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Roles of Aag,Alkbh2, and Alkbh3 in the Repair of Carboxymethylated and Ethylated Thymidine Lesions

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Abstract

Environmental and endogenous genotoxic agents can result in a variety of alkylated and carboxymethylated DNA lesions, including *N*3-ethylthymidine (*N*3-EtdT), O^2-EtdT, O^4-EtdT as well as *N*3-carboxymethylthymidine (*N*3-CMdT) and O^4-CMdT. By using non-replicative double-stranded vectors harboring a site-specifically incorporated DNA lesion, we assessed the potential roles of alkyladenine DNA glycosylase (Aag), alkylation repair protein B homologue 2 (Alkbh2) or Alkbh3 in modulating the effects of *N*3-EtdT, O^2-EtdT, O^4-EtdT, *N*3-CMdT or O^4-CMdT on DNA transcription in mammalian cells. We found thatthe depletion of Aagdid not significantly change the transcriptional inhibitory or mutagenic properties ofall five examined lesions, suggesting a negligible role of Aag in the repair of these DNA adducts in mammalian cells. In addition, our results revealed that *N*3-EtdT, but not other lesions, could be repaired by Alkbh2 andAlkbh3 in mammalian cells.Furthermore, we demonstraed the direct reversal of *N*3-EtdT by purified human Alkbh2 protein*in vitro*.These findings provided important new insights into the repair of the carboxymethylated and alkylated thymidine lesions in mammalian cells.

Human cells are constantly exposed to various endogenous and exogenous agents that can induce damage to genomic DNA[1]. Unrepaired DNA lesions may inhibit DNA replication and transcription as well as induce mutations in these processes, which may eventually result in the development of cancer and other human diseases[2,3].

DNA alkylation damage is generally unavoidable due to theubiquitouspresence of environmental and endogenous alkylating agents[4]. The*N*3, O^2, and O^4 of thymine are among the major alkylation sites in DNA and the resulting alkylated thymine lesions have been readily detected in various mammalian cells and tissues[5-9]. In particular, the regioisomeric

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Additional information

Supplementary information is available in the online version of the paper.

Competing Financial Interests

The authors declare no competing financial interests.

*N*3-ethylthymidine (*N*3-EtdT), O^2-EtdT, and O^4-EtdT (Figure 1) lesions have been detected at significantly higher levels in leukocyte and saliva DNA of smokers than nonsmokers, and thus have been proposed as prospective biomarkers for ethylation damage and for cancer risk assessment[8,9].

N-nitroso compounds (NOCs) represent another common type of DNA damaging agents that are widely present in processed meat, tobacco smoke, and other external and internal sources[10-12]. Metabolic activation of many NOCs can cause the formation of carboxymethylating agents (*e.g.*, diazoacetate) which can modify DNA to give a battery ofcarboxymethylated DNA adducts[13-15]. It was recently reported that *N*3-carboxymethylthymidine (*N*3-CMdT) and O^4-CMdT (Figure 1) are the major carboxymethylated thymidine lesions formed in isolated DNA exposed to diazoacetate, and may contribute to diazoacetate-induced signature mutations in *TP*53 gene found in gastrointestinal cancer[14,16-18].

Several studies have examinedthe roles of translesion synthesis DNA polymerases and nucleotide excision repair (NER) proteins in modulating the effects of these DNA lesions on DNA replication or transcription *in vitro* and in cells[17-23]. For instance, it was found that DNA polymerase V is required for the error-prone bypass of all three regioisomeric EtdT lesions and the error-free bypass of *N*3-CMdT in *Escherichia coli* cells[18,21]. In addition, depletion of transcription-coupled NER proteins can exacerbate the inhibitory and mutagenic effects of *N*3-EtdT, *N*3-CMdT and O^4-CMdT on DNA transcription in mammalian cells[19,20].

The *E.coli*N3-methyladenine DNA glycosylase II (AlkA) and its mammalian ortholog, alkyladenine DNA glycosylase (Aag), recognize and excise a chemically diverse array of alkylated DNA lesions[24-27]. In this vein, crude extractsof *E.coli* cells inducedfor the adaptive response remove O^2-methyl-2′-deoxycytidine (O^2-MedC) and O^2-MedT from DNA and these activities are dependent on the presence of AlkA[28].On the other hand, *E.coli* AlkB, a dioxygenase that requires non-heme Fe(II), molecular oxygen, and 2-oxoglutarate as cofactors,canrepaira variety of alkylated DNA lesions including*N*3-methyl-2′-deoxycytidine (*N*3-MedC) and *N*3-MedT[29-34]. Among the nine mammalian AlkB homologs (i.e., Alkbh1-Alkbh8 and FTO), Alkbh2 and Alkbh3 were observed to be capable of reversing directly some simple *N*-alkylated DNA lesions[29,35,36]. Moreover, deficiency in these two genes confers elevated sensitivities of mammalian cells toward alkylating agents[29,36-38].Therefore,it is important toinvestigatethe involvement ofAag, Alkbh2, and Alkbh3inthe repair of*N*3-EtdT, O^2-EtdT, O^4-EtdT, *N*3-CMdT and O^4-CMdT lesions in mammalian cells.

RESULTS AND DISCUSSION

Using an established competitive transcription and adduct bypassassay(Figure 2a)[39], we examined the potential roles of Aag, Alkbh2 and Alkbh3 in modulating the deleterious effects of *N*3-EtdT, O^2-EtdT, O^4-EtdT, *N*3-CMdT and O^4-CMdT on transcription in mammalian cells. Briefly, non-replicating double-stranded plasmids containing a site-specifically incorporated DNA lesion or unmodified dT were constructed, and co-transfected

with a non-lesion competitor into mammalian cells that are proficient or deficient in Aag, Alkbh2, or Alkbh3(Supplementary Figure S1). The RNA products were extracted from the cells, and treated with DNaseI to eliminate residual DNA contamination. The transcripts of interest were amplified by reverse transcription PCR (RT-PCR), and the resulting RT-PCR products were restriction digested to generate short oligodeoxyribonucleotides (ODN) fragments for polyacrylamide gel electrophoresis (PAGE)and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses. With the use of two sets of restriction enzymes, *i.e.*, NcoI/SfaNI and MlucI/Cac8I[19,20], restriction fragments with single nucleotide differences at the lesion site were resolved by PAGE analysis (Figure 2b-e). The identities of the restriction fragments of interest were also confirmed by LC-MS/MS analysis (Supplementary Figures S2-S3).

Our results showed that the fivelesions substantially inhibit DNA transcription and induce mutations, whereas depletion of Aag had a negligible effect on transcriptional alterations induced by these DNA lesions in mammalian cells (Figures 2b-e, and 3a-b, Supplementary Table S2 and S3). We also found that the absence of Alkbh2 or Alkbh3did not lead to a statistically significantchange ($P > 0.05$) in the transcriptional blockageor mutagenic properties ofO^2-EtdT, O^4-EtdT, *N*3-CMdT and O^4-CMdT in mammalian cells (Figure 4a-b, Supplementary Figure S4, and Supplementary Table S4 and S5). In addition,the removal of Alkbh2 or Alkbh3fromthe wild-type background conferredno obvious impact on the bypass of *N*3-EtdT, but could lead to an altered mutational signature of *N*3-EtdT(Figure 4a-b, Supplementary Figure S4, and Supplementary Tables S4 and S5).We found that transcriptional bypass of *N*3-EtdT induced A✧Cand A✧U mutations at markedly higher frequencies (~11% and ~26%, respectively) in Alkbh2-deficient cells than in the repair-proficient cells (~6% and ~20%, respectively)(Figure 4a-b, Supplementary Figure S4, and Supplementary Table S5). We also observed that *N*3-EtdT induced a substantially higher degree of A✧U mutation (~31%) in Alkbh3-deficient cells than in the wild-type background (Figure 4a-b, Supplementary Figure S4, and Supplementary Table S5). These results suggest that,in mammalian cells, Alkbh2 and Alkbh3 may be involved in the removal of *N*3-EtdT, but not O^2-EtdT, O^4-EtdT, *N*3-CMdT or O^4-CMdT.

We also investigated the repair activity of Alkbh2orAlkbh3toward*N*3-EtdT and the other four lesions using an *in vitro*DNA repair assay. To this end, we incubated a 12-mer lesion-bearing oligodeoxynucleotide,or a duplex DNA with a single lesionlocated in the same sequence context, with purified human Alkbh2or Alkbh3proteins in HEPES buffer containing the requisite cofactors including2-oxoglutarate and ascorbate. We subsequently monitored the products of the *in vitro* repair reaction by using LC-MS and MS/MS analysis. In the reaction without the DNA repair protein, we observed the monoisotopic peak for the $[M-3H]^{3-}$ ion (*m/z*1233.15) of the 12-mer substrate containing the unaltered *N*3-EtdTlesion (Figure 5a, b). In the presence of the Alkbh2 protein, we found that the *N*3-EtdT, when located in the double-stranded but not single-stranded DNA, was partially converted to unmodified thymidine (the $[M-3H]^{3-}$ ion at $m/z = 1223.79$) and an intermediate product with the methylene functionality in the ethyl group being oxidized to its hydroxymethylene derivative (i.e., HO-*N*3-EtdT, the $[M-3H]^{3-}$ ion at $m/z = 1238.44$) (Figure 5a, cand Supplementary Figure S5a). In addition, we did not observe any repair activity of Alkbh2 protein towardO^2-EtdT, O^4-EtdT, *N*3-CMdT or O^4-CMdT *in vitro* (Supplementary Figures

S6and S7), which isin agreement with our cellular repair results.Unexpectedly, the presence of purified Alkbh3 protein did not result in detectablelevel of repair of *N*3-EtdT-bearing DNA in the *in vitro* reaction under our experimental conditions(Supplementary Figure S5b).As a control, incubation of *N*3-MedC-bearing DNA with the Alkbh2 or Alkbh3 protein produced the undamaged product(Supplementary Figure S8), which is consistent with previous findings that *N*3-MedC is a substrate forthese repair proteins[36,37].

A previousstudy suggested thatthe bypass of a strongly blocking lesion (*e.g.N*3-MedT) by AlkB can confer distinct effects on miscoding properties of the DNA lesion during DNA replication in *E.coli* cells[30]. In this vein, the absence of AlkB did not considerably change the inhibitory effect of *N*3-MedT on DNA replication, but couldlead to a markedincrease in T→A transversion in *E.coli* cells[30].In line with this previous finding, our results demonstrated that the mammalian AlkB homologues (i.e., Alkbh2andAlkbh3) could manifest a significant change in the effect of *N*3-EtdT on the fidelity, but not the efficiency, of DNA transcription in mammalian cells. In this respect, we found that depletion of Alkbh2leads to elevated frequencies of A→U and A→C mutations, whereas depletion of Alkbh3can only increase A→Umutation opposite the *N*3-EtdT lesion.Further studies are needed to elucidate the distinct impacts of Alkbh2 and Alkbh3 on the mutagenic properties of *N*3-EtdT during transcription in mammalian cells in the future.

We alsofound that Alkbh2 is capable of repairing the *N*3-EtdT lesion within double-stranded DNA*in vitro* by a direct reversal mechanism.In keeping with our findings, previous *in vitro* studies showed that both the*E.coli*AlkB and the human Alkbh2 demethylate *N*3-MedT in DNA, but that Alkbh3 is much less efficient at repairing *N*3-MedT than the other two proteins[40].In addition, it was reported that *N*3-MedC is much more efficiently removed by Alkbh2 from double-stranded DNA than from single-stranded DNA [36]. We found that Alkbh2 is less efficient at removing *N*3-EtdT from double-stranded DNA than *N*3-MedC from single-stranded DNA under our *in vitro* repair conditions, indicating that *N*3-EtdT may not be as good a substrate as *N*3-MedC for Alkbh2. On the other hand, we did not observe the direct reversal of *N*3-EtdT by purified human Alkbh3 protein under the conditions tested.suggesting that other DNA repair cofactor(s)may be required for Alkbh3-mediated removal of *N*3-EtdT in mammalian cells. In this vein, a previous study showed that ASCC3 helicase can interact with Alkbh3 to facilitate DNA alkylation repair [41].

Different from *N*3-EtdT, we found that O^2-EtdT, O^4-EtdT and O^4-CMdT lesions are not substrates for Alkbh2orAlkbh3*in vitro*or in mammalian cells. Our results further supported the notion that AlkB and its mammalian homologs repair only those alkylated DNA lesions that are attached via the nitrogen atoms, but not oxygen atoms of the nucleobases[30,31,36,37,40,42]. On the other hand, we found that neither Alkbh2 nor Alkbh3 is involved in the repair of *N*3-CMdT *in vitro*or in mammalian cells. The distinct repair activities of the mammalian AlkB homologues for*N*3-EtdT and *N*3-CMdT may be attributed to the unique chemical properties of these two lesions. These results suggest that the addition of a hydrophilic and bulky carboxyl group, but not a hydrophobic and relatively small ethyl group, to the *N*3 position of thymine may inhibit the binding of the lesion to the active site of mammalian AlkB homologue (*e.g.*Alkbh2 or Alkbh3).

In conclusion,we systematically investigated, for the first time, the potential roles of Aag, Alkbh2 and Alkbh3 in the repair of regioisomeric ethylated and carboxymethylated thymidine lesions. Our results revealed thatAag displayed a negligible role in the transcriptional alterations induced by *N*3-EtdT,O^2-EtdT, O^4-EtdT, *N*3-CMdT and O^4-CMdT, suggesting that Aag is not involved in repairing these lesions in mammalian cells. We also found that,among these five DNA adducts, only *N*3-EtdT constitutes a substrate for the mammalian AlkB homologues (*i.e.*, Alkbh2 or Alkbh3). Together, these findings provide important new insights into the repair of DNA lesions induced by alkylating and carboxymethylating agents in mammalian cells.

METHODS

Materials

All chemicals, enzymes, [γ-^{32}P]ATP, and unmodified ODNs unless otherwise specifiedwere purchased fromSigma-Aldrich, New England BioLabs, Perkin-Elmer and Integrated DNA Technologies, respectively. Purified human Alkbh2 and Alkbh3 proteins as well as the mouse embryonic fibroblast (MEF) cells that are proficient or deficient in Alkbh2 or Alkbh3were prepared as described previously [36,38]. The $Aag^{+/+}$ and $Aag^{-/-}$MEF cells were kindlyprovided by Prof. Leona Samson[43,44]. Cells were grown in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin(Invitrogen),and 100 μg/mL streptomycin (ATCC) in a 37°C incubator with 5% CO_2.

Genotyping

DNA was isolated from MEF cells using a high-salt method [45], and the targeted *Alkbh2*,*Alkbh3*, or *Aag*deletions were confirmed by PCR analysis as described previously [25,38]. The primers are listed in Supplementary Table S1, and the PCR amplification with Phusion high-fidelity DNA polymerase (New England Biolabs) started at98°C for 2 min; then, 35 cycles at 98°C for 10 s, 58°C for 30 s, and 72°C for 5 s, and a final 5-min extension at 72°C. The PCR products were seperated on 2% (w/v) agarose gelsin the presence ofSYBR Safe DNA Gel Stain by electrophoresis, and then visualized underUVA light.

In vivo transcription assay

Non-replicating pTGFP-Hha10 plasmids containing a single site-specific lesion (i.e., *N*3-EtdT, O^2-EtdT, O^4-EtdT, *N*3-CMdT or O^4-CMdT)as well as the undamaged control and competitor plasmid substrates were prepared as described previously[19,20,46]. The *N*3- or O^2-EtdT-bearing plasmids were premixed with the competitor vector at a molar ratio of 12:1 (lesion/competitor), and the other lesion-bearing or lesion-free control plasmids were mixed individually with the competitor vector at a molar ratio of 3:1. The mixed DNA templates were then transfected into the $Aag^{-/-}$, $Alkbh2^{-/-}$ or $Alkbh3^{-/-}$ cells as well as the corresponding wild-type cells following the previously published procedures[19,20,46].

RNA extraction and RT-PCR

The RNAproducts arising from *in vivo* transcription were extracted using Total RNA Kit I (Omega) and were further treated witha DNA-free kit (Ambion) to eliminate DNA contamination following the manufacturer's instructions. The transcripts of interest were reverse transcribed and the resulting cDNA products were PCR amplified as described elsewhere[19,20,46].

PAGE analysis

For NcoI/SfaNI-mediated restriction digestion/postlabeling assay, a portion of the RT-PCR products was treated with 5 U NcoI and 1 U shrimp alkaline phosphatase in 10 μLof 1 × NEB buffer 3.1 at 37°C for 1 h and then at 70°C for 20 min. The dephosphorylated restriction fragments were radiolabeled with 5 U T4 polynucleotide kinase and ATP (50 pmol cold, premixed with 1.66 pmol [γ-^{32}P]ATP) and were further digested with 2 U SfaNI in 20 μLof 1 × NEB buffer 3.1 at 37°C for 2 h. The resulting DNA mixtures were resolved by 30% native PAGE (acrylamide:bis-acrylamide=19:1) and quantified by phosphorimager analysis[19,20,46]. The MluCI/Cac8I-mediated restriction digestion/postlabeling assay was conducted in a similar fashion and the detailed experimental procedures were described recently[19,20,46]. The frequency of base misincorporation and the relative bypass efficiency (RBE) of DNA lesions were determined as described previously[19,20,46].

LC-MS/MS analysis

LC-MS/MS identification of transcription products was performed as previously described[19,20,46]. Briefly, RT-PCR products were treated with 50 U MluCI, 25 U Cac8Iand 20 U shrimp alkaline phosphatase in 200 μLof 1 × CutSmart buffer at 37°C for 4 h. After phenol/chloroform extraction and ethanol precipitation, the DNA pellet was desalted with 70% ethanol and subjected to LC-MS/MS analysis following previously described procedures [19,20,46]. The LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific) was set up for monitoring the fragmentation of the $[M\text{-}3H]^{3-}$ ions of the complementary 10-mer ODNs, i.e., d(AATTATAGCM), where 'M' designates A, T, C, or G.

In vitro DNA repair assay

For *N*3-EtdT, O^2-EtdT, O^4-EtdT, *N*3-CMdT or O^4-CMdT, a 12-mer ODN d(5′-ATGGCGXGCTAT-3′) ('X' indicates a lesion) or a duplex DNA, i.e., d(5′-ATGGCGXGCTAT-3′)/d(5′-TTATAGCACGCCATGG-3′) was used as DNA substrate for *in vitro* repair studies. In addition, a 17-mer *N*3-CMdC-bearing ODN d(5′-GCGCAAAXCTAGAGCTC-3′) was employed as a control DNA substrate for Alkbh2- and Alkbh3-mediated repair reactions *in vitro*[36]. The DNA substrate (10 pmol) was incubated with purified Alkbh2 or Alkbh3 protein (50 pmol) at 37°C for 2h in a reaction mixture containing 50 mM HEPES-KOH, 75 μM $Fe(NH_4)_2(SO_4)_2$, 1 mM α-ketoglutarate, 2 mM ascorbate, 50 μg/ml bovine serum albumin. $MgCl_2$(10 mM) was added to optimize the Alkbh2-mediated repair reaction as described elsewhere[37]. The proteins in the reaction mixture were removed by phenol/chloroform extraction, and the DNA products were ethanol precipitated in the presence of 2.5 mg carrier DNA (*e.g.* pTGFP-Hha10 plasmid DNA).

After desalting with 70% ethanol, the DNA pellet was dissolved in water and subjected to LC-MS/MS analysis using the similar procedures as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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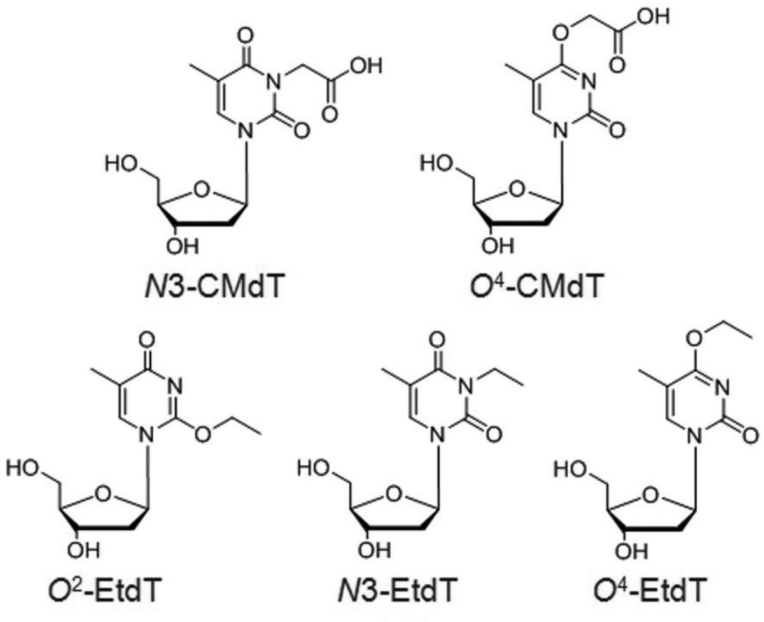

Figure 1.
Chemical structures of *N*3-EtdT, O^2-EtdT, O^4-EtdT, *N*3-CMdT, and O^4-CMdT.

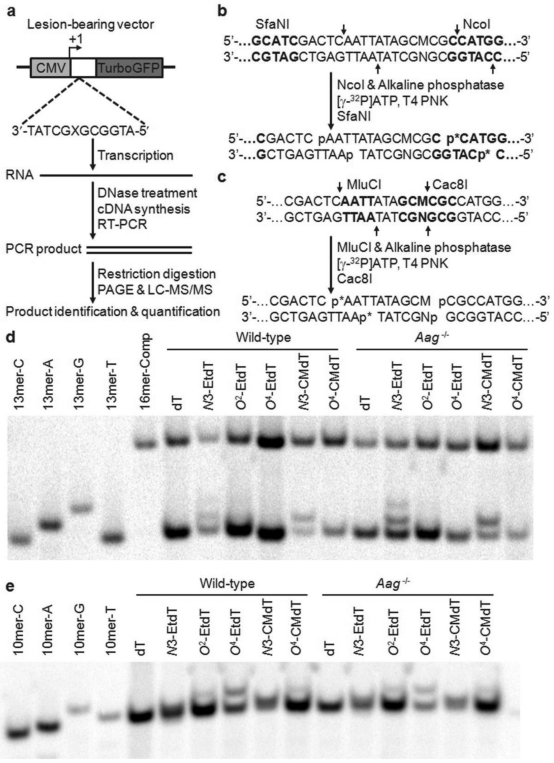

Figure 2.

In vivo transcription assay. (**a**) A schematic diagram illustrating the procedures for assessing the impact of the DNA lesions on DNA transcription.‘X’ indicates *N*3-EtdT, O^2-EtdT, O^4-EtdT, *N*3-CMdT, O^4-CMdT or dT, which was located on the transcribed strand of *TurboGFP* gene downstream of the CMV promoter. The +1 transcription start sites are indicated by arrowhead.Not shown is the lesion-free competitor vector, which has three more nucleotides near the lesion region than the undamaged control vector and is co-transcribed with the control or lesion-containing plasmids in mammalian cells. (**b-c**) Sample processing for NcoI/SfaNI-(**b**) or MlucI/Cac8I-(**c**) mediated restriction digestion/postlabeling assay (p* indicates ^{32}P-labeled phosphate group). (**d-e**) Representativegel images showing the NcoI/SfaNI-(**d**) or MlucI/Cac8I-(**e**) produced restriction fragments of interest. The restriction fragment arising from the competitor vector, i.e., d(5′-CATGGCGATATGCTAT-3′), is designated as ‘16mer-Comp’; ‘13mer-C’, ‘13mer-A’, ‘13mer-G’, and ‘13mer-T’represent the standard synthetic ODNsd(5′-CATGGCGNGCTAT-3′), where ‘N’ is C, A, G, and T, respectively.‘10mer-C’, ‘10mer-A’, ‘10mer-G’, and ‘10mer-T’represent the standard synthetic ODNs d(5′-AATTATAGCM-3′), where ‘M’ is C, A, G, and T, respectively.

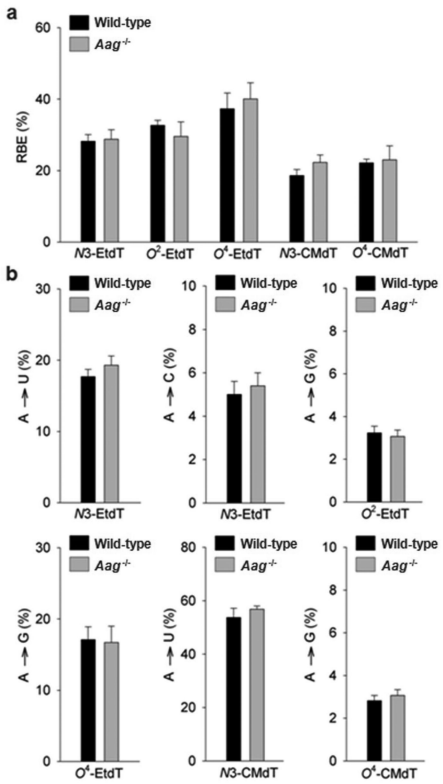

Figure 3.

Relative bypass efficiencies (RBEs) (a) and mutagenic potentials (**b**) of *N*3-EtdT, O^2-EtdT, O^4-EtdT, *N*3-CMdT, and O^4-CMdT during transcription in mammalian cellsthat are proficient (i.e., 'Wild-type') or deficient in Aag.The data represent the mean and standard error of the mean of results from three independent experiments.

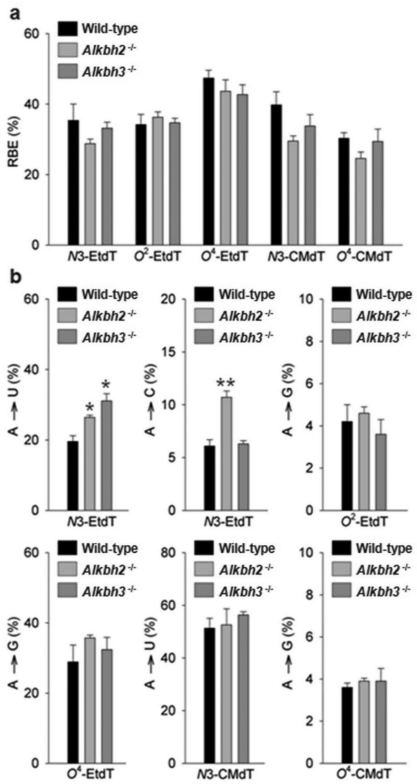

Figure 4.

Bypass efficiencies (**a**) and mutagenic potentials (**b**) of *N*3-EtdT, O^2-EtdT, O^4-EtdT, *N*3-CMdT, and O^4-CMdT during transcription in mammalian cellsthat are proficient or deficient in Alkbh2 or Alkbh3.The data represent the mean and standard error of the mean results from three independent experiments.'*', $P < 0.05$; '**', $P < 0.01$. The *P* values were calculated by using unpaired two-tailed Student's *t*-test.

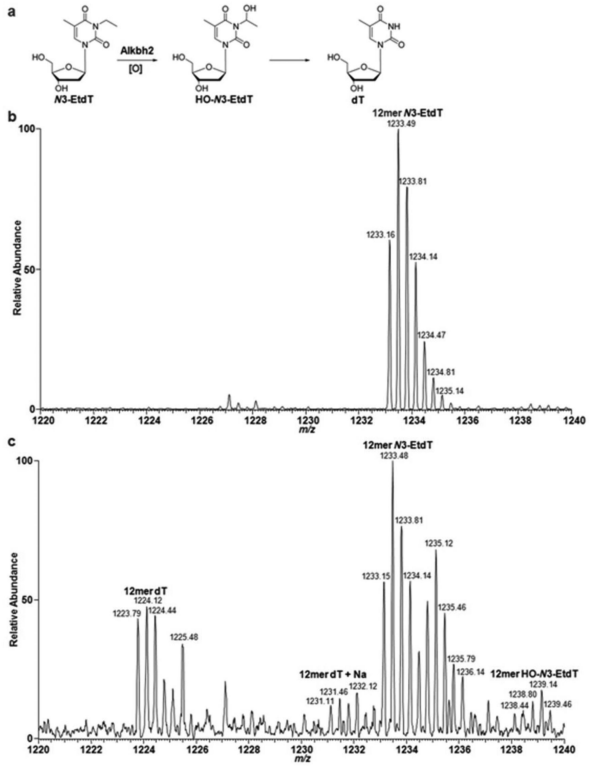

Figure 5.

Repair of *N*3-EtdT by Alkbh2*in vitro*.(**a**) A schematic diagram illustrating the repair of *N*3-EtdT by Alkbh2. (**b**,**c**) LC-MS for monitoring the products from the *in vitro* incubation reactions of a duplex DNA, that is, d(5′-ATGGCG(*N*3-EtdT)GCTAT-3′)/d(5′-TTATAGCACGCCATGG-3′) in the absence (**b**) or presence (**c**) of purified human Alkbh2 protein. Shown are the high-resolution “ultra zoom-scan” MS results for monitoring the $[M-3H]^{3-}$ ions of the initial 12mer *N*3-EtdT-bearing ODN (i.e., 12mer *N*3-EtdT) as well as its repaired product d(5′-ATGGCGTGCTAT-3′) (i.e., 12mer dT) and an intermediate product (i.e., 12merHO-*N*3-EtdT).