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LC-MS/MS for Assessing the Incorporation and Repair of N^2-Alkyl-2′-deoxyguanosine in Genomic DNA

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Abstract

Understanding the occurrence, repair, and biological consequences of DNA damage is important in environmental toxicology and risk assessment. The most common way to assess DNA damage elicited by exogenous sources in a laboratory setting is to expose cells or experimental animals with chemicals that modify DNA. Owing to the lack of reaction specificities of DNA damaging agents, the approach frequently does not allow for induction of a specific DNA lesion. Herein, we employed metabolic labeling to selectively incorporate N^2-methyl-dG (N^2-MedG) and N^2-*n*-butyl-dG (N^2-*n*BudG) into genomic DNA of cultured mammalian cells, and investigated how the levels of the two lesions in cellular DNA are modulated by different DNA repair factors. Our results revealed that nucleotide excision repair (NER) exert moderate effects on the removal of N^2- MedG and N^2-*n*BudG from genomic DNA. We also observed that DNA polymerases κ and η contribute

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Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.2c00101.
Supplementary experimental conditions, synthesis and mass spectrometric characterizations of N^2-MedG, the calibration curve for N^2-MedG, and LC-MS/MS quantification results for N^2-MedG and N^2-*n*BudG (PDF)

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.chemrestox.2c00101

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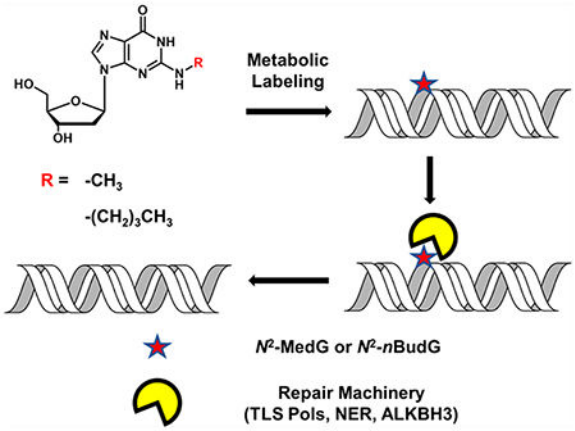
to the incorporation of N^2-MedG into genomic DNA and modulate its repair in human cells. In addition, loss of ALKBH3 resulted in higher frequencies of N^2-MedG and N^2-*n*BuG incorporation into genomic DNA, suggesting a role of oxidative dealkylation in the reversal of these lesions. Together, our study provided new insights into the repair of minor-groove N^2-alkyl-dG lesions in mammalian cells.

Graphical Abstract



INTRODUCTION

In all domains of life, genetic information has to be faithfully transmitted during cell division.[1] However, DNA is susceptible to chemical modifications induced by various endogenous and exogenous DNA damaging agents.[2,3] If not properly repaired, DNA lesions can impede DNA replication and transcription and introduce mutations to nascent DNA or RNA, which may contribute to cancer initiation.[4,5] Hence, it is important to have a comprehensive understanding about how various structurally defined DNA lesions are repaired.

Multidisciplinary efforts have been devoted toward developing reliable approaches to study DNA damage and repair.[6,7] Among them, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the most widely adopted.[8,9] Numerous DNA lesions have been reliably quantified using LC-MS/MS coupled with the stable isotope-dilution technique.[6,10] To study the formation and repair of DNA adducts in a laboratory setting, researchers frequently expose experimental animals and cultured cells to DNA damaging agents.[6,11] This approach not only enables accurate quantifications of known DNA lesions but also leads to the discovery of novel DNA modifications elicited by certain chemicals, which facilitate risk assessment.[9,11] Because of the lack of chemical specificity of the DNA damaging agents, the approach, however, frequently does not permit the induction of only one specific type of lesion in DNA.[12–14]

Alternatively, modified nucleobases or nucleosides could be added to the cell culture medium to enable their metabolic incorporation into genomic DNA.[15–17] For instance, 6-thioguanine (^{S}G), 6-thio-2′-deoxyguanosine (^{S}dG), and 5-aza-2′-deoxycytidine were shown

to be efficiently incorporated into genomic DNA, and these modified nucleobases and nucleosides have been successfully used as chemotherapeutic agents.[15,18,19] Additionally, 5-ethynyl-2′-deoxyuridine (EdU) could be incorporated into genomic DNA and its "clickable" property allows for its further application in bioorthogonal assays.[17,20] Moreover, 5-hydroxymethyl-2′-deoxycytidine and 5-formyl-2′-deoxycytidine could exert antitumor effects through their deamination to the corresponding dU derivatives and subsequent incorporation of the deaminated nucleosides into genomic DNA of cancer cells.[21] While the incorporation of these modified nucleosides into genomic DNA may involve replicative DNA polymerases, translesion synthesis (TLS) DNA polymerases have been documented to enable the incorporation of some of the modified nucleosides into genomic DNA. For instance, polymerase (Pol) λ and Pol κ were found to promote the incorporation of N^6-methyl-2′-deoxyadenosine and N^2-substituted-dG into genomic DNA of mammalian cells, respectively.[22–24] Thus, metabolic labeling of modified nucleosides and nucleobases may serve as a useful tool for studying the repair of modified nucleosides in genomic DNA.

Minor-groove N^2 position of dG is susceptible to modifications by various alkylating agents. For instance, exposure to benzo[*a*]pyrene results in N^2-BPDE-dG through its metabolite benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE).[25] In addition, aldehydes can attack the N^2 position of guanine to yield N^2-alkyl-dG.[26–28] The levels of N^2-MedG in mouse tissues were increased from 1.2–1.7 per 10^6 nucleosides to 1.9–4.2 per 10^6 nucleosides following a 4-week exposure to 1.5 mg/kg of methanol, which can be metabolized to formaldehyde.[28] Previous research in our laboratory demonstrated that N^2-alkyl-dG could be efficiently and accurately bypassed in mammalian cells proficient in translesion synthesis; however, loss of Pol κ, Pol ι, or REV1 could result in mutagenic bypass of these lesions.[29,30] Moreover, Pol η promotes the transcriptional bypass of these minor-groove DNA lesions.[24] Bulky N^2-BPDE-dG is a known substrate for NER;[31] nonetheless, no systematic research has been conducted about how NER responds to N^2-alkyl-dG lesions differing in sizes. Moreover, while *Escherichia coli* AlkB protein was shown to be capable of reverting various *N*-alkylated DNA lesions,[32] it remains unclear whether minor-groove N^2-alkyl-dG lesions could be subjected to a similar repair mechanism in human cells.

By taking advantage of metabolic incorporation, herein, we examined systematically the repair of N^2-methyl- and N^2-*n*-butyl-2′-deoxyguanosine (N^2-MedG and N^2-*n*BudG) in mammalian genome and its modulation by TLS polymerases, NER machinery and oxidative dealkylation enzymes (Figure 1). In addition, a comparative study on N^2-MedG and N^2-nBudG facilitates the assessment about the effects of alkyl group size on the metabolic incorporation and repair of N^2- alkyl-dG lesions.

EXPERIMENTAL SECTIONS

Materials.

If not mentioned otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and all enzymes were obtained from New England Biolabs (Ipswich, WA). Uniformly ^{15}N-labeled N^2-MedG ([$^{15}N_5$]-N^2-MedG), N^2-*n*BudG, and d_9-N^2-*n*BudG were synthesized previously.[24,33] N^2-MedG was prepared following published procedures (Scheme S1 and Figure S1).[34,35] HEK293T cells with *POLH*, *POLI*, *POLK*, *REV1*, and *REV3L* genes being

individually ablated by CRISPR were described previously,[30,36] while ALKBH1, ALKBH2, and ALKBH3 single knockout cells were generated following published procedures.[30,36] Repair-competent Chinese hamster ovary cells (CHO-AA8) and the isogenic ERCC1 knockout cells (CHO-7–27) were kindly provided by Prof. Michael Seidman (National Institute of Aging, Bethesda, MD).[37] Repair-proficient human skin fibroblasts (GM00637) and its XPA-deficient counterpart (GM04429) were generous gifts from Prof. Gerd P. Pfeifer (Van Andel Institute, Grand Rapids, MI).

Incorporation of N^2-nBudG and N^2-MedG into Genomic DNA.

HEK293T cells, TLS polymerase-deficient cells, NER-deficient cells, and ALKBH1–3 knockout cells were seeded in 6-well plates at 37 °C in a 5% CO_2 atmosphere. N^2-MedG and N^2-*n*BudG were added to the culture medium at a final concentration of 10 μM. After incubation for 3 (for N^2-*n*BudG) or 16 h (for N^2-MedG), the cells were harvested immediately or cultured for another 3 or 8 h in fresh media without the modified nucleoside and harvested afterwards. Genomic DNA was extracted from cells using Qiagen DNeasy Blood & Tissue Kit, and approximately 6 μg of DNA was recovered from a single well of cells.

Enzymatic Digestion.

Extracted genomic DNA was subjected to enzymatic digestion following previously published procedures.[14,24,38] In brief, 1.0 μg of cellular DNA was digested with 10 units of nuclease P1 and 0.00125 unit of phosphodiesterase II in a buffer with 30 mM sodium acetate (pH 5.6), 1 mM $ZnCl_2$, and 2.5 nmol of *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA, adenosine deaminase inhibitor). The above mixture was incubated at 37 °C for 24 h. After then, 1.0 unit of alkaline phosphatase, 0.0025 unit of phosphodiesterase I and one tenth volume of 0.5 M Tris-HCl (pH 8.9) were added. The resulting mixture was incubated at 37 °C for another 4 h and subsequently neutralized with 1.0 M formic acid. The enzymes in the digestion mixture were then removed by chloroform extraction. The aqueous phase was dried in vacuo and reconstituted in water for LC-MS/MS analysis.

Online nLC-MS/MS Analysis of N^2-nBudG and N^2-MedG in Cellular DNA.

Online nLC-MS/MS analysis of N^2-*n*BudG was performed following our previously described procedures.[24] N^2-MedG was quantified in a similar way except that a slower gradient was employed. In brief, the separation was conducted on a Dionex Ultimate 3000 module (Thermo Fisher Scientific, Inc.) with a homemade trapping column (150 μm × 40 mm) and an analytical column (75 μm × 200 mm) packed with Magic C18 AQ (200 Å, 5 μm, Michrom BioResource, Auburn, CA) reversed-phase materials. Mobile phases A and B contained 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The sample was loaded onto the trapping column with mobile phase A at a flow rate of 2.5 μL/min in 8 min, and the analyte (N^2-MedG) and its corresponding stable isotope-labeled standard were subsequently eluted from the column by using a 30 min linear gradient of 0−95% mobile phase B at a flow rate of 300 nL/min.

The LC effluent was directed to a TSQ-Altis triple-quadrupole mass spectrometer operated in the multiple-reaction monitoring (MRM) mode. The MRM transitions included the

neutral loss of a 2-deoxyribose (116 Da) from the $[M + H]^+$ ions of N^2-*n*BudG (i.e., *m*/*z* 324 → 208) and N^2-MedG (i.e., *m*/*z* 282 → 166), as well as their stable isotope-labeled counterparts (i.e., *m*/*z* 333 → 217 for d_9-N^2- *n*BudG and *m*/*z* 287 → 171 for [$^{15}N_5$]-N^2-MedG) (Figures 2 and S3). The voltage for electrospray was 2.0 kV, and the temperature for the ion transfer tube was 275 °C. The widths for precursor and fragment ion isolation were both 0.7 *m*/*z* unit, and the collision energy was 20 V.

The calibration curve for the analysis of N^2-*n*BudG was described previously,[24] whereas the calibration curve for N^2-MedG was constructed by spiking 1.0 μg of calf thymus DNA with different amounts of an N^2-MedG-containing 12-mer ODN (5′-ATGGCGXGCTAT-3′, X = N^2-MedG) and a fixed amount (250 fmol) of [$^{15}N_5$]-N^2-MedG, followed by enzymatic digestion and LC-MS/MS analysis as mentioned above for the cellular DNA samples (Figure S2).

RESULTS

Pol κ and Pol η Incorporate N^2-MedG into Genomic DNA and Contribute to Its Repair.

To examine systematically the repair of N^2-MedG in mammalian cells, we extended our previously reported method for N^2-*n*BudG[24] to include the quantification of N^2-MedG (Figures 1−3). In this respect, the N^2-alkyl-dG derivatives could be uptaken into cells and metabolically activated to yield the corresponding nucleoside triphophates, which could be subsequently incorporated into genomic DNA by translesion synthesis DNA polymerases.[23,24] By using this method, we found that N^2-MedG was not detectable in genomic DNA isolated from HEK293T cells. After a 16-h incubation of HEK293T cells in a medium containing 10 μM N^2-MedG, we detected N^2-MedG at a level of approximately 90 modifications per 10^6 nucleosides (Figure 3 and Table S1). It is of note that we did not observe any apparent alterations in the growth or survival of HEK293T cells upon exposure to N^2-MedG. Markedly lower levels of N^2-MedG were detected in genomic DNA from isogenic cells depleted of Pol κ or Pol η, underscoring their roles in the incorporation of N^2-MedG into genomic DNA. Other TLS Pols tested (i.e., Pol ι, REV1 and Pol ζ) did not exhibit any appreciable roles in the incorporation of N^2-MedG into genomic DNA, as their depletion did not result in any diminutions in the level of N^2-MedG incorporated into genomic DNA (Figure 3 and Table S1).

Our time-dependent repair experiments suggest a possible role of TLS polymerases in the repair of N^2-MedG. Similar to our recently published result on N^2-*n*BudG,[24] Pol κ assumes an important role in the removal of N^2-MedG (Figure 3 and Table S1). In particular, while we observed a progressive decline in the level of N^2-MedG in genomic DNA of parental HEK293T cells at 0−8 h following exposure to the modified nucleoside, no appreciable drop in the level of N^2-MedG was detected for Pol κ-deficient cells (Figure 3 and Table S1). However, different from our findings made for N^2-*n*BudG,[24] Pol η may also be involved in the repair of the less bulky N^2- MedG (Figure 3 and Table S1). Deficiencies in other human TLS Pols investigated in this study, that is, Pol ι, REV1, and Pol ζ, did not alter appreciably the time-dependent decreases in the levels of N^2-MedG, suggesting the lack of involvement of these polymerases in the repair of N^2-MedG in genomic DNA (Figure 3 and Table S1).

Involvement of NER in the Removal of N^2-alkyl-dG from Human Cells.

We next conducted our time-dependent repair experiments in mammalian cells proficient in DNA repair or deficient in NER factors (Figure 4, Tables S1 and S2). We found that depletion of two NER proteins, that is, xeroderma pigmentosum complementation group A (XPA) protein and endonuclease ERCC1, both led to diminished removal of the two minor-groove N^2-alkyl-dG lesions, revealing the contributions of NER in the repair of these two lesions in mammalian cells.

Human ALKBH3 May Be Involved in the Removal of N^2-Alkyl-dG Lesions from the Nucleotide Pool.

Our quantification results revealed a potential role of human AlkB analog ALKBH3, but not ALKBH1 or ALKBH2, in the reversal of the N^2-alkyl-dG lesions in the nucleotide pool (Figure 5, Tables S1 and S2). We observed elevated level of N^2-MedG and N^2-*n*BudG in the genomic DNA of ALKBH3-depleted cells compared to isogenic HEK293T cells after exposure to the modified nucleosides, that is, 160 versus 90 N^2-MedG and 32 versus 21 N^2-*n*BudG per 10^6 nucleosides, from ALKBH3 knockout cells and WT cells, respectively. Similar to what we observed for parental HEK293T cells, the levels of N^2-MedG and N^2-*n*BudG exhibit progressive decline over time in ALKBH3-deficient cells, suggesting that the two lesions in genomic DNA can still be repaired in ALKBH3-deficient background. Hence, increased levels of the N^2-MedG and N^2-*n*BudG observed in ALKBH3-deficient cells may arise from their diminished removal from the nucleotide pool.

DISCUSSION

Substantial efforts have been made to assess the occurrence and repair of structurally defined DNA lesions.[6,9,39] Exposing laboratory animals or cultured cells with DNA damaging agents is widely adopted and represents one of the most reliable and efficient ways to discover novel DNA lesions or quantify DNA lesions.[6,11] After removing exogenous chemicals in diet or cell culture medium, researchers can further assess the repair of DNA lesions of interest by monitoring time-dependent decreases in the levels of DNA lesions. The method may provide important knowledge about the occurrence and repair of DNA lesions induced by carcinogenic chemicals, thereby informing risk assessment.[9,11] The method, however, poses limitations sometimes. In particular, chemical exposure often gives rise to a wide spectrum of lesions. For instance, exposure to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a carcinogen found in tobacco and its combustion products, can induce a number of alkylation adducts formed on the nucleobases and backbone phosphates in DNA.[12,13,40] Metabolic labeling of genomic DNA with a structurally defined nucleoside can overcome this limitation by enabling selective incorporation of the modified nucleoside without the complicating effects of other DNA lesions.[22−24]

In this study, we employed a metabolic labeling method to introduce minor-groove N^2-MedG and N^2-*n*BudG into genomic DNA. After their incorporation into genomic DNA, we interrogated how their removals are modulated by DNA repair machinery and TLS polymerases. We found that Pol κ and Pol η play important roles in the incorporation of N^2-MedG into the human genome (Figure 3 and Table S1). Interestingly, both Pols also

contribute to the repair of N^2-MedG (Figure 3 and Table S1). Pol η is known to confer intrinsic resistance to chemotherapy,[41] while Pol κ-deficient mouse embryonic fibroblasts display decreased NER of UV-induced DNA lesions.[42] Taken these previous findings with our results together, Pol κ and η may function in DNA repair, which further expands their roles beyond translesion synthesis. It remains unclear why Pol η does not prominently modulate the repair of the more bulky N^2-*n*BudG.[24]

NER is a versatile DNA repair pathway that removes a plethora of lesions from DNA.[43] Bulky BPDE adducts formed at the N^2 position of dG is a good substrate for NER.[31] Recently Patel and co-workers[27] demonstrated that NER is required to protect mammalian cells against formaldehyde toxicity and N^2-dG adduct induced by the aldehyde. In line with these previous findings, our results unveil that NER contributes to the repair of both N^2-MedG and N^2-*n*BudG (Figure 4, Tables S1 and S2).

The AlkB family of Fe(II)- and α-ketoglutarate-dependent dioxygenases perform direct reversal by oxidative dealkylation that removes various alkyl adducts.[44] It was first found in *E. coli* and was considered as “adaptive response” protein that protects against alkylation.[45–47] AlkB has many substrates, from simple alkyl chain adducts to small exocyclic etheno adducts.[32,44,48,49] Human genome encodes nine AlkB homo-logues (i.e., ALKBH1–8, FTO), and, among them, ALKBH2 and ALKBH3 are DNA repair enzymes.[44,50–52] Our results showed that N^2-MedG and N^2-*n*BudG are elevated in genomic DNA of ALKBH3-depleted cells compared to parental repair-proficient cells after exposure to these two modified nucleosides in culture medium (Figure 5, Tables S1 and S2). Nevertheless, the level of N^2-alkyl-dG in genomic DNA of ALKBH3-deficient cells decreased gradually with time, in a manner similar to that found for parental HEK293T cells (Figure 5, Tables S1 and S2). Hence, ALKBH3 may reverse N^2-alkyl-dG in the nucleotide pool prior to its incorporation into genomic DNA, and its depletion may lead to high levels of N^2-alkyl-dG in the nucleotide pool, which may ultimately result in the incorporation of a higher level of N^2-alkyl-dG into genomic DNA.

Taken together, we employed a metabolic labeling method to incorporate minor-groove N^2-MedG and N^2-*n*BudG into genomic DNA. We also conducted a systematic repair study on these two lesions. Our results revealed the roles of TLS polymerases, NER and ALKBH3 in the repair of these lesions in mammalian cells. It will be interesting to examine, in the future, whether the method can also be employed for assessing the metabolic incorporation and repair of bulky aromatic hydrocarbon-induced N^2-dG adducts, for example, N^2-BPDE-dG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DEDICATION

This paper is dedicated to Prof. Lawrence J. Marnett on the occasion of the 35th anniversary of *Chemical Research in Toxicology* for his outstanding contributions to the chemical toxicology field and his unwavering support of young scientists in the field.

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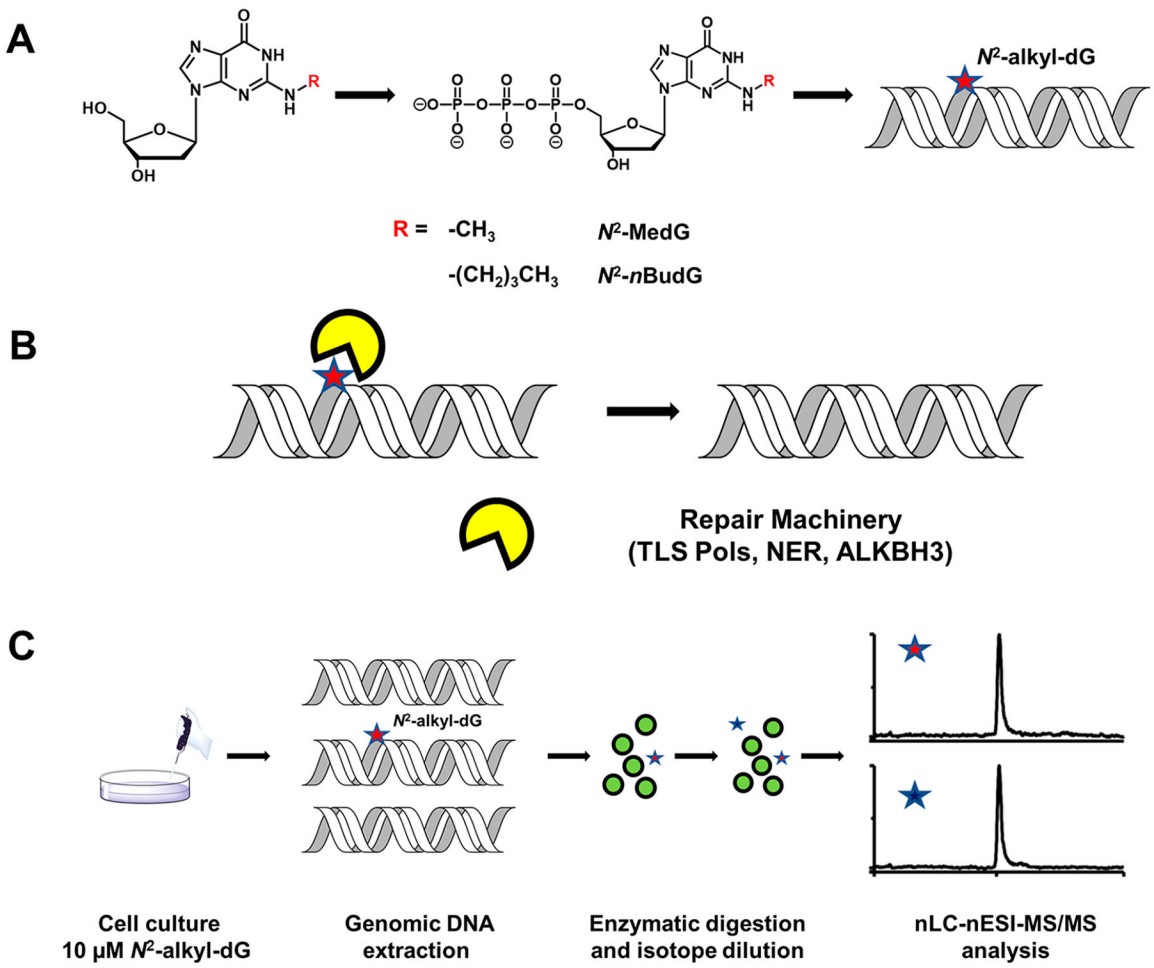

Figure 1.

LC-MS/MS for assessing the incorporation and repair of N^2-alkyl-dG in genomic DNA. (A) Genomic incorporation of N^2-MedG and N^2-*n*BudG. (B) Repair of N^2-MedG and N^2-*n*BudG. (C) Schematic diagram showing the experimental workflow. Cells proficient or deficient in DNA repair were exposed with 10 μM of N^2-MedG or N^2-*n*BudG, and then incubated in fresh medium without the modified nucleosides for 3 or 8 h. The cells were harvested and genomic DNA extracted. Oligodeoxynucleotides containing a site-specifically inserted and stable isotope-labeled N^2-MedG or N^2-*n*BudG were spiked into the genomic DNA, which were subsequently digested to mononucleosides. The nucleoside mixtures were subjected to LC-MS/MS analysis.

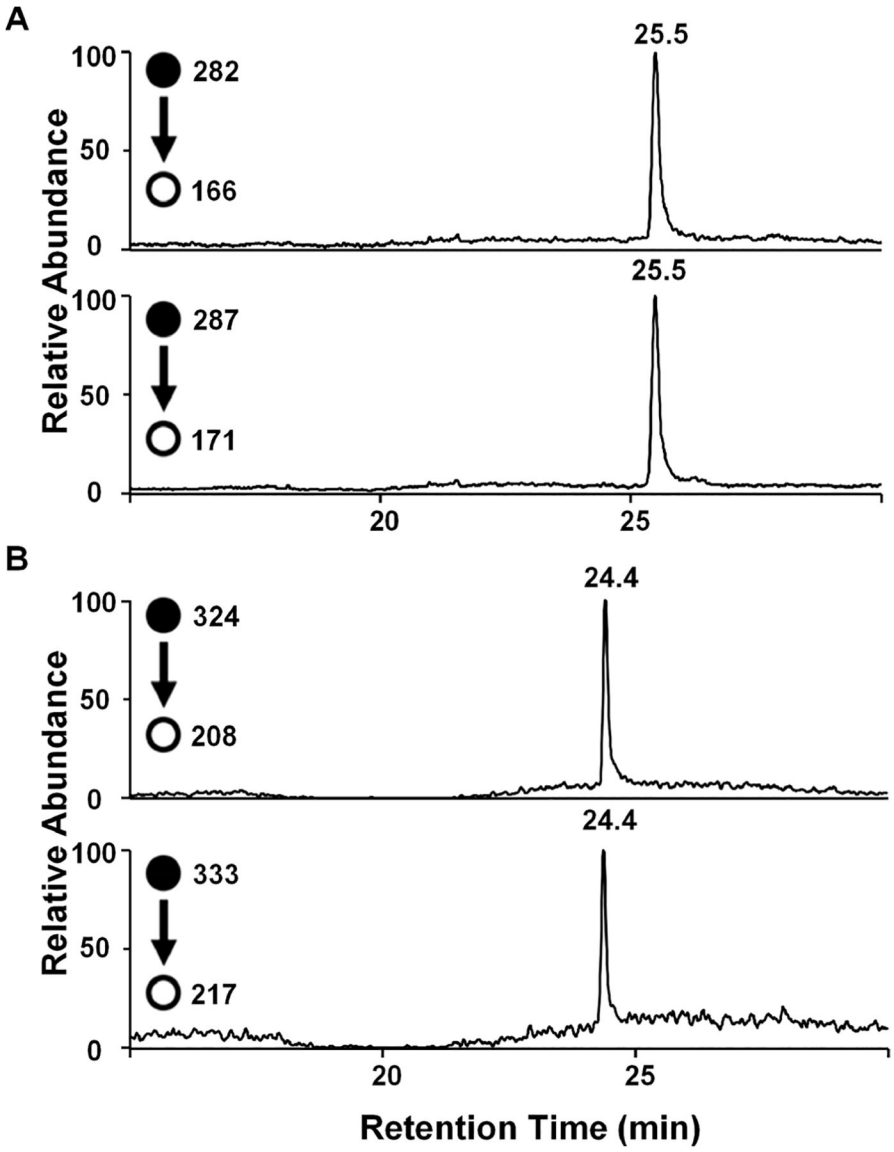

Figure 2.

Representative selected-reaction monitoring chromatograms of the *m*/*z* 282 → 166 (A, top panel), 287 → 171 (A, bottom panel), 324 → 208 (B, top panel), and 333 → 217 (B, bottom panel) transitions for the $[M + H]^+$ ions of the unlabeled and stable isotope-labeled N^2-MedG (A) and N^2-*n*BudG (B), respectively, in the digested nucleosides of DNA extracted from CHO-AA8 cells treated with 10 μM N^2-MedG for 16 h or N^2-*n*BudG for 3 h, respectively.

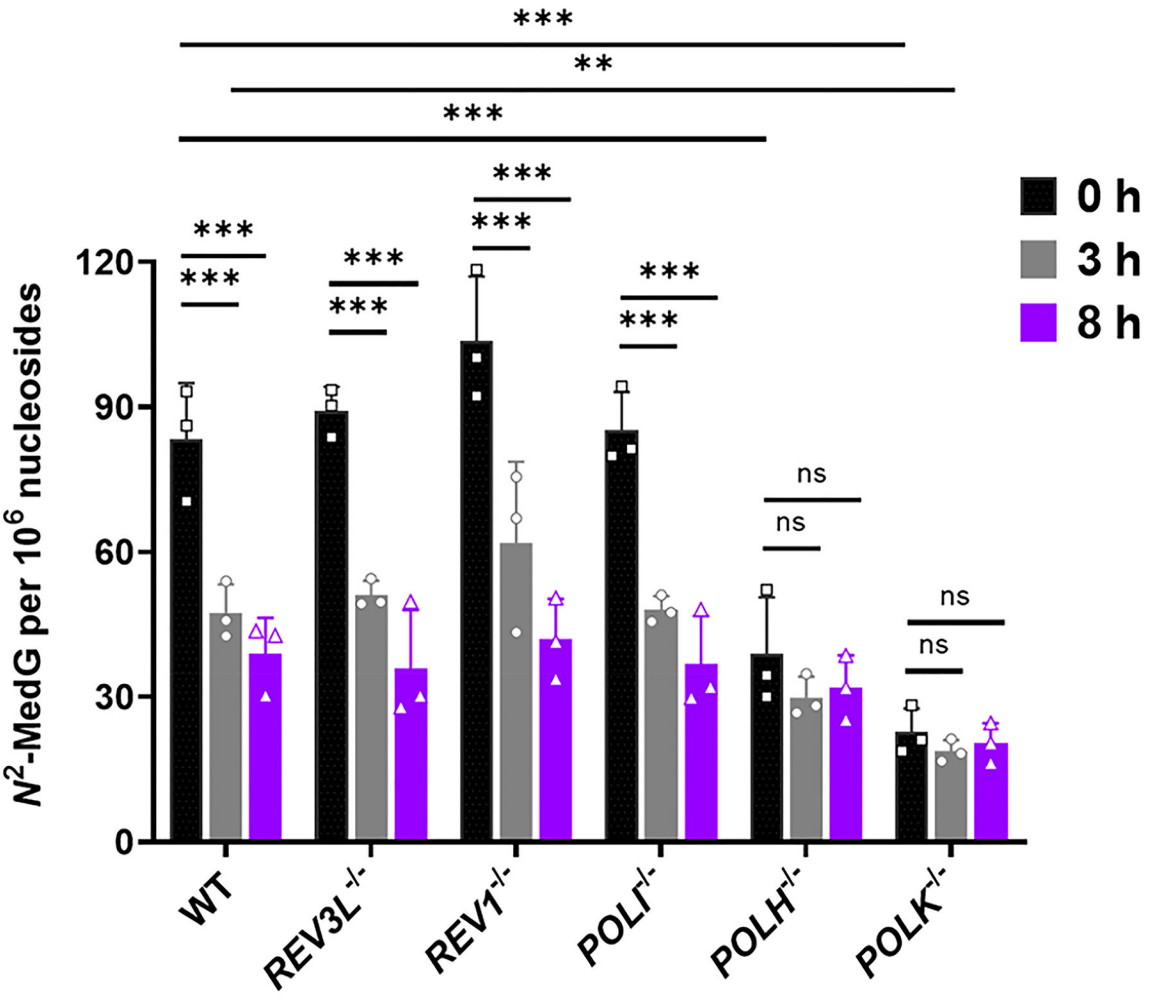

Figure 3.

Frequencies of N^2-MedG in cellular DNA isolated from parental and TLS polymerase-depleted HEK293T cells. All cells were exposed to 10 μM of N^2-MedG for 16 h. The cells were then harvested immediately, or after incubation in fresh media for another 3 or 8 h. The data represent the mean ± SD of results obtained from three independent experiments. ns, $p > 0.05$; *, $0.01 < p < 0.05$; **,$0.001 < p < 0.01$; ***, $p < 0.001$. The p values were calculated by one-way ANOVA with Tuckey's multiple comparisons test.

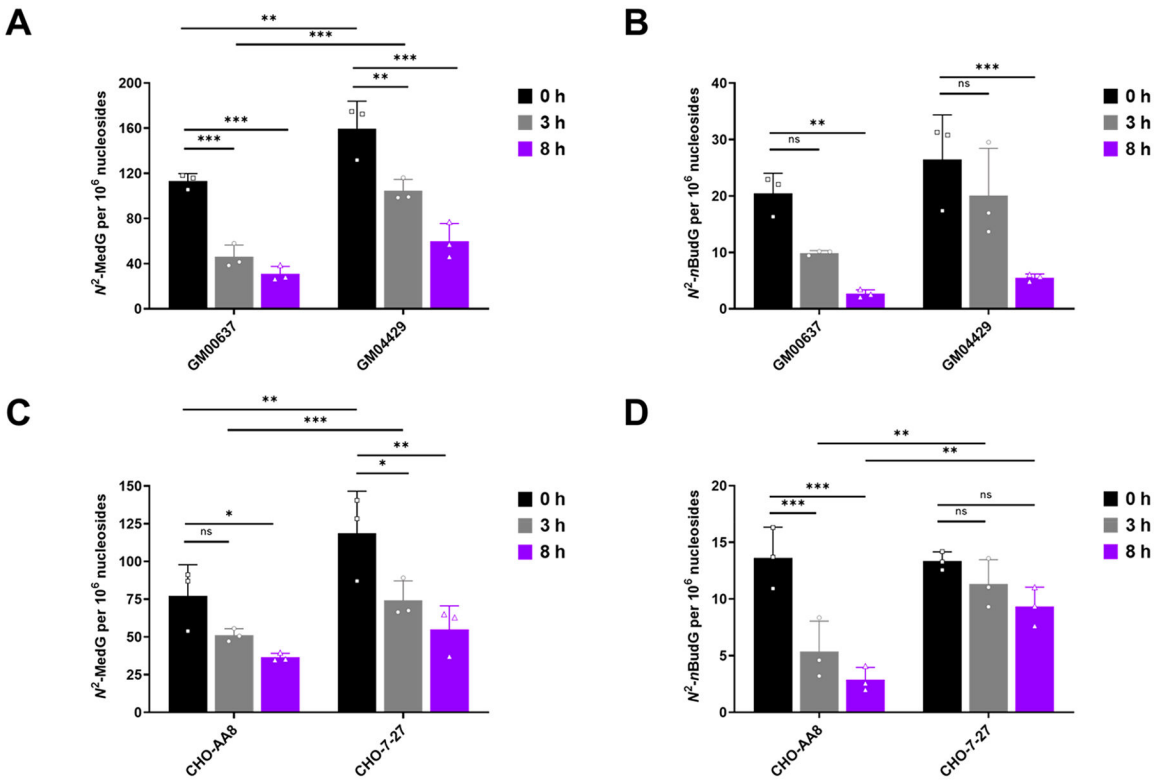

Figure 4.

LC-MS/MS results of N^2-MedG (A, C) and N^2-*n*BudG (B, D) in cellular DNA isolated from NER-competent and -deficient cells. After a 16-h exposure to 10 μM of N^2-MedG or 3-h exposure to 10 μM of N^2-*n*BudG, the cells were then harvested immediately, or cultured in fresh medium without the modified nucleosides for another 3 or 8 h. The data represent the mean ± SD of results obtained from three biological replicates. ns, $p > 0.05$; *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$. The p values were calculated by one-way ANOVA with Tuckey's multiple comparisons test.

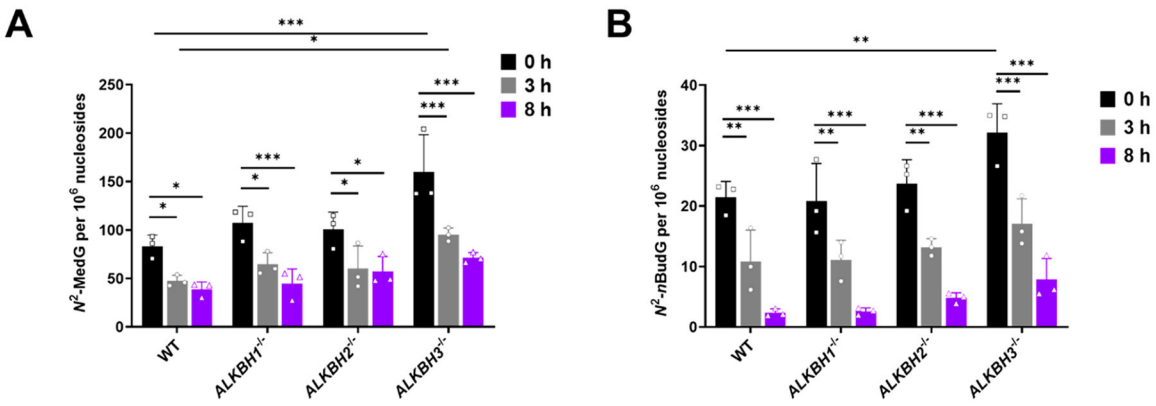

Figure 5.

Elevated level of N^2-MedG (A) and N^2-*n*BudG (B) were observed in cellular DNA isolated from ALKBH3-depleted cells. After a 16-h exposure to 10 μM of N^2-MedG or 3-h exposure to 10 μM of N^2-*n*BudG, respectively, isogenic HEK293T cells and ALKBH1−3 individually knockout cells were then harvested for DNA extraction immediately, or after incubation in fresh media without N^2-MedG or N^2-*n*BudG for another 3 or 8 h. The data represent the mean ± SD of results obtained from three biological replicates. *, 0.01< p < 0.05; **, 0.001< p < 0.01; ***, p <0.001. The p values were calculated by one-way ANOVA with Tuckey's multiple comparisons test.