In vitro reactions of butadiene monoxide with single- and double-stranded DNA: characterization and quantitation of several purine and pyrimidine adducts

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We have previously shown that butadiene monoxide (BM), the primary metabolite of 1,3-butadiene, reacted with nucleosides to form alkylation products that exhibited different rates of formation and different stabilities under in vitro physiological conditions. In the present study, BM was reacted with single-stranded (ss) and double-stranded (ds) calf thymus DNA and the alkylation products were characterized after enzymatic hydrolysis of the DNA. The primary products were regiosomeric N-7-guanine adducts. N-3-(2-hydroxy-3-buten-1-yl)adenine and N-3-(1-hydroxy-3-buten-2-yl)adenine, which were depurinated from the DNA more rapidly than the N-7-guanine adducts, were also formed. In addition, N°-(2-hydroxy-3-buten-1-yl)deoxyadenosine and N°-(1-hydroxy-3-buten-2-yl)deoxyadenosine were detected and evidence was obtained that these adducts were formed by Dimroth rearrangement of the corresponding N-1-deoxyadenosine adducts, not while in the DNA, but following the release of the N-1-alkylated nucleosides by enzymatic hydrolysis. N-3-(2-hydroxy-3-buten-1-yl)deoxyuridine adducts, which were apparently formed subsequent to deamination reactions of the corresponding deoxyguanosine adducts, were also detected and were stable in the DNA. Adduct formation was linearly dependent upon BM concentration (10–1000 mM), with adduct ratios being similar at the various BM concentrations. At a high BM concentration (750 mM), the adducts were formed in a linear fashion for up to 8 h in both ssDNA and dsDNA. However, the rates of formation of the N-3-deoxyuridine and N°-deoxyadenosine adducts increased 10- to 20-fold in ssDNA versus dsDNA, whereas the N-7-guanine adducts increased only slightly, presumably due to differences in hydrogen bonding in ssDNA versus dsDNA. These results may contribute to a better understanding of the molecular mechanisms of mutagenesis and carcinogenesis of both BM and its parent compound, 1,3-butadiene.

Introduction

1,3-Butadiene is a petrochemical used in the production of synthetic rubber and plastic and has been detected in cigarette smoke, automobile exhaust and in some gasoline formulations. Epidemiological studies have found excess mortality from lymphatic and hematopoietic cancers associated with occupational exposure to butadiene (1,2). Butadiene is a multisite carcinogen in rodents, with mice being more susceptible than rats (3,4). This carcinogenicity has been attributed to the metabolites of butadiene, based on the finding that butadiene is mutagenic in the Salmonella typhimurium test only in the presence of the S9 liver fraction of rats treated with phenobarbital or Aroclor 1254 (5). Butadiene monoxide (BM, 3,4-epoxy-1-butene), the primary metabolite formed in both in vitro and in vivo systems (6–10), is directly mutagenic in these tests, as is its further metabolite diepoxybutane (11,12). BM is also carcinogenic in mouse skin painting studies (13,14).

Butadiene is genotoxic in a number of other in vitro and in vivo systems. An increase in mutational frequency was seen in the hprt locus of lymphocytes from exposed B6C3F1 mice and in the tk locus of cultured human TK6 cells (15,16). An increase in hprt mutant frequency was also seen in the peripheral lymphocytes of non-smoking workers exposed to butadiene over controls (17). Butadiene, BM and diepoxidebutane all induce increases in sister chromatid exchanges in cultured human lymphocytes (18). Butadiene produced increases in sister chromatid exchanges, micronuclei and chromosomal aberrations in the bone marrow of mice, but showed no effect in rats (19,20). Short-term inhalation exposure of rats and mice to high concentrations of radiolabeled 1,3-[14C]butadiene resulted in radiolabel covalently bound to liver DNA in both species (21). However, differences in the types of DNA adducts formed, their stabilities and repair rates in susceptible tissues are unknown. Butadiene and BM have been shown to induce mutations at both G:C and A:T base pairs (16,22), suggesting that alkylation of any of the nucleoside bases of DNA may be involved in butadiene-induced mutagenesis.

Many studies have been undertaken to identify the alkylation products of BM with DNA. In 1984, Citti et al. (23) identified the major reaction products between BM and guanosine, deoxyguanosine and calf thymus (CT) double-stranded (ds)DNA as N-7-(2-hydroxy-3-buten-1-yl)guanine and its regioisomer N-7-(1-hydroxy-3-buten-2-yl)guanine. N-1-(1-hydroxy-3-buten-2-yl)guanine has also been detected using mass spectrometry techniques (24) and N°-alkyladenine adducts were detected by an HPLC/32P-post-labeling procedure (25). We have structurally characterized 29 adducts formed under in vitro physiological conditions (pH 7.4, 37°C) between BM and the four nucleoside bases of DNA (26–29). Initial characterization of the pyrimidine adducts was performed with the deoxynucleoside bases of DNA, whereas the N-7-guanine adducts, which were formed at lower rates than the N-7-guanosine adducts,
were stable at 37°C, pH 7.4, while the N-7-guanosine adducts were unstable and hydrolyzed the N-glycosidic bond with half-lives of 50–90 h. The N-1-adenosine adducts were the initial products of the reaction of BM and adenosine. Under in vitro physiological conditions, these adducts either undergo Dimroth rearrangement to yield N⁶-adenosine adducts or deaminate to form N-1-inosine adducts. Reaction of BM with deoxyctydine initially formed N-3 and O² adducts of deoxyctydine; the N-3-deoxyctydine adducts deaminated to N-3-deoxyuridine adducts, which were then stable. Reaction of BM and thymidine formed N-3-thymidine adducts, which were also stable at 37°C, pH 7.4. Recently, Tretyakova et al. have identified N-1- and N-3-adenine and N-7-guanine adducts from the reaction of BM with CT DNA, after acid hydrolysis of the DNA (30).

The present study describes the characterization of adducts formed by the reaction of BM with CT single-stranded (ss)DNA and dsDNA after enzymatic hydrolysis of the DNA using nuclease P1, DNase I, phosphodiesterase I and alkaline phosphatase. This enzymatic hydrolysis method is advantageous over acid hydrolysis because it allows the detection of both pyrimidine and purine adducts. Both ssDNA and dsDNA were used so that the effects of base pairing and hydrogen bonding on adduct formation can be determined.

Materials and methods

Materials

Racemic BM and trifluoroacetic acid were purchased from Aldrich (Milwaukee, WI). CT DNA, 2′-deoxyguanosine, 2′-deoxyadenosine, 2′-deoxyctydine, thymidine, 2′-deoxyuridine, 2′-deoxyinosine, adenosine, alkaline phosphatase (calf intestine) and phosphodiesterase I (bovine intestinal mucosa and type VI Croatalus atrox venom) were purchased from Sigma (St Louis, MO). Nuclease P1 (Penicillium citrinum) and DNase I (bovine pancreas, grade II) were purchased from Boehringer Mannheim (Indianapolis, IN). HPLC grade acetonitrile (ACN) was purchased from EM Science (Gibbstown, NJ). All other chemicals were of the highest grade commercially available.

BM–DNA adduct formation and enzymatic hydrolysis of adducted DNA

CT DNA (2 mg/ml) was dissolved in 50 mM Tris buffer containing 1 mM MgCl₂, pH 7.2, and rehydrated overnight at 4°C. ssDNA was made from the DNA by heating the DNA to 100°C for 5 min and rapidly cooling the solution on ice (31). At room temperature, BM (0.75 mmol/ml) was added to either ssDNA or dsDNA and the mixture reacted for 24 h at a 37°C Dubnoff shaking water bath. The reaction was stopped by precipitating the DNA from the reaction medium on ice with 0.1 vol 3 M sodium acetate and 3 vol ice-cold ethanol and centrifuging for 15 min at 3000 r.p.m. The supernatant was removed and the pellet was washed with 1 vol ice-cold 70% aqueous ethanol and the wash was added to the supernatant. The pellet was air dried for 15 min and redissolved in 50 mM Tris buffer. The DNA was then enzymatically hydrolyzed by a modified method (32) at 37°C for 24 h, using nuclease P1 (24 U/ml), alkaline phosphatase (3 U/ml), DNase I (10 U/ml) and phosphodiesterase I (0.3 U/ml, snake venom or bovine). The enzymatic hydrolysis was stopped by heating the sample at 100°C for 5 min, followed by centrifugation and filtration to remove the denatured enzyme protein. Each supernatant and enzymatically hydrolyzed DNA sample was concentrated by speed vacuum lyophilization and analyzed by HPLC.

In some experiments, neutral thermal hydrolysis was performed as previously described (32) to release all unstable N⁷- and N⁵-alkylpyrimines by heating the alkylated DNA at 100°C for 30 min in 50 mM Tris buffer, pH 7.2. The partially apurinic DNA was then precipitated with ethanol/acetate and enzymatically hydrolyzed as described above.

Acid-catalyzed hydrolysis of BM–DNA and BM–deoxyadenosine

Reaction of BM with DNA (described above) or deoxyadenosine (described below) was carried out. The reactions were stopped by extracting unreacted BM three times with 3 vol ethyl ether. Mild acidic hydrolysis was performed by adding 0.1 vol 1 N HCl to the alkylated DNA or deoxyadenosine in Tris buffer and heating the sample at 75°C for 20 min to release the purine bases (32). The samples were cooled to room temperature and the pH was adjusted to 7.2 with potassium hydroxide and stored at 4°C before analysis to prevent deamination/rearrangement. Samples were concentrated by speed vacuum lyophilization prior to analysis by HPLC. The retention times of the BM–N⁷-1-adenine regioisomers were 7.6 and 10.3 min while the retention times of the BM–N⁵-1-adenine regioisomers were 21.2 and 22.0 min (see below).

Synthesis of BM–DNA adduct standards

DNA adduct standards of BM were synthesized as previously described (26–29) by reacting deoxyxynucleosides or purine bases (10 mM) with BM (0.75 mmol/ml) in 50 mM Tris buffer, pH 7.2, containing 1 mM MgCl₂, for 24 h at a shaking water bath. Unreacted BM was removed by extracting three times with 3 vol ethyl ether. Adducts were analyzed by HPLC using the methods described below.

Some N⁷-alkylguanosines underwent imidazole ring-opening under mild alkaline conditions as indicated by UV spectra (λmax 272 nm) distinct from those of intact N⁷-alkylguanosines (28). Briefly, deoxyguanosine and BM were dissolved in acetic acid, reacted for 5 h at 50°C and then precipitated on ice by adding 1 vol acetic acid and 3 vol ethyl ether. The precipitate was re-disolved in 50 mM Tris buffer, containing 1 mM MgCl₂. The N⁷-deoxyguanosine adduct mixture was then incubated under alkaline conditions (pH 10 with KOH) at 37°C for 24 h and the pH was then adjusted back to neutrality. The alkaline incubation conditions resulted in disappearance of the four BM–N⁷-deoxyguanosine adducts (HPLC retention times 17.9, 20.4, 21.9 and 27.0 min) and appearance of four new peaks (retention times 17.5, 18.5, 20.1 and 21.7 min). The new peaks were tentatively characterized as ring-opened N⁷-deoxyguanosine adducts based upon their λmax of 272 nm, which is consistent with the reported λmax for imidazole ring-opened N⁷-ethylguanosine (33).

HPLC analysis and purification of BM–DNA adducts

Analytical separations of acid and enzymatically hydrolyzed reaction mixtures were performed by reverse phase HPLC, with a 20 µl injection volume onto a Beckman C₁₈ reverse phase analytical column (250×4.6 mm i.d.) using a Beckman gradient-controlled HPLC system (Irvine, CA) equipped with a Beckman diode array detector and UV detection at 260 and 280 nm. Analytical HPLC separations employed a linear gradient program starting at 10 min from 10 to 30% pump B over 3 min, pumping 30% B for 4 min, increasing from 30 to 60% pump B over 3 min, pumping at 60% B for 4 min, then increasing from 60 to 100% pump B over 3 min (pump A: 1% ACN, pH 2.5; pump B: 10% ACN, pH 2.5), at a flow rate of 1 ml/min.

Purification of adducts from the reaction of BM with DNA for positive ion fast atom bombardment mass spectrometry (FAB/MS) analysis was accomplished by injecting 50–100 µl of reaction mixture onto the column described above using the same mobile phases and gradient system as used for analytical separations. All adducts were collected as they eluted off the column, concentrated by lyophilization and re-injected onto the same column pumping isocratic 50% pump B and again collected as the adducts eluted off the column. The adducts were concentrated by lyophilization. The molecular ions were then determined for each purified adduct by FAB/MS on a Kratos MS 50 mass spectrometer equipped with a Kratos-DS 50 data system (Manchester, UK). An Ion Tech FAB gun utilizing xenon as the FAB gas was used with a direct insertion FAB probe. Spectra were performed in a glycerol matrix and were matrix subtracted.

Identification of BM–DNA adducts

The UV spectra of the products of the reaction between CT DNA and BM were obtained at pH 2.5 using a Beckman diode array detector in the mobile phases described above. The spectra and retention times of the products were compared with those obtained with the authentic standards (26–29). Molecular ions obtained by FAB/MS were used to confirm adduct identities. Dependence of DNA adduct formation on incubation time and BM concentration

ssDNA or dsDNA (2 mg/ml) and BM (10, 25, 50, 100, 250, 500 or 1000 mM) were reacted in 50 mM Tris buffer, pH 7.2, containing 1 mM MgCl₂, in a shaking water bath at 37°C. The reactions were stopped after 24 h by precipitating the DNA as described above. Analysis for adduct formation was performed by HPLC following enzymatic hydrolysis and concentration as described above. ssDNA or dsDNA (2 mg/ml) was also reacted with an excess of BM (750 mM) for 24 h at 37°C. Samples were withdrawn at timed intervals, the DNA precipitated, enzymatically hydrolyzed and concentrated as described above. Adducts were analyzed by HPLC to determine their time dependence.

DNA adduct quantitation

Adduct concentration in enzymatically hydrolyzed DNA was determined by measuring UV absorbance at 260 nm and comparing peak areas with a standard curve prepared using the reference standard (1–1000 µg/ml, r > 0.99) into the HPLC. DNA adduct standards were concentrated by speed vacuum lyophilization prior to analysis by HPLC. The retention times of the BM–N⁷-1-adenine regioisomers were 7.6 and 10.3 min while the retention times of the BM–N⁵-1-adenine regioisomers were 21.2 and 22.0 min (see below).
and molecular ions determined by FAB/MS (Table I). The two standards which had been previously characterized (26–29), identified by their UV spectra, co-elution with authentic sites were probably involved in base pairing of dsDNA. In contrast, the later eluting peaks (III–VI) increased 10- to 20-fold in peak areas in ssDNA, suggesting that these dsDNA. The amounts and ratios of adducts formed were altered. In products was observed with ssDNA and dsDNA; however, enzymatic hydrolysis (Figure 1A and B). A similar profile of products not seen in control DNA incubations also followed the formation of N6-deoxyguanosine adducts, which occur with half-lives of <3 h under in vitro physiological conditions (pH 7.4, 37°C).

Peak V was identified as co-eluting diastereomers of N6-(2-hydroxy-3-buten-1-yl)deoxyuridine based on its UV spectra (λmax 266 nm) and co-elution with authentic standards. The small shoulder on peak V had an identical UV spectrum and co-eluted with a single diastereomer of the other regioisomer, N6-(1-hydroxy-3-buten-2-yl)deoxyuridine. VI appears to be a result of several adducts co-eluting, including N1-(1-hydroxy-3-buten-2-yl)deoxyinosine and the second diastereomer of N6-(1-hydroxy-3-buten-2-yl)deoxyadenosine. VII and VIII are regioisomeric N3-(2-hydroxy-3-buten-1-yl)adenine and N3-(1-hydroxy-3-buten-2-yl)adenine.

Butadiene monoxide purine and pyrimidine adducts

Deoxyadenosine and N3-deoxyuridine adducts, respectively. Concentration of total nucleosides was measured by comparing sample peak areas of unmodified deoxyctydine and thymidine with standard curves prepared using the standard 2'-deoxyxynucleoside bases (10–2000 μg/ml, r > 0.99). These nucleosides were used for quantitation because they were the least impacted by adduct formation. Adduct concentration was normalized to the thymidine concentration of each sample. Total nucleoside concentrations were then estimated by assuming equivalent concentrations for thymidine and deoxyadenosine and for deoxyctydine and deoxyguanosine. The method was verified using unalkylated samples of CT DNA; the G+C content obtained experimentally (40–43%) was similar to the published (34) G+C content (42%).

Results

Reaction of racemic BM with CT DNA in Tris buffer, pH 7.2, at 37°C for 24 h, followed by enzymatic hydrolysis to the deoxynucleoside bases resulted in the formation of a number of products not seen in control DNA incubations also followed by enzymatic hydrolysis (Figure 1A and B). A similar profile of products was observed with ssDNA and dsDNA; however, the amounts and ratios of adducts formed were altered. In dsDNA, the earlier eluting products (I and II) were predominant, increasing only slightly in peak areas in ssDNA versus dsDNA. In contrast, the later eluting peaks (III–VI) increased 10- to 20-fold in peak areas in ssDNA, suggesting that these sites were probably involved in base pairing of dsDNA.

Adducts formed in the reaction of BM with CT DNA were identified by their UV spectra, co-elution with authentic standards which had been previously characterized (26–29), and molecular ions determined by FAB/MS (Table I). The two largest product peaks, I and II, were identified as N7-(2-hydroxy-3-buten-1-yl)guanine and N7-(1-hydroxy-3-buten-2-yl)guanine, respectively (Figure 2). These adducts were identified based on their UV spectra, with a λmax of 250 nm, their co-elution with authentic standards and by FAB/MS of the purified products from the DNA reaction which produced an M+1 ion of m/z 222 for both adducts (Table I). The corresponding deoxyguanosine adducts were inherently unstable and spontaneously depurinated from DNA with a half-life of 50 h (23). Approximately 25% of the N7-guanine adducts were depurinated during the 24 h, 37°C reaction period, as measured in the supernatant after ethanol/acetate precipitation of the DNA (Figure 1C). The remaining 75% was released as N7-alkylated guanine adducts during the enzymatic hydrolysis step and/or the subsequent heating step to precipitate the proteins. The N7-guanine adducts were completely depurinated from the DNA following neutral thermal hydrolysis, with none remaining in the DNA as determined by enzymatic hydrolysis of the DNA after the neutral thermal hydrolysis step (data not shown).

Peaks III and IV were identified as diastereomers of N3- (2-hydroxy-3-buten-1-yl)deoxyuridine (Figures 1 and 2). These adducts were identified by their UV spectra (λmax 263 nm), which were identical to previously characterized standards (28), their co-elution with these standards and by FAB/MS of peak IV, which gave an M+1 ion of m/z 299, as expected for a deoxyuridine adduct of BM and in contrast to an M+1 ion of m/z 298 for a deoxyxystidine adduct of BM (28). We have previously shown that the N3-deoxyuridin adducts are formed by hydrolytic depurination of the corresponding N3-deoxyctydine adducts, which occur with half-lives of <3 h under in vitro physiological conditions (pH 7.4, 37°C).

Fig. 1. HPLC chromatogram of CT ssDNA, control or reacted with BM for 24 h at 37°C and precipitated with ice cold ethanol/acetic acid. (A) Control DNA. (B) Precipitated DNA enzymatically hydrolyzed with nuclease P1, DNase I, alkaline phosphatase and snake venom phosphodiesterase. (C) Supernatant following precipitation of the BM-reacted DNA. I and II are N7-(2-hydroxy-3-buten-1-yl)guanine and N7-(1-hydroxy-3-buten-2-y1)guanine, respectively. III and IV are diastereomers of N3-(2-hydroxy-3-buten-1-yl)deoxyuridine. V is diastereomeric N6-(2-hydroxy-3-buten-1-y1)deoxyadenosine, while the small shoulder on V is the regioisomer N6-(1-hydroxy-3-buten-2-yl)deoxyadenosine. VI appears to be a result of several adducts co-eluting, including N1-(1-hydroxy-3-buten-2-yl)deoxyinosine and the second diastereomer of N6-(1-hydroxy-3-buten-2-yl)deoxyadenosine. VII and VIII are regioisomeric N3-(2-hydroxy-3-buten-1-yl)adenine and N3-(1-hydroxy-3-buten-2-yl)adenine.
Table I. Products of the reaction of BM with CT DNA

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Identity</th>
<th>UV λ&lt;sub&gt;max&lt;/sub&gt; (pH 2.5)</th>
<th>m/z (M+1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 13.9</td>
<td>N-7-(2-hydroxy-3-buten-1-yl)guanine</td>
<td>250 nm</td>
<td>222</td>
</tr>
<tr>
<td>II 17.3</td>
<td>N-7-(1-hydroxy-3-buten-2-yl)guanine</td>
<td>250 nm</td>
<td>222</td>
</tr>
<tr>
<td>III 31.8</td>
<td>N-3-(2-hydroxy-3-buten-1-yl)deoxyuridine</td>
<td>263 nm</td>
<td>NA</td>
</tr>
<tr>
<td>IV 32.3</td>
<td>N-3-(2-hydroxy-3-buten-1-yl)deoxyuridine</td>
<td>263 nm</td>
<td>299</td>
</tr>
<tr>
<td>V 33.0</td>
<td>N&lt;sup&gt;8&lt;/sup&gt;-deoxyadenosine, both regioisomers</td>
<td>266 nm</td>
<td>322</td>
</tr>
<tr>
<td>VI 33.9</td>
<td>Unresolvable mixture of adducts</td>
<td>265 nm</td>
<td>NA</td>
</tr>
<tr>
<td>VII 11.7</td>
<td>N-3-(2-hydroxy-3-buten-1-yl)adenine</td>
<td>276 nm</td>
<td>ND</td>
</tr>
<tr>
<td>VIII 16.5</td>
<td>N-3-(1-hydroxy-3-buten-2-yl)adenine</td>
<td>276 nm</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Retention times were obtained from different experiments using the same HPLC method. NA, molecular ions were not detectable above background noise due to very small amount of sample available; ND, not determined.*

**Fig. 2.** Structures of adducts identified from the reaction of BM with CT DNA. HPLC co-elution with synthetic standards, UV spectroscopy and FAB/MS were used to identify I and II as regioisomeric N-7-guanine adducts of BM. Similarly, III and IV were identified as diastereoisomers of N-3-(2-hydroxy-3-buten-1-yl)deoxyuridine. Tentative assignments for VII and VIII were based on the previously reported regioselectivity and elution patterns of N-3-(2-hydroxy-3-buten-1-yl)adenine and N-3-(1-hydroxy-3-buten-2-yl)adenine (31).**

and ssDNA at 37°C for either 4 or 18 h. The 4 h reaction time was selected because it favored the formation of N-1-deoxyadenosine adducts upon the reaction of BM with deoxyadenosine with relatively little Dimroth rearrangement, while the 18 h reaction time was selected because it favored rearrangement to yield the N<sup>6</sup>-deoxyadenosine adducts (27), with the 18 h time point yielding predominantly N<sup>6</sup>-adenine adducts. Acid hydrolysis of the reaction products of BM with deoxyadenosine after the 4 or 18 h incubation demonstrated hydrolysis of the deoxynucleosides to both the N-1- and N<sup>6</sup>-adenine adducts. Acid hydrolysis of the alkylated ssDNA following a 4 or 18 h incubation at 37°C resulted in detection of the N-1-adenine adducts, but no N<sup>6</sup>-adenine adducts, with a time-dependent increase in adduct formation (data not
shown). These results provide strong evidence that Dimroth rearrangement did not occur in the DNA whether it reacted with BM for 4 or 18 h.

Tretjakova et al. (30) reported that the N-1-(2-hydroxy-3-buten-1-yl)adenine regioisomer was formed to a greater extent than N-1-(1-hydroxy-3-buten-2-yl)adenine in reactions of BM with DNA, in a ratio of 1.5:1. We find a similar ratio of 2.2:1 for the N-1-deoxyadenosine regioisomers formed in reactions between BM and deoxyadenosine at 4 and 18 h, with or without acid hydrolysis to the N-1-adenine regioisomers, and the same ratio for the N\textsuperscript{6}-deoxyadenosine regioisomers in ssDNA after enzymatic hydrolysis or for the N-1-adenine regioisomers after acid hydrolysis of BM-reacted ssDNA.

Peak VI contained more than one adduct based on UV spectral analysis. Both the leading and the trailing edges of peak VI had UV spectral characteristics similar to N-1-inosine adducts formed with BM (27) and N-1-deoxyinosine adducts with a \(\lambda_{\text{max}}\) of 252 nm (data not shown). The center of the peak had a spectral shape consistent with N\textsuperscript{6}-deoxyadenosine and/or N-3-deoxyuridine with a \(\lambda_{\text{max}}\) of 264 nm. The standard of the later eluting diastereomer of N\textsuperscript{6}-(1-hydroxy-3-buten-2-yl)deoxyadenosine co-eluted with the center of peak VI. Authentic standards of N-3-(1-hydroxy-3-buten-2-yl)deoxyuridine (28) also co-eluted with peak VI. Extensive HPLC method development did not separate these components any further and FAB/MS analysis of peak VI did not produce any definitive molecular ions above background.

The N-3-adenine adducts of BM, VII and VIII, were detected in the combined ethanol/acetate supernatant from the DNA precipitation step (Figure 1C). These adducts were identified based on their UV spectra (\(\lambda_{\text{max}}\) 276 nm) and co-elution with the major products of the reaction between BM and adenine, which have been characterized as N-3-(2-hydroxy-3-buten-1-yl)adenine and N-3-(1-hydroxy-3-buten-2-yl)adenine (30). These adducts were not detected in neutral thermal hydrolysates of the DNA, suggesting that they were more unstable than the N-7-guanine adducts and were completely depurinated from the DNA during the reaction period of 24 h at 37°C. These adducts increased ~2-fold in peak area in ssDNA versus dsDNA. Tretjakova et al. (30) reported a ratio of 2.2:1 favoring the N-3-(1-hydroxy-3-buten-2-yl)adenine isomer over the N-3-(2-hydroxy-3-buten-1-yl)adenine isomer, with these adduct levels being ~10-fold lower than those of the N-7-guanine adducts. We observed a similar ratio of 1.5:1 between the two N-3-adenine adducts, favoring the later eluting product (VIII). Therefore, we tentatively identified these adducts as regioisomeric N-3-adenine adducts based on their elution order and their regioselectivity. We did not quantitate these adducts because we have no pure standards for these products.

Imidazole ring-opened N-7-deoxyguanosine adducts of BM were synthesized in order to investigate the possible detection of these adducts in CT DNA under the enzymatic hydrolysis conditions. However, no evidence for formation of these adducts was obtained.

Attempts to hydrolyze the DNA with only nuclease P1, DNase I and alkaline phosphatase, as previously described for ethylated and methylated DNA (32), resulted in detection of many more peaks than are shown in Figure 1. These peaks did not co-elute with the previously characterized standards formed by the reactions of BM and the deoxynucleoside bases of DNA (26–29). Ultimately, the unidentified products were characterized as incomplete digestion products. These products were probably caused by the bulky alkyl groups which may have blocked the enzymatic digestion; these products were not detected in the unalkylated controls. Peak VI did not co-elute with the previously characterized standards for DNA, in a ratio of 1.5:1. We find a similar ratio of 2.2:1 for the N-1-deoxyadenosine regioisomers formed in reactions between BM and deoxyadenosine at 4 and 18 h, with or without acid hydrolysis to the N-1-adenine regioisomers, and the same ratio for the N\textsuperscript{6}-deoxyadenosine regioisomers in ssDNA after enzymatic hydrolysis or for the N-1-adenine regioisomers after acid hydrolysis of BM-reacted ssDNA.

Fig. 3. Effect of BM concentration on the formation of adducts when dsDNA or ssDNA (2 mg/ml) was reacted with BM for 24 h at 37°C. The various BM-DNA adduct concentrations were determined using HPLC as described in Materials and methods. BM concentrations up to 1000 mM had linear characteristics.

CT DNA (ssDNA and dsDNA, 2 mg/ml) was reacted with varying concentrations of BM (10, 25, 50, 100, 250, 500 and 1000 mM) at 37°C for 24 h in 50 mM Bis–Tris buffer, pH 7.2, in order to investigate the BM concentration dependence of adduct formation (Figure 3). Adduct formation with BM concentrations up to 1000 mM was linear. All adducts were detected at BM concentrations of \(\geq 10\) mM in ssDNA and at BM concentrations of \(\geq 100\) mM in dsDNA. The ratios of adducts formed were similar over all the BM concentrations used. When adducts were quantitated at 25, 100 and 500 mM BM, the N-7-guanine adducts predominated with dsDNA and the concentrations of these adducts increased only slightly with ssDNA (Table II). The N\textsuperscript{6}-deoxyadenosine adducts (V) and N-3-deoxyuridine adducts (III and IV) were detected to a much lesser extent with dsDNA, but showed a 10- to 20-fold increase with ssDNA (Table II).

When CT DNA (ssDNA and dsDNA, 2 mg/ml) was reacted with a high concentration of BM (750 mM) under in vitro physiological conditions (pH 7.2, 37°C), all of the adducts were formed in a roughly linear fashion (Figure 4), except for the N-7-guanine adducts, which plateaued at time points \(>8\) h. This plateau may be due to increased depurination of N-7-guanine adducts at later time points. The rates of formation of
Table II. Quantitation of adducts formed in the reaction of BM with CT ssDNA and dsDNA

<table>
<thead>
<tr>
<th>Adduct</th>
<th>BM concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 mM</td>
</tr>
<tr>
<td></td>
<td>dsDNA</td>
</tr>
<tr>
<td>I</td>
<td>1/280</td>
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*The various BM–DNA adduct concentrations were determined using HPLC as described in Materials and methods. Adduct levels are expressed as adducts per unmodified base.

Discussion

We have previously characterized the adducts of BM with guanosine, adenosine, deoxycytidine and thymidine (26–29). This study describes the identification of some of these adducts following enzymatic hydrolysis of CT DNA reacted in vitro with BM. The adducts were formed in a time- and BM concentration-dependent manner. These results confirmed what was reported previously with the nucleoside (26–29) and DNA (36,42,43), all of which increased in animals exposed to 1,3-butadiene (16,22).

We have identified as additional adducts diastereomeric N6-(2-hydroxy-3-buten-1-yl)deoxyadenosine and N6-(1-hydroxy-3-buten-2-yl)deoxyadenosine. These adducts were previously detected in 32P-post-labeling studies of alkylated CT DNA (25). Studies examining the reaction of other epoxides, including styrene oxide, cyanoethylene oxide and propylene oxide (36,42,43), also reported N6-deoxyadenosine adducts in enzyme-hydrolyzed CT DNA. However, a recent study produced the N1-adenine adducts from CT DNA reactions with BM after acid hydrolysis of the adduct from DNA (30). We have previously demonstrated that the N1-adenosine adducts are the precursors of N6-adenosine adducts through Dimroth rearrangement (27). In this study, we subjected BM-alkylated DNA and deoxyadenosine to enzymatic and acid hydrolysis. We found that enzymatic hydrolysis of alkylated DNA resulted in products that co-eluted with N6-deoxyadenosine standards, while producing no products which co-eluted with N1-deoxyadenosine products. In contrast, acid hydrolysis of alkylated DNA produced products that co-eluted with acid-hydrolyzed N1-deoxyadenosine, but no products co-eluting with acid-hydrolyzed N6-deoxyadenosine. These results suggested that the N1-adenine adducts were stable in DNA and did not rearrange under the acidic conditions of hydrolysis, whereas they converted to the N6-deoxyadenosine adducts when the free deoxynucleosides are released by enzymatic hydrolysis. This is consistent with previous studies showing the Dimroth rearrangement only occurs readily under neutral to mild alkaline conditions (27,44,45) and may explain the discrepancies between studies which form one adduct or the other. The results also demonstrated that the N6-deoxyadenosine adducts were not formed directly in DNA under our in vitro physiologic conditions. In a study by Segal et al. (46) Dimroth rearrangement of 1-(2-carboxyethyl)adenine did not occur in ssDNA under neutral conditions (pH 7.5, 37°C, 18 h) followed
Butadiene monoxide purine and pyrimidine adducts

by acid hydrolysis, but complete rearrangement occurred when the alkylated DNA was incubated at pH 11.7, 37°C, 18 h, followed by acid hydrolysis. The N-1 adducts of adenosine may be important mutagenic precursors due to the role of these positions in normal hydrogen bonding within the DNA. The N-1-adenosine adducts of BM have also been shown to undergo hydrolytic deamination to N-1-inosine adducts (27). The N-1-deoxyinosine adducts tentatively identified in this study may also be important mutagenic precursors because they are stable adducts in the absence of repair and may have altered base pairing properties than the precursor adenine. While there are repair enzymes which remove inosine/hypoxanthine from DNA, it is presently unknown whether these enzymes recognize N-1-alkylated bases.

This study is the first to report the formation of the diastereomeric pyrimidine adducts N-3-(2-hydroxy-3-buten-1-yl)deoxyuridines in DNA (Figure 1, peaks III and IV). The regioisomeric N-3-(1-hydroxy-3-buten-2-yl)deoxyuridine adducts may also be present as a component of peak VI. These adducts were previously characterized from the reaction of BM with deoxyctytidine (28) and were formed from deamination of the corresponding N-3-deoxycytidylate adducts. N-3-deoxyuridine adducts were formed in CT DNA with other reactive epoxides, including ethylene oxide, propylene oxide and 2-cycanoethylene oxide (36,42,47,48). These adducts may be important mutagenic precursors due to their stability (28) and because they have different hydrogen bonding characteristics and pair differently than the parent base, cytosine, in addition to the possibility that the alkylation may entirely block DNA replication enzymes. Uracil DNA N-glycosylase is a repair enzyme that releases uracil from DNA. While it is able to recognize a number of oxidative DNA base modifications such as 5-hydroxymethyl uracil, were not substrates for the enzyme (49). Thus, there are structural limitations to the repair enzyme and repair of 3-alkyl-deoxyuridine by a specific glycosylase (49). Therefore, there are structural limitations to the repair enzyme and repair of 3-alkyl-deoxyuridine by a specific glycosylase is presently unknown.

Reactions of aliphatic epoxides with DNA have previously been investigated (36,42,43,45,50). In those studies, N-7-guanine adducts were the primary products while substitution at the N2 of guanine, N-3 of cytosine/uracil and N-1, N-3 and N0 of adenine were also observed. These are the same positions of substitution reported in this study, with the exception of the N6-guanine adducts, which may not be formed in large enough quantities to be detectable by UV with our HPLC method.

In conclusion, this study describes a method using enzymatic hydrolysis/UV detection to analyze the adducts formed by BM in ssDNA and dsDNA. This method results in complete digestion of the alkylated DNA, which was not the case using fewer enzymatic components. We have confirmed the formation of regioisomeric N-7-guanine adducts, N-1- and N0-deoxyadenosine adducts and N-3-adenine adducts of BM with CT DNA. In addition, we have identified the formation of diastereomeric N-3-deoxycytidine adducts. The N0-deoxyadenosine and N-3-deoxyuridine adducts increased 10- to 20-fold in ssDNA versus dsDNA due to the involvement of the N-1 position of adenine and the N-3-position of cytosine in base pairing in dsDNA. The modest increase in the N-7-guanine adducts in ssDNA versus dsDNA is consistent with the lack of involvement of this site in base pairing. Identification and quantitation of the BM adducts formed with DNA may improve our understanding of the mechanism(s) of mutagenicity of BM and its parent compound, 1,3-butanediene.

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