ζ vs the replicative Pol δ, to catalyze the bypass and extension past an O^6-CMG adduct with primer extension and steady-state kinetics studies. Human Pol η, κ, ι and δ bypassed O^6-CMG and elongated primers beyond O^6-CMG in the T_{48} template. In contrast, human Pol ζ was stalled and unable to overcome the adduct.

Table 1. Steady-state kinetics for incorporation by TLS polymerases of most frequently inserted dNTPs opposite G or O^6-CMG

Polymerase	Entry	Template[a]	incoming dNTP	k_{cat} [min^{-1}]	K_M [μM]	k_{cat}/K_M [$\mu M^{-1}min^{-1}$]	f_{inc}[b]
Eta (η)	1	G	dCTP	8.6 ± 0.1	0.2 ± 0.02	43.1 ± 6.2	
	2	CMG	dCTP	1.3 ± 0.03	3.7 ± 0.6	0.36 ± 0.1	1
	3	CMG	dTTP	0.6 ± 0.02	252 ± 21	0.0025 ± 0.001	0.007
	4	CMG	dGTP	0.6 ± 0.02	180 ± 18	0.0035 ± 0.001	0.01
	5	CMG	dATP	2.8 ± 0.09	31.5 ± 4.5	0.088 ± 0.02	0.25
Kappa (κ)	6	G	dCTP	8.2 ± 0.2	4.2 ± 0.6	1.93 ± 0.3	
	7	CMG	dCTP	0.5 ± 0.02	64.7 ± 10.0	0.0072 ± 0.002	
Iota (ι)	8	G	dCTP	0.6 ± 0.02	16.3 ± 2.2	0.035 ± 0.007	
	9	CMG	dCTP	0.10 ± 0.01	713 ± 124	0.0002 ± 0.0001	1
	10	CMG	dTTP	0.10 ± 0.01	119 ± 18	0.0011 ± 0.0004	6
	11	CMG	dATP	0.10 ± 0.01	781 ± 187	0.0001 ± 0.00005	0.6

[a]Template: G = T_{48G} and CMG = T_{48CMG}.

[b]$f_{inc} = (k_{cat}/K_M)_{(CMG\ template\ incom.\ dCTP)} / (k_{cat}/K_M)_{(CMG\ template\ incom.\ dNTP)}$ within each set of polymerase experiments

In a functional yeast assay described previously, a plasmid containing *p53* cDNA treated with KDA was used to transform yeast cells. Subsequent DNA sequencing from the mutated colonies revealed increased GC→TA transversion mutations relative to the *N*-methyl-*N*-nitrosourea (MNU) treatment, which predominantly generates O^6-MeG adducts and GC→AT transitions.[27-29] The increased GC→TA transversion mutations are potentially attributed to the presence of O^6-CMG. Our present finding suggests a role for Pol η in the cellular bypass of O^6-CMG due to its high proficiency in bypassing and extending from O^6-CMG. Moreover, Pol η misincorporates dAMP more frequently for O^6-CMG relative to other O^6-alkylG adducts, which might explain increased GC→TA transversion mutations. Amongst all Pols tested, Pol η most efficiently bypassed O^6-CMG, incorporating nucleotides in the order dCMP > dAMP > dGMP > dTMP. The preference for incorporation of dAMP over dTMP stands in contrast to previous results for Pol η bypass of O^6-MeG or O^6-BnG. In those examples, dTMP was preferentially incorporated and dAMP incorporation was not significant.[24] Furthermore, recent crystal structures of DNA containing O^6-CMG suggest a chemical basis for mispairing of O^6-CMG with T in a Watson-Crick- or high-wobble-type pair.[30,31] Additionally, *in silico* models of Pol η in complex with O^6-CMG containing DNA suggest the Pol η active site may accommodate O^6-CMG:T.[30] The preference for dATP misincorporation over dTMP observed herein was therefore surprising and has only been observed for O^6-alkylguanine adducts previously in the bypass of the bulky nitrosamine-derived pyridyoxobutyl adduct O^6-PobG, for which Pol η incorporated dNTPs in the order dCMP > dGMP > dAMP > dTMP.[24] Apart from being proficient at incorporating dCMP and dAMP opposite O^6-CMG, Pol η was also most efficient in extending from the terminal mismatches comprised of O^6-CMG paired opposite canonical bases.

Pol κ was the second most efficient Pol for O^6-CMG bypass. Pol κ is usually considered to be a good extender Pol and is the most faithful amongst the Y-family Pols. It is additionally proficient in error-free bypass of certain bulky adducts.[19,44] We observed that O^6-CMG was bypassed by Pol κ exclusively error-free by incorporating dCMP. However, for other O^6-alkylG adducts examined in previous studies, including O^6-MeG, O^6-PobG, and O^6-BnG, Pol κ was found to be more promiscuous, incorporating between two and four different nucleotides, but always favoring dCTP at a similar rate as observed here for O^6-CMG. Additionally, the proficiency and specificity with which Pol κ extended after bypassing O^6-CMG was similar as it was for O^6-MeG.[24,45] In addition to the bypass, Pol κ could also perform error free extension and extend primers with a terminal C paired opposite O^6-CMG. The extension efficiency from the C:O^6-CMG pair was around 4.5-fold higher compared to incorporation of dCMP opposite O^6-CMG, but was in the same range as previously reported for the extension from O^6-MeG.[24,45] These data indicate a potentially important miscoding difference between carboxymethyl and other O^6-alkyl-G adducts, whereby the carboxyl group avoids interfering with the correct insertion of dCMP by Pol κ.

In addition to Pol η and Pol κ, Pol ι also bypassed O^6-CMG, however, bypass efficiencies were lower than for the other TLS Pols. Pol ι only incorporated a few bases, not elongating the primer to full-length. This low primer extension capacity is consistent with previous observations for Pol ι bypassing adducts such as O^6-MeG, O^6-BnG, 3-methylcytosine, T-T dimers or an abasic site.[24,46,47] Despite the limited activity of Pol ι, it could be shown that the enzyme displayed a preference for incorporating dTMP over dCMP opposite O^6-CMG, which corresponds as well with previous observations for the bypass of O^6-alkylG adducts by Pol ι.[24,48]

Table 2. PLS steady-state kinetics for incorporation of dCMP by polymerase η, κ or ζ.

Polymerase	Entry	Primer[a]	Template[b]	k_{cat} [min^{-1}]	K_M [μM]	k_{cat}/K_M [μM^{-1}min^{-1}]	f_{ext} [c]
Eta (η)	1	C	G	4.7 ± 0.2	1.1 ± 0.2	4.25 ± 0.9	
	2	C	CMG	1.8 ± 0.1	2.5 ± 0.5	0.71 ± 0.1	1
	3	T	CMG	0.6 ± 0.01	8.1 ± 0.7	0.077 ± 0.014	0.1
	4	G	CMG	0.5 ± 0.003	16.6 ± 3.0	0.033 ± 0.001	0.045
	5	A	CMG	0.5 ± 0.008	7.0 ± 0.8	0.064 ± 0.01	0.09
Kappa (κ)	6	C	G	12.6 ± 0.4	8.2 ± 1.2	1.54 ± 0.3	
	7	C	CMG	2.2 ± 0.13	67.7 ± 12.2	0.032 ± 0.01	
Zeta (ζ)	8	C	G	0.19 ± 0.03	4.1 ± 0.4	0.045 ± 0.006	
	9	C	CMG	0.05 ± 0.002	16.7 ± 2.7	0.0028 ± 0.0006	1
	10	T	CMG	0.06 ± 0.002	23.8 ± 3.8	0.0023 ± 0.0005	0.82
	11	G	CMG	0.09 ± 0.003	78.3 ± 12.4	0.0011 ± 0.0003	0.40
	12	A	CMG	0.09 ± 0.004	37.4 ± 6.0	0.0025 ± 0.0006	0.88

[a]Primer: P_{20N} where N indicates G, A, T or C. [b]Template: G = T_{48G} and CMG = T_{48CMG}

[c]$f_{ext} = (k_{cat}/K_M)_{(C:CMG)} / (k_{cat}/K_M)_{(X:CMG)}$ within each set of polymerase experiments

The 6-fold preference for dTMP incorporation opposite O^6-CMG by Pol ι was similar to what has been observed previously for O^6-MeG in the template, also with the same enzyme.[24,48] For O^6-MeG, there is a crystal structure that suggests the basis of this preference is the formation of a Hoogsteen pair. On the basis of the similar coding preference of O^6-CMG and O^6-MeG, a similar interaction could be invoked. Such a model would contradict the predictions suggested by the only available crystal structure of O^6-CMG, which suggests that T:O^6-CMG forms a Watson-Crick- or high-wobble-type pair.[30,31] However, the latter is derived from a structure of the free duplex and does not account for the important influences of the enzyme. In contrast to Pol η and κ, Pol ι had no PLS activity, suggesting that its role, if any, in O^6-CMG bypass may be for the insertion rather than extension step.

In comparison to other TLS Pols, Pol ζ is worse at adduct bypass and seems more proficient in elongating from terminal mismatches.[19,26,44] Indeed, we found that it did not bypass O^6-CMG, but readily extended from all primers terminated with canonical bases paired with the adduct. This observation is fully consistent with a predominant function of ζ being to extend after mismatches or TLS. [19,26,44] It had similar efficiency in extending from the matched, C terminated primer, as well as from the mismatched T- and A-terminated primers paired with O^6-CMG-containing template.

How O^6-CMG is handled by Y- and B-Family Pols, on the basis of relative rates of each process, is summarized in Figure 4. Pol δ incorporates either dCMP or dTMP opposite O^6-CMG and subsequently elongates after the damage, resulting in either error-free bypass or a GC→AT transition mutation (Figure 4, D). As the elongation might be impaired it seems possible that after incorporation of a nucleotide by Pol δ, Pol ζ or κ could take over in order to avoid stalling of the replication machinery. In case Pol δ is unable to process the damage and stalls, the most likely TLS process would be error free incorporation of dCMP opposite O^6-CMG by Pol η and κ. Subsequently, Pol η, Pol κ or Pol ζ would elongate the primer from the C:O^6-CMG mismatch (Figure 4, A). Although error-free bypass of O^6-CMG seems to be the most prevalent scenario, there is also a potentially relevant rate of error. Incorporation of dAMP opposite O^6-CMG by Pol η, and subsequent elongation by Pol η or ζ would be anticipated to result in a GC→TA transversion mutation (Figure 4, C). Furthermore dTMP incorporation by Pol η or Pol ι and subsequent elongation could generate GC→AT transitions mutations (Figure 4, B).

This work provides the first characterization of O^6-CMG bypass by DNA polymerases. Five different human Y- and B-family polymerases were examined. The bypass studies were enabled on the basis of a newly described method for the chemical synthesis of a phosphoramidite reagent for the convenient synthesis of O^6-CMG containing ODNs. O^6-CMG was bypassed most efficiently via incorporation of dCMP by Pol η; however, based on the steady-state kinetic data, there is a 25% chance of dAMP incorporation opposite O^6-CMG by Pol η, generating potential GC→TA transversion mutations. Experiments were carried out under identical conditions, assuming equal concentrations of each Pol, however, the relative contributions and biological relevance of each event will depended on relative enzyme expression levels, and their activity and accessibility to the replication machinery.

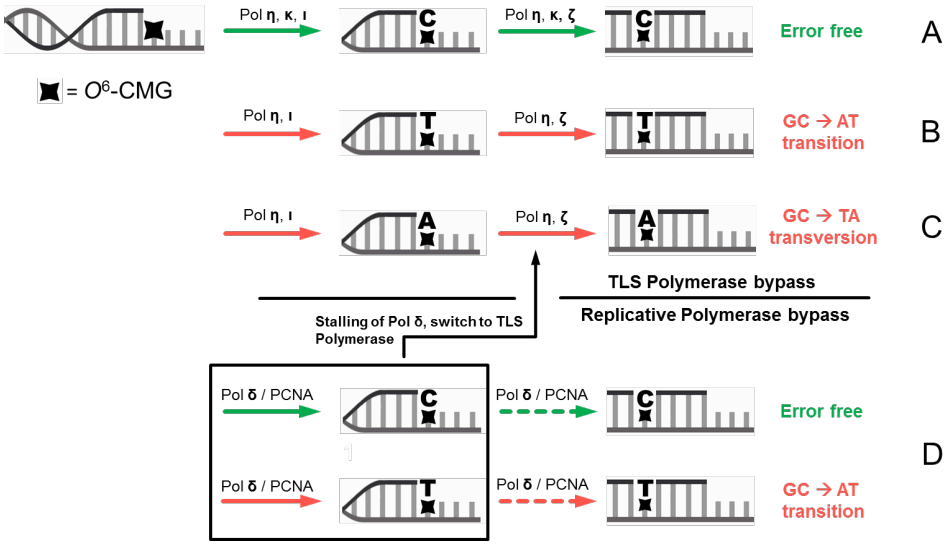


Figure 4. Model for DNA replication by human polymerases in the presence of O^6-CMG derived on the basis of kinetic data obtained in this study. (A) Error-free or error-prone bypass by incorporation of dCMP or dTMP and impaired elongation by replicative Pol δ. If replication is stalled before O^6-CMG or after incorporation of a nucleotide by Pol δ, TLS Polymerases are expected to take over the catalysis of DNA synthesis. (B) Error-free bypass and elongation, predominately by Pol η and κ (C) Incorporation of dTMP by Pol η or ι followed by PLS by Pol η or ζ resulting in GC→AT transition. (D) Error-prone incorporation of dAMP predominately by Pol η and subsequent elongation by Pol η or Pol ζ generating GC→TA transversions. Error-free pathways are shown in green, mutagenic pathways in red.

Therefore, the kinetic data provided from this study offers an important starting point for quantitative modeling of the propensity for mutation to result from O^6-CMG formation as a function of biological characteristics of the progenitor cell.

ASSOCIATED CONTENT

Supporting Information. Synthesis details and characterization of nucleoside intermediates 1-6, HPLC trace and mass spectrometry analysis of O^6-CMG containing 48mer DNA and human Pol ζ analysis are described in the Supporting Information.

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Notes

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