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1,3-Butadiene-induced adenine DNA adducts are genotoxic but only weakly mutagenic when replicated in *Escherichia coli* of various repair and replication backgrounds

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Abstract

The adverse effects of the human carcinogen 1,3-butadiene (BD) are believed to be mediated by its DNA-reactive metabolites such as 3,4-epoxybut-1-ene (EB) and 1,2,3,4-diepoxybutane (DEB). The specific DNA adducts responsible for toxic and mutagenic effects of BD, however, have yet to be identified. Recent *in vitro* polymerase bypass studies of BD-induced adenine (BD-dA) adducts show that DEB-induced N^6,N^6-DHB-dA (DHB = 2,3-dihydroxybutan-1,4-diyl) and 1,N^6-γ-HMHP-dA (HMHP = 2-hydroxy-3-hydroxymethylpropan-1,3-diyl) adducts block replicative DNA polymerases but are bypassed by human polymerases η and κ, leading to point mutations and

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Author Contributions

N.T. and J.M.E. conceived the study. S.-c.C. designed, performed and analyzed the experiments, and wrote the paper. J.W. analyzed the crude next-generation sequencing data. U.I.S. synthesized the BD-induced lesion-containing oligonucleotides. All authors reviewed the results and approved the final version of the manuscript.

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Supporting Information. The supporting information is available free of charge on the ACS Publication website.

Results on the error rate of the conserved sequence, lesion bypass efficiency of εA and 8-oxoG, and mutation frequency and specificity of εA, 8-oxoG and the negative control (A). (PDF)

deletions. In contrast, EB-induced N^6-HB-dA (HB = 2-hydroxy-3-buten-1-yl) does not block DNA synthesis and is non-mutagenic. In the present study, we employed a newly established *in vivo* lesion-induced mutagenesis/genotoxicity assay via next-generation sequencing to evaluate the *in vivo* biological consequences of S-N^6-HB-dA, R,R-N^6,N^6-DHB-dA, S,S-N^6,N^6-DHB-dA and R,S-1,N^6-γ-HMHP-dA. In addition, the effects of AlkB-mediated direct reversal repair, MutM and MutY catalyzed base excision repair, and DinB translesion synthesis on the BD-dA adducts in bacterial cells were investigated. BD-dA adducts showed the expected inhibition of DNA replication *in vivo*, but were not substantively mutagenic in any of the genetic environments investigated. This result is in contrast with previous *in vitro* observations and opens the possibility that *E. coli* repair and bypass systems other than the ones studied here are able to minimize the mutagenic properties of BD-dA adducts.

Graphical Abstract
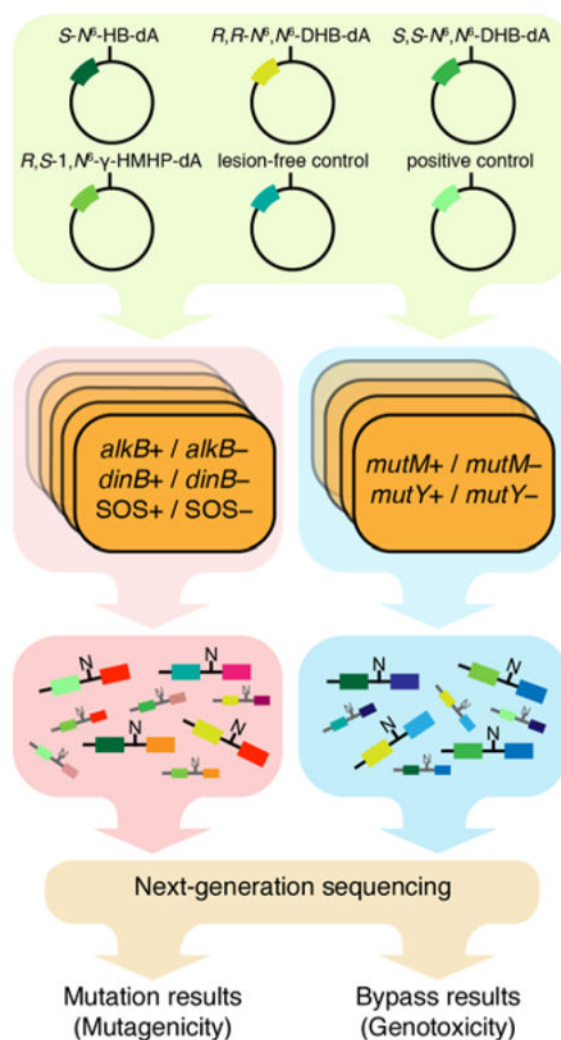
INTRODUCTION

1,3-Butadiene (BD) is a common air pollutant found in automobile emissions and cigarette smoke.[1, 2] It is also a high volume industrial chemical used in the production of synthetic rubber and plastics.[3] Multiple epidemiological studies have found that the inhalation of BD is associated with increased risks of leukemia, lymphatic and hematopoietic cancers and hence, the International Agency for Research on Cancer has classified BD as a human carcinogen.[4] Understanding the carcinogenic mechanism of BD is of great interest to occupational safety and, more broadly, to public health.

The carcinogenicity of BD is believed to be mediated by its epoxide metabolites, which have been shown to be direct-acting mutagens.[4–7] BD is metabolically activated by cytochrome P450 to form 3,4-epoxy-1-butene (EB), 1,2,3,4-diepoxybutane (DEB) and 3,4-epoxy-1,2-butanediol (EBD), which are capable of alkylating DNA to give a range of nucleobase adducts, some of which are shown in Figure 1*A*.[4, 5, 8–11] DEB is considered the ultimate carcinogenic species because it is 50 to 100-fold more genotoxic and mutagenic than EB and EBD.[7, 11–14] The higher genotoxicity and mutagenicity of DEB have been hypothesized to be due to its bifunctional alkylating ability, which gives it the ability to form DNA–DNA crosslinks and exocyclic adducts.[9, 11, 15–17] One DEB-induced intrastrand crosslinked adduct has been shown to be genotoxic and mutagenic.[18, 19]

Although the role of EB and DEB in BD-induced mutagenesis is clear and several DNA adducts have been detected, the adduct that is most likely responsible for the mutagenic effect of BD is yet to be determined.[4] Exposure to BD and its metabolites induces a large number of point mutations at A:T base pairs, suggesting the potential importance of the adenine adducts.[4, 20, 21] Specifically, a statistically significant increase in A:T→G:C and A:T→T:A mutations was observed in B6C3F1 *laci* transgenic mice exposed to BD.[20] In human TK6 lymphoblasts, DEB induced A:T→T:A transversions and partial deletions, while EB induced A:T→T:A transversions, as well as G:C→A:T transitions.[20]

Numerous BD-induced adenine (BD-dA) adducts have been identified.[8, 9, 22] The N^6-substituted adenine adducts are of particular interest because of their hydrolytic stability in DNA, potentially leading to their accumulation *in vivo*. N^6-(2-hydroxy-3-buten-1-yl)-dA (N^6-HB-dA) is an adduct formed by the alkylation of adenine with EB (Fig. 1*A*).[23] DEB can form three types of exocyclic adenine lesions: N^6,N^6-(2,3-dihydroxybutan-1,4-diyl)-2′-deoxyadenosine (N^6,N^6-DHB-dA), 1,N^6-(2-hydroxy-3-hydroxymethylpropan-1,3-diyl)-2′-deoxyadenosine (1,N^6-γ-HMHP-dA), and 1,N^6-(1-hydroxymethyl-2-hydroxypropan-1,3-diyl)-2′-deoxyadenosine (1,N^6-α-HMHP-dA) (Fig. 1*A*).[9] At physiological temperature and pH, 1,N^6-γ-HMHP-dA and 1,N^6-α-HMHP-dA can interconvert, with the equilibrium favoring the presence of 1,N^6-α-HMHP-dA.[9] These four adducts were detected when calf thymus DNA was allowed to react with BD epoxides, although only small amounts of

N^6,N^6-DHB-dA were detected.[9, 22] 1,N^6-γ-HMHP-dA adducts have also been detected in tissues of laboratory mice treated with BD by inhalation.[24]

Even though some of these adenine lesions have been found to be mutagenic *in vitro*,[25, 26] their mutagenicity in living cells has not been investigated. The goal of this study was to evaluate the *in vivo* genotoxicity and mutagenicity of N^6-substituted BD-dA adducts in *Escherichia coli* cells. Previously, we have shown that while the *in vitro* bypass of *S*-N^6-HB-dA is largely error-free, the bypass of *R,S*-1,N^6-γ-HMHP-dA and *R,R*-N^6,N^6-DHB-dA are highly mutagenic.[25, 26] Two translesion synthesis (TLS) polymerases, hPol η and κ, which are specialized polymerases capable of catalyzing DNA synthesis past various DNA adducts, can misincorporate A or G opposite *R,S*-1,N^6-γ-HMHP-dA[25] and A, G or C opposite *R,R*-N^6,N^6-DHB-dA.[26] If such bypass events occurred *in vivo*, these misinsertions could explain the aforementioned BD-induced A→T and A→G mutations.

Knowing how lesions are repaired is also critical for understanding the biological consequences of the lesions. In particular, we investigated here the ability of two repair pathways to reverse the biological effects of the BD-dA adducts: the direct reversal pathway of AlkB and the base excision pathway mediated by MutM and MutY; all of these enzymes have human homologs with similar substrate specificities.[27–30] AlkB is an iron(II)- and α-ketoglutarate-dependent dioxygenase capable of repairing many alkylated DNA damages, including ethenoadenine (εA) and ethanoadenine (EA), which are structurally similar to 1,N^6-γ-HMHP-dA and 1,N^6-α-HMHP-dA.[31, 32] MutM (also known as Fapy glycosylase (Fpg)) and MutY are primarily known for preventing the G:C→T:A mutation caused by 7,8-dihydro-8-oxoguanine (8-oxoG).[33] MutM removes 8-oxoG when it is paired with cytosine and MutY excises adenine when it is mispaired with 8-oxoG. However, MutM and MutY have been shown to have a broader substrate specificity beyond removing 8-oxoG and A.[33] Furthermore, since our previous results show that the *in vitro* bypass of the BD-dA adducts by hPol η and κ can be mutagenic,[25, 26] we investigated whether similar observations could be made from *in vivo* TLS. We focused on the TLS by DinB because it is the *E. coli* homolog of hPol κ.[34]

A high-throughput methodology allowing for the simultaneous analysis of the biological properties of multiple samples was necessary to complete this large-scale investigation. Recently, we successfully incorporated next-generation sequencing into our site-specific mutagenesis assay,[35] allowing for a concurrent analysis of multiple lesions in multiple cell strains.[36] In the present study, we applied the same methodology to investigate polymerase bypass and mutagenesis mediated by the four BD-dA adducts (Fig. 1*B*) in various repair/replication backgrounds (Fig. 2). The adducts hindered replication to various degrees but, counter to our previous *in vitro* results with human polymerases,[25, 26] none of the adducts were significantly mutagenic in the repair/replication backgrounds investigated. Sometimes, the lack of significant mutagenicity of a DNA adduct *in vivo* is attributable to DNA repair systems that counter the effect of an otherwise mutagenic adduct. We found, however, that neither AlkB, MutM nor MutY appeared to play a role in the repair of the BD-dA lesions since the mutagenicities and genotoxicities of the lesions were not significantly different in the presence or absence of these repair enzymes. Our observations suggest that as yet

undiscovered repair or replication factors suppress the mutagenicity of BD-dA adducts in living cells, limiting their biological impact following exposure to BD.

EXPERIMENTAL PROCEDURES

Oligonucleotide synthesis

All lesion-free oligonucleotides were obtained from Integrative DNA Technologies. Lesion-containing oligonucleotide16-mers with the sequence 5′-GAAGACCTXGGCGTCC-3′, where X was either a S-N^6-HB-dA, R,R-N^6,N^6-DHB-dA, S,S-N^6,N^6-DHB-dA or R,S-1,N^6-γ-HMHP-dA lesion, were synthesized with the post-oligomerization synthesis method described previously.[37] Control oligonucleotides with the same sequence but an εA or 8-oxoG at position X were synthesized as described elsewhere.[31, 38] The purity of the oligonucleotides was evaluated by Electrospray Ionization Time-of-flight mass spectrometry (ESI-TOF MS).

In vivo lesion-induced mutagenesis assay with next-generation sequencing

The method of using next-generation sequencing to study lesion-induced mutagenesis in cells has been previously described in detail.[36] A brief overview of the method and variations from our original procedure are outlined here. The overall procedure can be broken down into four parts: genome construction, *in vivo* replication, library preparation and next-generation sequencing (Fig. 2).

Genome construction—Lesion-containing and lesion-free oligonucleotides (5′-GAAGACCTXGGCGTCC-3′, where X is a lesion or a regular base) were first ligated to barcode-containing oligonucleotides (5′-CACGGTB$_1$B$_2$B$_3$TGCTCTGAC-3′, where B$_1$B$_2$B$_3$ is a trinucleotide barcode unique to each lesion or control). The ligated products were then inserted into the EcoRI-lineralized M13mp7(L2) single-stranded bacteriophage genomic DNA. After purification, the relative concentrations of different constructed genomes were determined via the genome normalization procedure.[36]

In vivo replication—Preparation of the electrocompetent cells and induction of the SOS response were done as previously described.[36] For the investigations on AlkB and DinB, *E. coli* strains HK81 (as AB1157 but *nalA*; this is the wild-type strain), HK82 (as HK81 but *alkB*⁻), HK84 (as HK82 but *dinB*⁻), as well as the SOS-induced version of HK82 (HK82 SOS⁺) and HK84 (HK84 SOS⁺) were used. Each of these conditions was studied in three replicates to monitor biological variability. These conditions were chosen such that the effects of AlkB, DinB and SOS-induction could be deduced from pair-wise comparisons of each repair/replication state.

Constructed barcoded genomes with S-N^6-HB-dA, R,R-N^6,N^6-DHB-dA, S,S-N^6,N^6-DHB-dA, R,S-1,N^6-γ-HMHP-dA, εA or A were mixed together at a 3:3:3:3:1:1 ratio. For each electroporation, 250 fmol of the genome mixture was introduced into 150 μL of electrocompetent cells. The number of successful transformations was determined immediately after each electroporation. In this study, the obtained transformation efficiency (~4.7 ×10⁵ infective centers) was similar to those in past experiments. The transformed cells

were incubated for 6 h and the resulting progeny phage were amplified in SCS110 cells for another 7 h. Single-stranded DNA was isolated from progeny phage using the QIAprep Spin M13 Kit (Qiagen). A ~1 kb sequence containing the region of interest was PCR amplified using the same PCR condition as previously described (1 fmol of M13 template, 50 pmol of each PCR primers (forward: 5′-CGATTTCGGAACCACCATCAAACAGG-3′, reverse: 5′-TGAGAGTCTGGAGCAAACAAGAGAATCG-3′) and PfuTurbo polymerase (Agilent) for 25 cycles of 95°C for 30 sec, 68°C for 30 sec and 72°C for 1.25 min).[36]

A nearly identical procedure with a few modifications was followed for the study on MutM and MutY. *E. coli* strains AB1157 (wild-type), *mutM⁻*, *mutY⁻* and *mutM⁻/mutY⁻* were used and an additional 8-oxoG lesion was included as a positive control. Barcoded genomes containing *S*-N^6-HB-dA, *R,R*-N^6,N^6-DHB-dA, *S,S*-N^6,N^6-DHB-dA, *R,S*-1,N^6-γ-HMHP-dA, εA, 8-oxoG or A were mixed at a 2:2:2:3:1:2:1 ratio. A total of 280 fmol genome mixture and 150 μL electrocompotent cells were used in each electroporation. A similar transformation efficiency (~2.1 ×10^5 infective centers) was achieved. Progeny DNA was extracted from progeny phage after the 6 h incubation and 7 h SCS110 amplification. To avoid the possibility of PCR-induced artifacts, a slightly different PCR condition was used for amplifying the 1 kb region; the amount of template was lowered from 1 fmol to 0.01 fmol and the number of amplification cycles reduced from 25 to 20, while other settings remained the same as described above.

Library preparation—During the sequencing library preparation step, the PCR-amplified 1 kb products were randomly fragmented and tagged by using the Nextera DNA Sample Preparation Kit (Illumina) following manufacturer's instructions. A second set of barcodes was also appended to the fragmented DNA such that the samples from different repair/replication backgrounds and from different biological replicates were uniquely labeled.

Next-generation sequencing—Equal amounts of barcoded DNA fragments from different samples were combined and sequenced on an Illumina MiSeq sequencer. The sequencing data were processed and analyzed as described previously.[36] Briefly, reads from different repair/replication backgrounds and biological replicates were first separated into groups based on their barcodes. The adaptors were trimmed and the paired-end reads were concatenated. The sequencing reads from different lesions were further separated according to the lesion barcodes. Aligning sequencing reads from each lesion to the expected M13 sequence identified any mutation induced by the lesion, which was used to determine mutation frequency of the lesion. Lesion bypass efficiency was determined based on the change in number of sequencing reads from the lesion to that of the lesion-free control, since lesion that blocks replication would produce fewer progeny.

RESULTS

Next-generation sequencing provided the coverage and accuracy to study lesion-induced mutagenesis

As depicted in Figure 2, the strategy of investigating the biological consequences of the BD-dA lesions involved constructing barcoded lesion-containing genomes, allowing them to replicate inside cells with various repair/replication capabilities, and then sequencing the

libraries prepared from the progeny DNA to obtain the mutagenicity and genotoxicity data for individual lesions under various conditions. The capability of next-generation sequencing to perform massively parallel sequencing enabled the concurrent analysis of multiple samples, making this large-scale investigation manageable. In this study, samples from the AlkB and DinB study were analyzed in one sequencing run and samples from the MutM and MutY study were sequenced in a second run.

The depth of coverage at the lesion site and the sequencing accuracy were two critical factors influencing the reliability of the sequencing approach. For both sequencing runs, we were able to achieve on average a 26,000-fold coverage at the lesion site per experiment that analyzed one lesion in a single cellular environment. This high coverage at the lesion site indicated a large number of progeny genome was sequenced and thus, generating a statistically robust result. Even for strongly replication-blocking lesions, which produced less progeny DNA, we still obtained at least a 400-fold coverage. As for the sequencing accuracy, the error rate at the conserved bases surrounding the lesion site was ~0.5% for both sequencing runs (Supplementary Table S1 & S2). This is similar to the error rate in our previous studies.[36]

Genotoxicity of BD-dA lesions is affected by lesion structure and stereochemistry

Lesion genotoxicity is measured as a reduction in replication bypass efficiency in our assay. A lesion with potent genotoxicity would strongly block replication and, hence, result in low bypass efficiency. The bypass efficiencies of the positive controls (εA and 8-oxoG, Supplementary Fig. S1 & S2) were consistent with our previous observations,[31, 38] giving us confidence in our genotoxicity measurements.

The four BD-dA lesions induced various degrees of genotoxicity (Fig. 3). The rank of the four lesions by their genotoxicity was R,S-1,N^6-γ-HMHP-dA > S,S-N^6,N^6-DHB-dA > R,R-N^6,N^6-DHB-dA > S-N^6-HB-dA. R,S-1,N^6-γ-HMHP-dA had a bypass efficiency of only ~10% in the non-SOS-induced cells, while S-N^6-HB-dA was not a replication block (Fig. 3A). The same ranking of genotoxicity was observed for cells lacking MutM and MutY (Fig. 3B), although the bypass efficiencies of R,R-N^6,N^6-DHB-dA and S,S-N^6,N^6-DHB-dA were lower; this discrepancy, however, could be due to the genome normalization procedure.

When comparing the bypass efficiencies of the same lesions under different repair/replication conditions, we found that SOS-induction strikingly increased the bypass efficiency of R,S-1,N^6-γ-HMHP-dA from 9% to 42% in $alkB^-/dinB^+$ cells and from 10% to 31% in $alkB^-/dinB^-$ cells (Fig. 3A). Some of this increase in bypass could be attributed to the DinB TLS polymerase because the bypass efficiency of R,S-1,N^6-γ-HMHP-dA in the $alkB^-/dinB^-$/SOS$^+$ background was lower than that in the $alkB^-/dinB^+$/SOS$^+$ background (P = 0.04). The bypass of S,S-N^6,N^6-DHB-dA in the SOS-induced $alkB^-/dinB^+$ cells (73%) was slightly higher than that of the non-induced cells (62%), but this difference was not statistically significant (P = 0.07). In most of the other cases, the bypass efficiencies of a lesion were not significantly different across the different conditions studied, indicating that the repair enzymes (AlkB, MutM and MutY) and the TLS polymerase studied (DinB) did not play a major role in alleviating the genotoxicity of these replication blocking lesions.

BD-dA lesions induced few mutations under all repair and replication conditions investigated

By isolating progeny DNA from the lesion-containing genomes and analyzing the base compositions at and around the lesion site, we were able to gain insights into the *in vivo* mutagenicity of individual lesions under various repair and replication conditions. In the AlkB and DinB study, the mutation result of the positive control (εA, Supplementary Fig. S3) was consistent with our previous observations: εA was non-mutagenic in *alkB*+ background but highly mutagenic in *alkB*− background, producing primarily A→T transversions.[31] Furthermore, for the negative control with an unmodified A base at the lesion site, essentially 100% of the progeny had an A at the lesion site after replication in all the conditions studied, as expected (Supplementary Fig. S4). These control results gave us confidence in the validity of our method as applied to the BD-dA adducts.

We found that all of the four BD-dA lesions coded >97.4% as A at the lesion site after replication, in all five backgrounds with various AlkB, DinB and SOS response statuses (Fig. 4). Some of these lesions induced a very low level of mutations (~1%). *R,R-N*6,*N*6-DHB-dA induced ~0.5% A→G mutations under all five conditions and 0.5–0.7% A→T mutations in SOS-induced cells. The mutation pattern of *S,S-N*6,*N*6-DHB-dA was very similar to that of *R,R-N*6,*N*6-DHB-dA, but the *S,S*-isomer was slightly more mutagenic (~1.2% A→G mutations in all five conditions and 0.7–1.1% A→T mutations in SOS-induced cells). *R,S*-1,*N*6-γ-HMHP-dA caused ~1.5% A→T mutations across the five repair/replication backgrounds. Although these low levels of mutations were detectable, and are consistent with mutations seen *in vitro*, they were very close to the assay's limit of detection and should be interpreted with caution.

Similar observations were made in the MutM and MutY proficient or deficient cell strains (Fig. 5). More than 95.3% of the progeny genome from all four BD-dA lesions coded as A at the lesion site in all four of the MutM and MutY cell strains. A low level of G in all four strains and a low level of T in the *mutY*− and *mutM*−/*mutY*− were observed. However, low levels of G and T were also seen in the negative control with unmodified A at the lesion site (Supplementary Fig. S5). We concluded that the low levels of G and T were likely to be PCR artifacts. Nevertheless, a similar mutation pattern of each lesion observed in the AlkB and DinB experiment could also be seen in this study: compared to the negative control, *R,R-N*6,*N*6-DHB-dA and *S,S-N*6,*N*6-DHB-dA had a higher level of G (with *S,S-N*6,*N*6-DHB-dA being slightly more mutagenic) and *R,S*-1,*N*6-γ-HMHP-dA had a higher level of T. Results from the positive control (8-oxoG) confirmed the phenotype of the cell strains (Supplementary Fig. S6). In the strains lacking MutY, a 40–47% G→T mutation rate was observed for the 8-oxoG control. The MutM deficiency only had a modest impact in our experiment due to the known mechanism of action of this glycosylase: it only removes an 8-oxoG that is correctly paired with a C, which would have resulted in a non-mutagenic outcome in our assay.

In summary, none of the four BD-dA lesions appeared to be very mutagenic *in vivo* under a wide range of conditions studied, although *R,R-N*6,*N*6-DHB-dA and *S,S-N*6,*N*6-DHB-dA seemed to induce a low level (1–2%) of A→G mutations and *R,S*-1,*N*6-γ-HMHP-dA

appeared to cause (~1%) A→T mutations. More importantly, there was no significant variation in mutagenicity across the different repair and replication backgrounds.

DISCUSSION

In vitro replication and repair studies of DNA adducts is an important first step towards evaluating their biological consequences. *In vivo* site-specific mutagenesis experiments, which involve introducing the lesion of interest as part of a viral or plasmid molecule into a cell, explore the abilities of lesions to cause mutations, to evade repair, and to block the repertoire of replicative and other polymerases in a natural milieu. As one example, early work by us and others revealed that εA, which is mutagenic and toxic *in vitro*, shows few adverse biological effects *in vivo*.[39] Later we showed that the repair protein AlkB can repair εA by a unique and previously unseen direct repair mechanism.[31] That *in vitro* study next led to discovery of the enormously powerful influence AlkB has in countering the effects of agents that form εA *in vivo*. Accordingly, coordinated application of *in vitro* and *in vivo* studies often results in the discovery of biological factors that cause and counter the biological properties of DNA lesions.

In an attempt to identify the DNA adducts responsible for the adverse effects of BD, we investigated the *in vivo* genotoxicity and mutagenicity of the four structurally distinct BD-dA lesions: S-N^6-HB-dA, R,R-N^6,N^6-DHB-dA, S,S-N^6,N^6-DHB-dA and R,S-1,N^6-γ-HMHP-dA. These lesions have been previously evaluated *in vitro* with steady-state kinetics analyses and primer extension assays, but they had not been investigated *in vivo*.[25, 26] Furthermore, we also examined the ability of direct reversal repair (AlkB) and base-excision repair (MutM and MutY) to repair these lesions, as well as the effects of TLS by DinB polymerase. To make this large-scale investigation manageable, a next-generation lesion-induced mutagenesis assay was used, which allowed the concurrent analysis of many lesions and repair/replication backgrounds.[36]

In terms of genotoxicity, our results indicated that the four lesions hindered replication to different degrees (Fig. 3). Our finding that the S,S-N^6,N^6-DHB-dA adduct was more genotoxic than its R,R- stereoisomer is interesting as it is an example of how different stereoisomers can block replication differentially. One of the well-studied examples of different adduct stereoisomers having different biological effects is the benzo[*a*]pyrene adduct (B[*a*]P) (reviewed in [40]). It has been reported that the B[*a*]P G-*cis* adduct inhibits polymerase extension more strongly than the G-*trans* adduct, presumably due to greater steric conflict by the *cis* isomer blocking the incoming nucleotide.[41, 42] It has also been proposed that the different isomers may have different conformations within the active site of polymerase, which results in different interactions with the polymerase and, hence, different replication outcomes.[43] According to our previous NMR study, the structural differences between R,R- and S,S-N^6,N^6-DHB-dA in duplex DNA is subtle.[44] It would be interesting to study these adducts in the active site of a polymerase to determine exactly how the difference in genotoxicity arises.

Despite their often-strong genotoxicities (Fig. 3) and their ability to induce mutations *in vitro*,[25, 26] none of the BD-dA lesions studied were very mutagenic in *E. coli* under any of

the conditions that we investigated (Fig. 4 & 5). In our earlier *in vitro* investigations, several of these lesions were shown to have a high propensity for miscoding when replicated by human TLS polymerases.[25, 26] For example, the mutation frequency of *R,S*-1,N^6-γ-HMHP-dA ranged from 20–80%, depending on the polymerase, inducing A→T and A→C mutations.[25] We also found that upon primer extension, all four dNTPs were incorporated opposite *R,R*-N^6,N^6-DHB-dA at a similar frequency and the mutation rate could be as high as 60–80%.[26] These results show that many of these lesions are highly mutagenic *in vitro*. However, the same four BD-dA lesions induced few mutations in *E. coli*.

Interestingly, although these lesions appeared to induce only a very low level of mutations *in vivo*, the mutations they generated were consistent with the previously observed *in vitro* mutation patterns. NMR spectroscopy has shown that the N^6-substituent of *S*-N^6-HB-dA is accommodated in the major groove, and this modified base retains normal Watson-Crick base pairing with the correct complementary base, thymine.[45] Our previous *in vitro* study concluded that the bypass of *S*-N^6-HB-dA was not mutagenic,[26] which is consistent with our current *in vivo* observation.

As for the two N^6,N^6-DHB-dA lesions, NMR structural analysis showed that both stereoisomers significantly destabilized the DNA duplex by interfering with base stacking interactions and retaining only one hydrogen bond with the complementary thymine.[44] This decrease in stability was reflected in a similar decrease in melting points for both the *R,R*- and *S,S*-duplexes.[44, 46] Previously, we showed that all four bases can be inserted opposite *R,R*-N^6,N^6-DHB-dA when bypassed by TLS polymerases *in vitro*.[26] Here, we found that both N^6,N^6-DHB-dA lesions might induce low levels of A→G (~1%) and A→T (<1%) mutations. Interestingly, our results suggested that the two stereoisomers might have different degrees of mutagenicity and genotoxicity. Comparing the two N^6,N^6-DHB-dA lesions, the *S,S*-isomer has a slightly higher mutagenicity and genotoxicity. Even though two studies have found that both isomers reduce the melting point of duplex DNA to a similar extent, it was proposed that the different orientations of the hydroxyl groups on the DHB adducts could interact with DNA polymerases differently during TLS, thus leading to different outcomes.[44, 46] Investigation of the three stereoisomers of the DEB metabolites also found that the *S,S*-isomer is more cytotoxic and mutagenic than the *meso*- and *R,R*-isomers.[47] Our results here support the hypothesis that lesion stereospecificity modulates mutagenicity and genotoxicity, which has also been previously observed with the stereoisomers of three other BD adducts.[48, 49]

Previously, we found that the duplex with a *R,S*-1,N^6-γ-HMHP-dA:A mismatch had a greater thermal stability than the duplex with an A:A mismatch, suggesting this adduct may pair with an A to cause an A→T mutation.[46] Primer extension studies showed that *R,S*-1,N^6-γ-HMHP-dA could indeed introduce A→T as well as A→C mutations during *in vitro* TLS.[25] It was hypothesized that *R,S*-1,N^6-γ-HMHP-dA may adopt a *syn* confirmation, similar to εA, to form a Hoogsteen base pair with A or G.[25] We observed a similar A→T mutation pattern by *R,S*-1,N^6-γ-HMHP-dA in the current *in vivo* study, albeit at a low level (~1%).

Although the *in vivo* mutation patterns we observed in this study seem to be consistent with previous *in vitro* results and the A→G and A→T mutations are consistent with the known mutations induced by BD,[20] it should be kept in mind that the *in vivo* mutagenicity we observed was at a low level, which should be interpreted with caution. Even if these mutations were truly induced by the lesions, whether the low mutation levels have significant biological relevance needs to be investigated further. Many of the lesions that we have studied with our *in vivo* mutagenesis assay in the past have a mutagenicity of 30–50%.[31, 36, 38, 50] By contrast, the four lesions we have studied here have a mutagenicity of at most 1–2%, which would be considered to be non-mutagenic or weakly mutagenic.

Another important observation from this study was that in most of the cases, there were no significant differences in lesion genotoxicity or mutagenicity across the different genetic backgrounds evaluated (with the exception of the TLS polymerases, discussed below). The presence or absence of a specific repair enzyme or bypass polymerase (AlkB, MutM, MutY or DinB) did not make the lesions significantly more or less mutagenic or genotoxic. This result indicates that these repair and bypass mechanisms do not have an effect on the four BD-dA lesions under the conditions evaluated *in vivo*. Two of the exceptions where there were observable differences were that the bypass of R,S-1,N^6-γ-HMHP-dA was higher in the SOS-induced cells (Fig. 3*A*) and the two N^6,N^6-DHB-dA adducts induced a low level (~1 %) of A→T mutation in the SOS-induced cells (Fig. 4). Previously, our primer extension experiments found that hPol κ (the human homolog of DinB, or Pol IV) causes A→T mutations when bypassing R,R-N^6,N^6-DHB-dA.[26] It is possible that our observation of A→T mutations, albeit at a low level, by the two N^6,N^6-DHB-dA adducts in $dinB^+$/SOS$^+$ background (Fig. 4) is attributable to the previously discovered miscoding potential of hPol κ. Compared to the $dinB^+$/SOS$^+$ background, there was also a lower level of the A→T mutation by the two N^6,N^6-DHB-dA adducts in $dinB^-$/SOS$^+$ background (Fig. 4), which could be due to the action of other bypass polymerases in *E. coli*.

The contrast between *in vitro* and *in vivo* mutagenicities of the BD-dA lesions is stunning and warrants explanation; specifically, the *in vitro* bypass of R,R-N^6,N^6-DHB-dA and R,S-1,N^6-γ-HMHP-dA is very mutagenic but they were essentially non-mutagenic under any of the *in vivo* conditions we studied here. At least three possibilities could explain this inconsistency. First, there may be unknown repair mechanisms that efficiently repair these lesions *in vivo*. An example of this situation was seen with the εA lesion, as indicated above. This lesion was found to be mutagenic *in vitro*[51] but its mutation frequency was found to be low in wild-type cells.[39] This puzzle was eventually solved after εA was investigated in AlkB-proficient and deficient cells; εA was highly mutagenic and genotoxic in *alkB*⁻ cells but its mutagenicity and genotoxicity were almost completely eliminated in *alkB*⁺ background.[31] This observation led to the discovery that εA can be efficiently repaired by AlkB. A similar situation could exist for the BD-dA lesions. In our recent study, we have shown that BD-dA adducts are rapidly repaired by nuclear extracts from human cells, but the identity of the glycosylase involved is yet to be established.[52] However, the rapid repair hypothesis may be insufficient to explain why three of the BD-dA lesions were genotoxic but not mutagenic.

A second possibility is that there may be a collaborative effect of different TLS polymerases *in vivo*, which is not available in an *in vitro* bypass assay using a single-type polymerase. Cells have many different TLS polymerases at their disposal and these polymerases can work in concert to avoid mutagenic outcomes. In fact, we have shown that hPol ι can insert the correct base (T) opposite R,R-N^6,N^6-DHB-dA and R,S-1,N^6-γ-HMHP-dA, which can then be extended by other TLS polymerases such as hPol κ or hPol η.[25, 26] Similar cooperative partnerships of TLS polymerases have also been observed before.[53, 54] If such a polymerase switching mechanism were to occur in our *in vivo* experiment, it would explain the non-mutagenic bypass of these BD-dA lesions. Future studies will test these lesions in hPol ι deficient cells to prove or disapprove this hypothesis.

A third possibility is that there may be fundamental differences between the replicative polymerases in mammalian and bacterial cells, and/or between *in vitro* and *in vivo* replication conditions, causing these adducts to behave differently in the two systems. (To date, these BD-dA adducts have not been investigated as *in vitro* substrates for bacterial polymerases.) We have observed a similar example before with the ethanoadenine adduct (EA).[32] EA was found to be highly mutagenic in a primer extension assay with several mammalian polymerases[55] but it was essentially not miscoding in our *in vivo* study.[32] Interestingly, EA has several other similarities with the BD-dA lesions invested in this study. First, because of its saturated carbon bridge, EA is structurally more similar to R,S-1,N^6-γ-HMHP-dA than εA is. Second, EA exhibits potent genotoxicity without significant mutagenicity, which was also seen for three of the lesions in this study. In addition, two BD-induced guanine lesions have also been shown to block replication strongly but are essentially non-mutagenic.[49] Although it may not be very intuitive to picture how a lesion can block replication but retain the characteristics for correct base pairing, it has been shown before that incorporation of nucleotides can happen without the hydrogen bonds between the template and incoming nucleotide.[56, 57] While the two DHB-dA adducts have been shown to retain the ability to form one hydrogen bond with the complementary thymine,[44] both EA and R,S-1,N^6-γ-HMHP-dA lack the ability to form hydrogen bonds with thymine but are essentially not miscoding. It would be interesting to study how the R,S-1,N^6-γ-HMHP-dA interacts with polymerases to maintain faithful replication.

Our observation that the four BD-dA lesions were only weakly mutagenic *in vivo* is similar to the results from previous studies on several other BD-derived monoadducts, where their mutagenicities were all <1% when replicating in mammalian or *E. coli* cells.[48, 49] A BD-derived N1-deoxyinosine adduct is highly mutagenic *in vivo*, which is not surprising because this N1-deoxyinosine adduct is the deamination product of a BD-induced N1-dA lesion.[18] Deamination changes the coding property of a base and hence, strong mutagenicity is expected. It should also be noted that the N1-deoxyinosine adduct is yet to be detected *in vivo* following BD exposure.[18]

In conclusion, our *in vivo* results indicated that BD-dA adducts block replication to various extents in bacterial cells, and that some of the replication inhibition can be alleviated by the induction of TLS polymerases. None of these lesions were particularly mutagenic in living cells, a result that stands in contrast with the previous *in vitro* bypass data, which showed that the R,R-N^6,N^6-DHB-dA and R,S-1,N^6-γ-HMHP-dA adducts are highly mutagenic.

While the TLS polymerases enhanced the bypass of R,S-1,N^6-γ-HMHP-dA, none of the repair mechanisms investigated (AlkB, MutM and MutY) had a significant effect on the BD-dA adducts. The working hypothesis to come out of this study is that bacterial cells, perhaps in contrast with mammalian systems, have evolved repair systems not studied here, or high fidelity replication systems that enable tolerance of the bulky DNA lesions formed by BD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

ABBREVIATIONS

8-oxoG	7,8-dihydro-8-oxoguanine
A	adenine
B[a]P	benzo[a]pyrene
BD	1,3-butadiene
BD-dA	BD-induced adenine lesions
C	cytosine
DEB	1,2,3,4-diepoxybutane
EA	ethanoadenine
εA	ethenoadenine
EB	3,4-epoxybut-1-ene
EBD	3,4-epoxy-1,2-butanediol
G	guanine
N^6-HB-dA	N^6-(2-hydroxy-3-buten-1-yl)-dA
N^6,N^6-DHB-dA	N^6,N^6-(2,3-dihydroxybutan-1,4-diyl)-2′-deoxyadenosine
1,N^6-α-HMHP-dA	1,N^6-(1-hydroxymethyl-2-hydroxypropan-1,3-diyl)-2′-deoxyadenosine
1,N^6-γ-HMHP-dA	1,N^6-(2-hydroxy-3-hydroxymethylpropan-1,3-diyl)-2′-deoxyadenosine
SD	standard deviation

| **T** | thymine |
| **TLS** | translesion synthesis |

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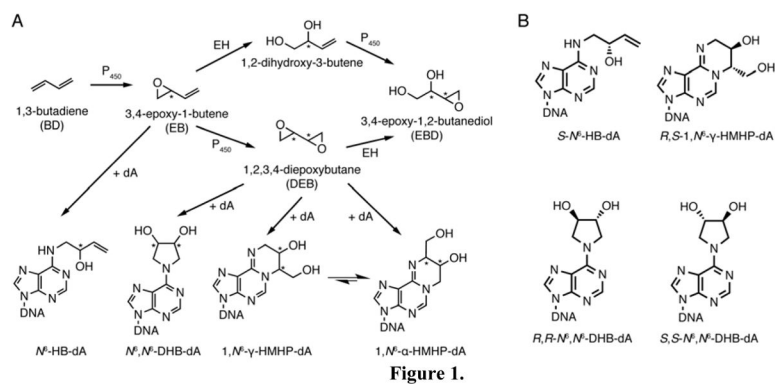
Figure 1.

Figure 1.
Metabolic activation of 1,3-butadiene and some adenine adducts it produces. *A*, 1,3-butadiene (BD) is metabolized by CYP2E1 to produce 3,4-epoxy-1-butene (EB). EB can react with adenine bases on DNA to form the N^6-HB-dA adduct. It can also undergo further oxidation by CYP2E1 to become 1,2,3,4-diepoxbutene (DEB) or be hydrolyzed to 1,2-dihydroxy-3-butene by epoxide hydrolase (EH). DEB can react with adenine to form a variety of adducts, including N^6,N^6-DHB-dA, 1,N^6-γ-HMHP-dA and 1,N^6-α-HMHP-dA. Under physiological conditions, 1,N^6-γ-HMHP-dA and 1,N^6-α-HMHP-dA can interconvert with the equilibrium shifting towards the latter. All of these adducts have stereoisomers. The asterisks denote stereocenters. Note that the adducts shown here are not an exclusive list of adducts that can be generated by BD. *B*, the structures of the four stereospecific BD-induced adenine adducts investigated in this current study.
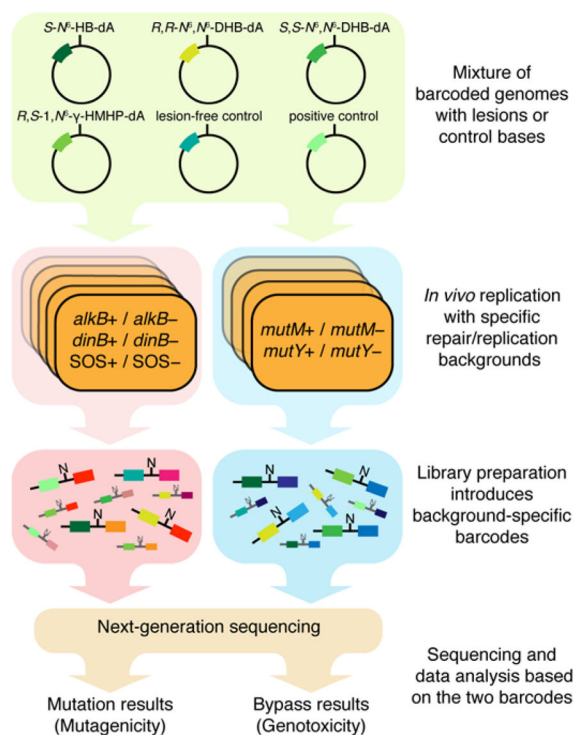
Figure 2.

Figure 2.
Overall procedure of the *in vivo* lesion-induced mutagenesis assay with next-generation sequencing. Single-stranded M13 lesion-containing and control genomes, each with a unique lesion barcode (line segments with different shades of green), were mixed together at a known ratio and introduced into *E. coli* host cells with a specific repair and replication capability. In part one of the study, the host cells had various AlkB, DinB and SOS-induction statuses. In the second part, host cells had different MutM and MutY statuses. Following *in vivo* replication, sequencing libraries were prepared from the amplified progeny genome. During this step, the progeny DNA from each repair/replication background received another unique barcode, designating the different backgrounds (line segments with different shades of red or blue). N represents the base at the site originally contained the lesion in the progeny DNA. The resulting DNA was pooled and sequenced. Lesion mutagenicity and genotoxicity under the various repair/replication conditions were obtained by analyzing the sequencing data based on the two sets of barcodes.
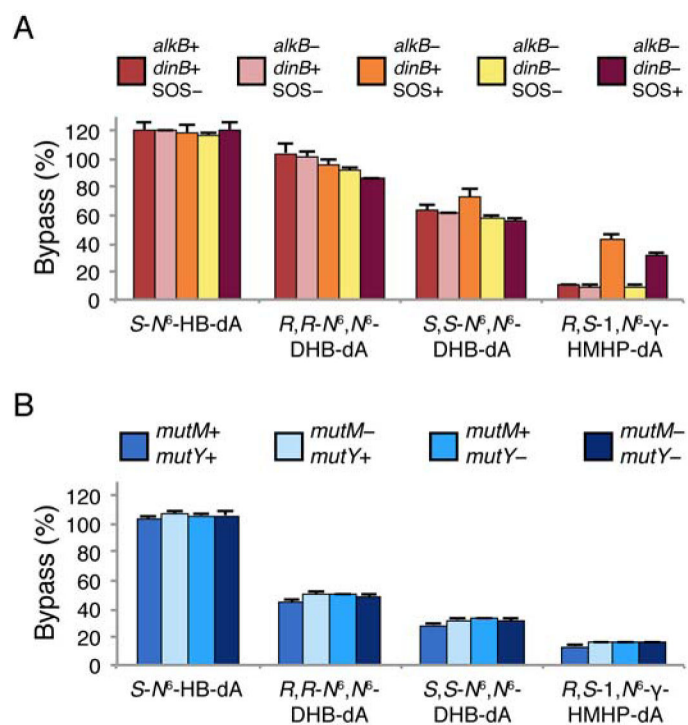
Figure 3.

Figure 3.
Lesion bypass efficiency of the four BD-induced adenine adducts. Lower bypass efficiency represents stronger lesion genotoxicity. *A*, bypass results in cells with different AlkB, DinB and SOS-induction statuses. *B*, bypass results in cells with different MutM and MutY statuses. Each graphed value corresponds to the mean of three independent biological replicates; error bars represent one SD.
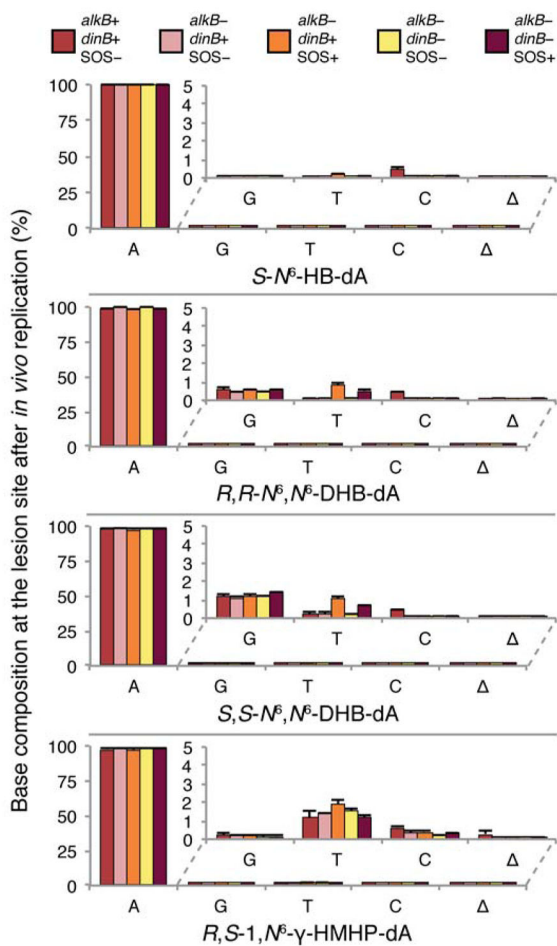
Figure 4.
Mutation frequency and specificity of the four BD-induced adenine adducts in cells with different AlkB, DinB and SOS-induction statuses. A, G, T and C represent the possible bases at the lesion site (N in Fig. 2) after *in vivo* replication and   denotes the occurrence of deletions at the lesion site. Each graphed value corresponds to the mean of three independent biological replicates; error bars represent one SD. To better show the low percentage events, the G, T, C and   results were zoomed-in and shown in the insets.
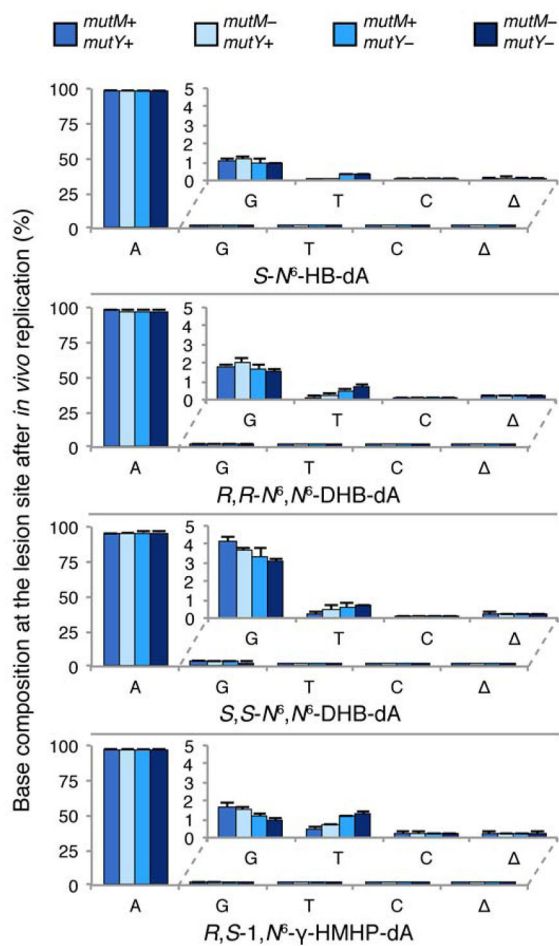
Figure 5.

Figure 5.
Mutation frequency and specificity of the four BD-induced adenine adducts in cells with different MutM and MutY statuses. A, G, T and C represent the possible bases at the lesion site (N in Fig. 2) after *in vivo* replication and   denotes the occurrence of deletions at the lesion site. Each graphed value corresponds to the mean of three independent biological replicates; error bars represent one SD. To better show the low percentage events, the G, T, C and   results were zoomed-in and shown in the insets.