O\textsuperscript{6}-alkyl-2'-deoxyguanosine (O\textsuperscript{6}-alkyl-dG) lesions are among the most mutagenic and prevalent alkylated DNA lesions that are associated with cancer initiation and progression. In this study, using a shuttle vector-based strand-specific PCR-competitive replication and adduct bypass assay in conjunction with tandem MS for product identification, we systematically assessed the repair and replicative bypass of a series of O\textsuperscript{6}-alkyl-dG lesions, with the alkyl group being a Me, Et, nPr, iPr, nBu, iBu, or sBu, in several human cell lines. We found that the extent of replication-blocking effects of these lesions is influenced by the size of the alkyl groups situated on the O\textsuperscript{6} position of the guanine base. We also noted involvement of distinct DNA repair pathways and translesion synthesis polymerases (Pols) in ameliorating the replication blockage effects elicited by the straight- and branched-chain O\textsuperscript{6}-alkyl-dG lesions. We observed that O\textsuperscript{6}-methylguanine DNA methyltransferase is effective in removing the O\textsuperscript{6} position of guanine, whereas repair of the branched-chain lesions relied on nucleotide excision repair. Moreover, these lesions were highly mutagenic during cellular replication and exclusively directed G→A mutations; Pol η and Pol ζ participated in error-prone bypass of the straight-chain lesions, whereas Pol κ preferentially incorporated the correct dCMP opposite the branched-chain lesions. Together, these results uncover key cellular proteins involved in repair and translesion synthesis of O\textsuperscript{6}-alkyl-dG lesions and provide a better understanding of the roles of these types of lesions in the etiology of human cancer.

Highly efficient and accurate DNA replication, which is critical for maintaining normal cellular functions, can be compromised by DNA damage (1). Many exogenous and endogenous agents can react with DNA to induce DNA damage, and alkylation constitutes a major and diverse type of DNA damage (2). Among the four exocyclic oxygen atoms of nucleobases, the O\textsuperscript{6} position of guanine is the most facile site to be alkylated (3). Appreciable levels of O\textsuperscript{6}-alkyl-2'-deoxyguanosine (O\textsuperscript{6}-alkyl-dG)\textsuperscript{2} could be detected in human tissues (4, 5). Moreover, O\textsuperscript{6}-alkyl-dG lesions alter the hydrogen-bonding property of the guanine base and give rise to high frequencies of G→A mutations; hence, these lesions are thought to be involved in the initiation and progression of human cancer (6, 7).

Cells are equipped with multiple mechanisms to repair the O\textsuperscript{6}-alkylguanine lesions. In this vein, human O\textsuperscript{6}-alkylguanine DNA alkyltransferase (MGMT) can directly transfer alkyl groups from the O\textsuperscript{6} position of guanine to Cys-145 in the protein, which leads to irreversible inactivation of MGMT (8–10). Therefore, the amount of active MGMT limits the capacity of cells to repair O\textsuperscript{6}-alkyl-dG lesions (11, 12). In addition, the efficiency of MGMT-mediated repair of O\textsuperscript{6}-alkyl-dG lesions decreases with the size of the alkyl groups (10, 13). Apart from direct lesion reversal, nucleotide excision repair (NER) is thought to be the predominant pathway for repairing bulky O\textsuperscript{6}-alkyl-dG adducts (7, 14).

Despite the presence of multiple DNA repair pathways, some DNA lesions evade repair and may stall replicative DNA polymerases (15, 16). Sustained stalling can result in replication fork collapse, which may give rise to DNA double-strand breaks, leading to genomic instability (17). To overcome the replication blockage effects of DNA lesions, mammalian cells have at least seven proteins with substantial translesion synthesis (TLS) activity that can synthesize DNA past the damaged nucleosides. These include four Y-family polymerases (Pol η, Pol θ, Pol κ, and REV1), one B-family polymerase (Pol ζ), and two A-family polymerases (Pol η and Pol ν) (18–22). TLS polymerases, despite being capable of employing damaged DNA as a template, often exhibit relatively low selectivity in nucleotide insertion and lack proofreading 3’→5’ exonuclease activities, which sometimes lead to significantly diminished fidelity and give rise to mutations in the genome (15, 23).

Several previous studies characterized the biological consequences of O\textsuperscript{6}-alkyl-dG lesions in Escherichia coli and mammalian cells (24–26). Although both O\textsuperscript{6}-methyl-dG (O\textsuperscript{6}-Me-dG) and O\textsuperscript{6}-ethyl-dG (O\textsuperscript{6}-Et-dG) may exert cytotoxic effects via mismatch repair-induced double-strand breaks (26), they do not significantly block DNA synthesis (27, 28). On the other hand, bulkier O\textsuperscript{6}-alkyl-dG lesions, including O\textsuperscript{6}-benzyl-dG (O\textsuperscript{6}-Bz-dG) and O\textsuperscript{6}-4-(3-pyridyl)-4-oxobut-1-yl-dG (O\textsuperscript{6}-POB-guanosine; O\textsuperscript{6}-nPr-dG, O\textsuperscript{6}-iso-propyl-2'-deoxyguanosine; O\textsuperscript{6}-Bu-dG, O\textsuperscript{6}-isobutyl-2'-deoxyguanosine; O\textsuperscript{6}-sBu-dG, O\textsuperscript{6}-sec-butyl-2'-deoxyguanosine; ODN, oligodeoxynucleotide; SSPCR-CRAB, strand-specific PCR-competitive replication and adduct bypass; SAP, shrimp alkaline phosphatase; XPA, xerodermia pigmentosum complementation group A.

This work was supported by National Institutes of Health Grant R01 ES025121. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Figs. 51–56.

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2 The abbreviations used are: O\textsuperscript{6}-alkyl-dG, O\textsuperscript{6}-alkyl-2'-deoxyguanosine; MGMT, O\textsuperscript{6}-alkylguanine DNA alkyltransferase; NER, nucleotide excision repair; TLS, translesion synthesis; Pol, polymerase; O\textsuperscript{6}-nPr-dG, O\textsuperscript{6}-n-propyl-2'-deoxyguanosine; O\textsuperscript{6}-Bz-dG, O\textsuperscript{6}-Bz-dG, O\textsuperscript{6}-4-(3-pyridyl)-4-oxobut-1-yl-dG (O\textsuperscript{6}-POB-guanosine; O\textsuperscript{6}-nPr-dG, O\textsuperscript{6}-iso-propyl-2'-deoxyguanosine; O\textsuperscript{6}-Bu-dG, O\textsuperscript{6}-isobutyl-2'-deoxyguanosine; O\textsuperscript{6}-sBu-dG, O\textsuperscript{6}-sec-butyl-2'-deoxyguanosine; ODN, oligodeoxynucleotide; SSPCR-CRAB, strand-specific PCR-competitive replication and adduct bypass; SAP, shrimp alkaline phosphatase; XPA, xeroderma pigmentosum complementation group A.
lesions are more blocking to DNA replication than their straight-chain counterparts (Fig. 2C).

Our results also showed that all O6-alkyl-dG lesions directed exclusively G→A mutation at high frequencies, indicating the unique miscoding attribute of this type of lesion. O6-Me-dG and O6-Et-dG induced more than 60% of G→A transitions, which is relatively higher than other O6-alkyl-dG lesions (Fig. 2D and Fig. S2). Moreover, the G→A mutation frequency of these lesions decreases with increasing size of the alkyl groups (from O6-nPr-dG to O6-sBu-dG).

**Distinct roles of MGMT and NER in repairing straight-chain versus branched-chain O6-alkyl-dG lesions**

MGMT is the key repair protein that directly removes alkyl groups from the O6 site of guanine (13). We found that treatment of cells with O6-BG (an MGMT-specific inhibitor) led to slightly reduced bypass efficiencies for all straight-chain O6-alkyl-dG lesions except O6-nBu-dG (Fig. 2, B and C). However, depletion of MGMT only slightly elevated the O6-Me-dG-induced G→A mutation, but no significant change was observed for other lesions (Fig. 2D and Fig. S3). In contrast, neither the bypass efficiencies nor mutation frequencies of any of the branched-chain lesions were affected by the inactivation of MGMT (Fig. 2, C and D), suggesting a lack of involvement of MGMT in repairing these lesions.

Apart from the MGMT protein, several studies indicated robust repair of O6-alkyl-dG by NER (14, 36). Therefore, we next examined whether the size and shape of the alkyl group influence the efficiency of NER-mediated repair of these lesions. To this end, we employed xeroderma pigmentosum complementation group A (XPA)-deficient GM04429 and repair-proficient GM00637 human skin fibroblasts for a replication experiment. Deficiency in XPA led to marginal reduction in the bypass efficiency of O6-nPr-dG and O6-nBu-dG, whereas no appreciable change in bypass efficiency was observed for O6-Me-dG or O6-Et-dG (Fig. 2C). Moreover, depletion of NER did not affect the mutation frequency for any of these lesions (Fig. 2D). Removal of MGMT in an XPA-deficient background further attenuated the bypass efficiency for O6-Me-dG and O6-Et-dG but not for O6-nPr-dG or O6-nBu-dG (Fig. 2C), confirming the capability of MGMT to repair the smaller O6-alkyl-dG lesions.

In contrast to the straight-chain lesions, loss of the NER pathway resulted in pronounced decreases in bypass efficiency and increases in mutation frequency for branched-chain lesions (Fig. 2, C and D). Moreover, elimination of MGMT in XPA-deficient cells did not give rise to any apparent changes in bypass efficiency or mutation frequency of these lesions (Fig. 2, C and D). These observations indicate that, although MGMT can effectively remove smaller straight-chain adducts (i.e. methyl and ethyl) from the O6 position of guanine, repair of branched-chain O6-alkyl-dG lesions relies heavily, if not exclusively, on the NER pathway.

**Involvement of various TLS polymerases in bypassing O6-alkyl-dG lesions**

TLS polymerases assume important roles in bypassing lesions that block replicative polymerases. By employing an
SSPCR-CRAB assay (Fig. 3, A and B, and Fig. S1), HEK293T cells, and isogenic cells where TLS polymerase was genetically ablated, we explored the individual or combined roles of various TLS polymerases in bypassing O\textsuperscript{6}-alkyl-dG lesions (37, 38).

O\textsuperscript{6}-Me-dG is unique in that no alteration in bypass efficiency was observed upon knockout of any TLS polymerases. Depletion of REV1 significantly decreased the bypass efficiency of all other O\textsuperscript{6}-alkyl-dG lesions, which is in agreement with the notion that REV1 plays an indispensable role as a scaffolding component for Y-family TLS polymerases (39, 40). In addition, individual ablation of Pol\textsubscript{\eta}/H9257 or Pol\textsubscript{\zeta}/H9259 conferred a pronounced reduction in bypass efficiency for all straight-chain lesions except O\textsuperscript{6}-Me-dG (Fig. 3C and Fig. S2).

Translesion bypass of the branched-chain lesions is more complex. Loss of all TLS polymerases except Pol\textsubscript{\zeta}/H9256 led to diminished bypass efficiency for O\textsuperscript{6}-iPr-dG and O\textsuperscript{6}-iBu-dG, whereas depletion of Pol\textsubscript{\eta}, \iota, \kappa, or \zeta alone did not change the bypass efficiency for O\textsuperscript{6}-sBu-dG (Fig. 3C). It is interesting to note that genetic ablation of Pol\textsubscript{\zeta} did not elicit any alterations in the bypass efficiency for any of the branched-chain lesions. Previous studies indicated that Pol\textsubscript{\zeta} predominantly serves as an “extender” during lesion bypass (41, 42); therefore, we postulate that this polymerase may not be effective in extending the nascent strand following nucleotide insertion opposite the branched-chain O\textsuperscript{6}-alkyl-dG lesions.

The above observations suggested cooperative and redundant roles of TLS polymerases in bypassing the O\textsuperscript{6}-alkyl-dG lesions. We further extended the study to the use of Pol\textsubscript{\eta}/Pol\textsubscript{\zeta} and Pol\textsubscript{\iota}/Pol\textsubscript{\kappa}–deficient cells. The bypass efficiency of O\textsuperscript{6}-Et-dG, O\textsuperscript{6}-nPr-dG, and O\textsuperscript{6}-nBu-dG in Pol\textsubscript{\eta}/Pol\textsubscript{\zeta}–deficient cells was almost identical to that in Pol\textsubscript{\eta}–deficient cells (Fig. 4A), indicating that Pol\textsubscript{\eta} and Pol\textsubscript{\zeta} may act sequentially to bypass the straight-chain O\textsuperscript{6}-alkyl-dG lesions. On the other hand, simultaneous removal of Pol\textsubscript{\iota} and Pol\textsubscript{\kappa} resulted in lower bypass efficiencies for the branched-chain O\textsuperscript{6}-alkyl-dG lesions relative to depletion of either polymerase (Fig. 4B and Fig. S2). Both polymerases were found to be effective in incorporating nucleotides opposite a variety of DNA lesions (42–44), whereas Pol\textsubscript{\kappa} can also exert an extension role in lesion bypass (45, 46). Therefore, we propose that Pol\textsubscript{\iota} and Pol\textsubscript{\kappa} may function separately in bypassing branched-chain lesions.

**The fidelity of TLS polymerases in bypassing O\textsuperscript{6}-alkyl-dG lesions**

Although genetic ablation of Pol\textsubscript{\eta} led to significantly reduced bypass efficiency for O\textsuperscript{6}-Et-dG, loss of this polymerase did not alter the mutation frequency of O\textsuperscript{6}-Et-dG, suggesting that Pol\textsubscript{\eta} and other polymerases have similar fidelity in nucleotide insertion opposite O\textsuperscript{6}-Et-dG. In contrast, loss of Pol\textsubscript{\eta} or Pol\textsubscript{\zeta} significantly decreased the mutation frequency of O\textsuperscript{6}-nPr-dG and O\textsuperscript{6}-nBu-dG (Fig. 3D and Fig. S2), revealing that
these two TLS polymerases promote error-prone bypass of the larger straight-chain lesions. No further diminution in mutation frequency was observed upon dual depletion of Pol η and Pol ζ (Fig. 4C), supporting cooperative roles of the two TLS polymerases in inaccurate bypass of O<sub>6</sub>-nPr-dG and O<sub>6</sub>-nBu-dG.

Although the absence of Pol ι or Pol κ did not result in any alteration in mutagenic properties for the straight-chain lesions, genetic ablation of Pol ι diminished the mutation frequency of O<sub>6</sub>-iPr-dG, O<sub>6</sub>-iBu-dG, and O<sub>6</sub>-sBu-dG, underscoring the role of Pol ι in misincorporating dTMP opposite the branched-chain lesions. However, elevated mutation frequencies were detected upon depletion of Pol κ (Fig. 3D and Fig. S2), reflecting the higher accuracy of Pol κ compared to other TLS polymerase(s) in bypassing branched-chain lesions. Interestingly, the mutation frequency of the three branched-chain lesions in Pol ι/Po κ-deficient cells returned to a similar level as in HEK293T cells (Fig. 4D).

Removal of REV1 resulted in markedly attenuated G→A mutation frequencies for the larger O<sub>6</sub>-alkyl-dG lesions (from O<sub>6</sub>-nPr-dG to O<sub>6</sub>-sBu-dG) (Fig. 3D and Fig. S2), which parallels the bypass efficiency results. Upon loss of the core TLS machinery, rescue of lesion-induced replication blockage effects may predominantly rely on the repair pathway, which leads to an appreciable decline in bypass efficiency and mutation frequency. In addition, we observed that mutation frequency decreased with the size of the alkyl groups in O<sub>6</sub>-alkyl-dG lesions. The finding suggested that O<sub>6</sub>-alkyl-dG lesions could be inaccurately bypassed by the polymerases we studied or by other TLS polymerases (i.e. Pol θ and Pol ν), whereas the larger lesions are more blocking and are therefore less mutagenic.

**Discussion**

Genomic DNA is under constant attack by both exogenous and endogenous genotoxic agents, which may modify the chemical structure of DNA to yield various DNA lesions (47). DNA lesions may perturb the efficiency and fidelity of DNA replication, compromising genome integrity. Some specialized repair proteins and TLS polymerases can protect cells by mitigating the deleterious effects conferred by DNA lesions (48, 49), whereas dysregulation of these systems may result in cell death, mutations, and even cancer (50).

Previous studies revealed the prevalence of O<sub>6</sub>-alkyl-dG lesions in cells and tissues of animals upon exposure to alkylating agents, which can cause direct DNA damage or modify the chemical structure of DNA to yield various DNA lesions. These lesions can be bypassed by TLS polymerases, which may play a critical role in maintaining genome integrity. However, the specific roles and mechanisms of TLS polymerases in bypassing various DNA lesions are not fully understood. Further research is needed to elucidate the mechanisms of TLS polymerases in bypassing DNA lesions and to develop strategies to modulate the activity of these enzymes for therapeutic purposes.
Repair and replication of O\(^6\)-alkylguanine lesions

In this study, we examined systematically how a series of O\(^6\)-alkyl-dG lesions with varying sizes (from methyl to butyl) and structures (n- and isopropyl; n-, iso-, or sec-butyl) of the alkyl groups perturb the efficiency and accuracy of DNA replication in human cells. Our findings unveiled the involvement of distinct pathways in repairing straight- versus branched-chain O\(^6\)-alkyl-dG lesions.

We observed significant replication blockage effects for all O\(^6\)-alkyl-dG lesions except O\(^6\)-Me-dG in human cells, with branched-chain lesions exerting stronger blockage effects than their straight-chain counterparts. In addition, all of these lesions induced a high frequency of G→A mutations (Figs. 2D and 3D). Both the bypass efficiency and mutation frequency dropped with the size of the alkyl group. These observations parallel previous findings (37, 52) and support the hypothesis that adduct size and shape can pronouncedly influence the efficiency of DNA replication.

The differences in repairing straight- and branched-chain O\(^6\)-alkyl-dG lesions

Several studies showed efficient repair of O\(^6\)-Me-dG and O\(^6\)-Et-dG lesions by MGMT in mammalian cells (14, 53). We found that treatment of cells with O\(^6\)-BG, an MGMT inhibitor (54), led to moderate decreases in bypass efficiency for most straight-chain O\(^6\)-alkyl-dG lesions but not for any of the branched-chain lesions (Fig. 2C). The observations are in accordance with previous findings that the binding activity and repair rate of MGMT decreased with the size of the adduct on the O\(^6\) position of guanine (55, 56). Although previous studies have suggested branched-chain O\(^6\)-alkyl-dG lesions to be poor but potential substrates for MGMT (31, 57, 58), our results indicated that MGMT lacks the ability to repair these lesions in human cells.

Previous studies have suggested the involvement of NER in repair of the bulkier O\(^6\)-alkyl-dG lesions (14, 59). We observed...
decreased bypass efficiency and elevated mutation frequency for all branched-chain lesions in XPA-deficient cells relative to repair-competent human skin fibroblasts, whereas loss of NER had a minimal impact on straight-chain lesions (Fig. 2, C and D). These results, therefore, furnished experimental evidence to support the role of NER in removing the branched-chain O6-alkyl-dG lesions in mammalian cells. Interestingly, we observed that depletion of MGMT led to increased bypass efficiency for O6-nBu-dG in repair-proficient cells but not in cells deficient in XPA (Fig. 2C). Inappropriate substrate binding by MGMT has been shown previously to impede NER repair (60); hence, this result suggests that MGMT binding may shield O6-nBu-dG from NER.

**Requirement of various TLS Pols in bypassing O6-alkyl-dG lesions**

Our replication studies using polymerase-deficient cells revealed the roles of various TLS polymerases in bypassing the O6-alkyl-dG lesions. Unlike O6-Me-dG, where loss of any TLS polymerase(s) did not exert any appreciable effect on its bypass efficiency, bypass of other bulkier O6-alkyl-dG lesions is substantially modulated by TLS polymerases. Thus, our results confirmed the previous finding that O6-Me-dG can be readily bypassed in mammalian cells (61).

We found that REV1 is involved in bypassing all lesions except O6-Me-dG (Fig. 3C), which is in line with the fact that REV1 can act as a scaffold protein in supporting the TLS mediated by other Y-family polymerases, including Pol η, Pol ι, and Pol κ (39, 40). We also observed that Pol η participated in bypassing most of the O6-alkyl-dG lesions (Fig. 3C), which is consistent with the results from the *in vitro* biochemical assay showing Pol η to be the most efficient TLS polymerase in nucleotide incorporation opposite O6-alkyl-dG (29). Moreover, genetic ablation of Pol ζ led to reduced bypass efficiency for the straight-chain O6-Pr-dG, O6-nPr-dG, and O6-nBu-dG, albeit to lower degrees than observed for Pol η-deficient cells. Further depletion of Pol η in the Pol η-deficient background did not elicit any further diminutions in bypass efficiency (Fig. 4A), underscoring the key role of Pol η in bypassing straight-chain lesions. In light of previous findings regarding the sequential actions of TLS polymerases in lesion bypass (42, 62), we postulate that Pol η may incorporate a nucleotide opposite straight-chain lesions, which is followed by extension of the nascent strand by Pol ξ or other polymerases (e.g. Pol κ).

Different from what we observed regarding the roles of Pol η and Pol ζ in promoting efficient bypass of straight-chain O6-alkyl-dG lesions, Pol ι or Pol κ appear to be important in bypassing branched-chain O6-iPr-dG and O6-sBu-dG lesions. Previous studies revealed functions of Pol ι and Pol κ in supporting accurate bypass of minor-groove N2-dG lesions in mammalian cells (63, 64). Our results showed that concurrent removal of Pol ι and Pol κ conferred a more pronounced reduction in the bypass efficiency of O6-iPr-dG, O6-sBu-dG, and O6-sBu-dG than depletion of either polymerase alone, indicating separate roles of these two polymerases in bypassing branched-chain lesions. The observation is reminiscent of other studies showing different activities of Pol ι and Pol κ in translesion synthesis of cis,syn cyclobutane pyrimidine dimers (65) and abasic sites (66).

**Impact of TLS Pols in modulating the mutagenic properties of O6-alkyl-dG lesions**

Our study also revealed roles of TLS polymerase in influencing the mutagenic potentials of O6-alkyl-dG lesions. Along this line, Choi *et al.* (67) reported error-free bypass of O6-alkyl-dG lesions by recombinant human REV1, which was attributed to its unique dCMP transferase activity. However, we found that loss of REV1 conferred a pronounced decrease in G→A mutation frequency for all O6-alkyl-dG lesions except O6-Me-dG and O6-Et-dG (Fig. 3D). This finding, therefore, suggests that REV1 may not assume a catalytic role in nucleotide incorporation opposite the lesion.

Loss of Pol ζ or Pol Ω led to markedly diminished G→A mutation for O6-nPr-dG and O6-nBu-dG lesions, which supports roles of these two polymerases in mediating error-prone nucleotide insertion opposite these lesions. Previous studies revealed cooperative roles of Pol ζ and Pol Ω in error-free TLS of cis,syn cyclobutane pyrimidine dimers (68). Simultaneous ablation of Pol ζ and Pol Ω further attenuated the bypass efficiency of the two lesions to the levels observed in REV1-deficient cells. We therefore propose a model for cooperative bypass of the straight-chain O6-alkyl-dG lesions by Pol η, Pol ζ, and REV1. REV1 may bind to the lesion site, which enables recruitment and stabilization of Pol η and Pol ζ to facilitate initial nucleotide insertion and subsequent strand extension, respectively.

It is worth noting that the mutation frequency of O6-Me-dG and O6-Et-dG detected in our experiment was higher than what was observed previously (24, 26). The differences could be attributed to the differences in intrachromosomal and episomal replication of the lesion-harboring DNA. Specifically, the Chinese hamster ovary and HEK293 cells used in the previous studies are not permissive for simian virus 40 (SV40)–dependent episomal replication; therefore, the lesion-containing vector needs to be integrated into the host chromosome for its replication (24, 69). In contrast, the existence of SV40 T large anti in HEK293T and human skin fibroblast cells used in this study enables facile replication of the pTGFP-Hha10 plasmid, which contains an SV40 replication origin (70, 71), prior to lesion repair.

TLS of the branched-chain O6-alkyl-dG lesions involves Pol ι and Pol κ. In particular, we found significant drops in mutation frequency for these lesions in Pol ι–deficient cells. Our observation is in accordance with *in vitro* studies showing a strong preference of Pol ι for misincorporating dTTP opposite O6-alkyl-dG lesions (29, 72). Previous structural studies have demonstrated that, unlike Pol η and Pol κ, which employ Watson–Crick base-pairing, Pol ι utilizes Hoogsteen base-pairing for DNA synthesis opposite template purines (73, 74). The unique base-pairing property of Pol ι may lead to preferential misinsertion of dTTP opposite O6-alkyl-dG. It is worth noting that Pol κ is more accurate than other TLS polymerases in bypassing branched-chain lesions. Although *in vitro* findings revealed that Pol κ is able to incorporate dCMP and dTMP efficiently opposite the straight-chain O6-Me-dG, a recent study showed error-free bypass of O6-carboxymethylguanine by human Pol κ.
Repair and replication of O⁶-alkylguanine lesions

(72). These results indicate a unique nucleotide selectivity of Pol κ upon bypassing the highly blocking branched-chain O⁶-alkyl-dG lesions. Moreover, previous studies have suggested a role of Pol κ in NER (75, 76). Hence, Pol κ may also participate in repair of O⁶-alkyl-dG lesions via the NER pathway, especially those carrying the branched-chain alkyl groups (Fig. 2C). However, future studies are needed for understanding the exact molecular mechanism underlying involvement of Pol κ in ameliorating the mutagenic effects elicited by branched-chain O⁶-alkyl-dG lesions.

Together, our systematic cellular study of a large group of structurally defined O⁶-alkyl-dG lesions provided novel insights into their impact on the efficiency and fidelity of DNA replication in human cells. We confirmed the replication blockage and mutagenic effects of O⁶-alkyl-dG lesions and revealed the involvement of distinct repair pathways and TLS polymerases in the removal and replicative bypass of straight-chain versus branched-chain O⁶-alkyl-dG lesions. Hence, the results of this study help fill the gap between the mutagenic mechanism and the carcinogenic effects of O⁶-alkyl-dG lesions.

Experimental procedures

Materials

All chemicals, unless otherwise specified, were from Sigma-Aldrich (St. Louis, MO), and all enzymes were from New England Biolabs (Ipswich, MA). 1,1,1,3,3,3-Hexafluoro-2-propanol was obtained from Oakwood Products Inc. (West Columbia, SC), and [γ-³²P]ATP was acquired from PerkinElmer Life Sciences. All unmodified ODNs were obtained from Integrated DNA Technologies (Coralville, IA). HEK293T cells deficient in TLS polymerases were produced previously using the CRISPR-Cas9 genome editing method (37, 38).

Construction of lesion-containing and lesion-free control plasmids

The lesion-containing as well as the lesion-free control and competitor genomes were prepared following procedures published previously (77). Briefly, the parental vector was digested with Nt.BstNBI to generate a gapped plasmid, followed by annealing with a 25-mer complementary ODN in 100 mM NiCl₂. The gapped plasmid was then isolated using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). The residual unreplicated plasmid was further removed by DpnI digestion and exonuclease III cleavage, following procedures reported previously (35).

O⁶-benzylguanine treatment

The treatment with O⁶-benzylguanine was conducted as described previously (54) with minor modifications. One day prior to treatment, human skin fibroblast cells were plated at a density of 8 × 10⁴ cells in 24-well plates. After culture for 24 h, the cells were incubated with fresh medium containing 20 μM O⁶-benzylguanine (O⁶-BG) for 12 h. The medium was then removed, and the cells washed three times with PBS to eliminate residual O⁶-BG. The cells were recovered in fresh medium for 2 h and were subsequently transfected with plasmids for the cellular replication assay or harvested for Western blot analysis.

Western blotting

Total protein was extracted from the cell pellet using ice-cold CellLytic M cell lysis reagent (Sigma-Aldrich) containing 1% (v/v) protease inhibitor mixture (Sigma-Aldrich). Proteins were resolved by electrophoresis in 10% SDS-PAGE and transferred to nitrocellulose membranes. The blotted membranes were blocked for 1 h in PBS-T (PBS containing 0.1% Tween 20) plus 5% powdered nonfat dry milk, followed by incubation with primary antibodies against human MGMT (R&D Systems, goat polyclonal, 1:10,000) or mouse m-IgG (Santa Cruz Biotechnology, mouse monoclonal, 1:5,000) for 2 h. The membranes were then washed three times with PBS-T and incubated for 1 h with peroxidase-labeled donkey anti-goat IgG-HRP (Santa Cruz Biotechnology; 1:10,000) or mouse m-IgGk BP-HRP (Santa Cruz Biotechnology; 1:10,000) (Fig. 54). Amersham Biosciences ECL Prime Western blot detection reagent (GE Healthcare) was used to visualize the protein bands.

PCR amplification and PAGE analyses

The progeny genomes were amplified by nested PCR following procedures published previously (38). The final PCR amplifications were separated using 1% agarose gel, purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific), and stored at −20 °C until use. For PAGE analysis, 150 ng of the PCR fragments was treated with 5 units of NcoI and 1 unit of shrimp alkaline phosphatase (SAP) at 37 °C in 10 μl of NEB buffer 3 for 1 h, followed by heat treatment at 80 °C for 20 min to deactivate the SAP. To the above mixture were then added 1.25 μCi (0.5 pmol) of [γ-³²P]ATP and 5 units of T4 polynucleotide
kinase. The reaction was continued at 37 °C for 30 min, followed by heating at 75 °C for 20 min to deactivate the T4 polynucleotide kinase. To the above mixture was then added 2.0 unit SfaNI, and the solution was incubated at 37 °C for 1.5 h. The digestion was subsequently terminated by addition of 20 μl of formamide gel loading buffer. The above restriction digestion yielded a 16-mer radiolabeled fragment for the competitor genome and 13-mer fragments for the control or lesion-carrying genome (Figs. 2A and 3A). The digestion products were separated using 30% native polyacrylamide gel (acrylamide:bi-sacrylamide, 19:1) and quantified by phosphorimaging analysis (Figs. 2B and 3B). The relative bypass efficiency and mutation frequency were used to represent the effects of DNA lesions on replication efficiency and fidelity, respectively, where the relative bypass efficiency values were calculated from the ratios of (lesion signal/competitor signal)/(nonlesion control signal/competitor signal).

Identification of mutagenic products by LC-MS/MS

The replication products were also identified using LC-MS/MS analysis. Briefly, 2 μg of the PCR products described above were digested with 30 units of SfaNI and 15 units of SAP in 150 μl of NEB buffer 3 at 37 °C for 2 h, followed by deactivation of the phosphatase by heating at 80 °C for 20 min. To the mixture was added 50 units of NcoI, and the solution was incubated at 37 °C for another 2 h. The resulting solution was extracted once with 2.5 volumes of phenol/chloroform/isoamyl alcohol (25:24:1, v/v), and to the aqueous layer were subsequently added 50 units of NcoI, and the solution was incubated at 37 °C for 2 h, followed by deactivation of the T4 polynucleotide kinase. The reaction was continued at 37 °C for 30 min, followed by heating at 75 °C for 20 min to deactivate the T4 polynucleotide kinase. The digestion was subsequently terminated by addition of 20 μl of formamide gel loading buffer. The above restriction digestion yielded a 16-mer radiolabeled fragment for the competitor genome and 13-mer fragments for the control or lesion-carrying genome (Figs. 2A and 3A). The digestion products were separated using 30% native polyacrylamide gel (acrylamide:bi-sacrylamide, 19:1) and quantified by phosphorimaging analysis (Figs. 2B and 3B). The relative bypass efficiency and mutation frequency were used to represent the effects of DNA lesions on replication efficiency and fidelity, respectively, where the relative bypass efficiency values were calculated from the ratios of (lesion signal/competitor signal)/(nonlesion control signal/competitor signal).

Author contributions—H. D. formal analysis; H. D. and P. W. investigation; H. D. writing-original draft; P. W. resources; P. W. methodology; L. L., resources; Y. W. conceptualization; Y. W. supervision; H. D. formal analysis; H. D. and P. W. investigation; H. D. writing-original draft; P. W. resources; P. W. methodology; L. L., resources; Y. W. conceptualization; Y. W. supervision; H. D. writing-original draft; P. W. resources; P. W. methodology.

Note added in proof—In the version of this article that was published as a Paper in Press on June 5, 2019, Lin Li was inadvertently omitted from the author line. This error has now been corrected.

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