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Regio- and stereospecific DNA adduct formation in mouse lung at N^6 and N7 position of adenine and guanine after 1,3-butadiene inhalation exposure

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Butadiene monoepoxide (BMO) alkylated guanine N7 and adenine N^6 adducts were prepared and enriched by solid phase extraction and HPLC. The purified adducts were analysed by a modified ^{32}P-postlabelling assay, which utilized one dimensional TLC chromatography and a subsequent HPLC analysis with UV and radioactivity detectors. *In vitro* with Ct-DNA the formation of N7-dGMP and N^6-dAMP adducts were linear at a concentration range of 44 to 870 nmol of BMO per mg DNA at physiological pH. N7-dGMP and N^6-dAMP adducts were formed in a ratio of 200:1. In dGMP and in dAMP 48 % and 86 % of adducts were covalently bound to the C-2 carbon of BMO. CD-1 mice were inhalation exposed to butadiene for 5 days and 6 h per day. The N7-dGMP adduct level in lung samples of animals exposed to 200, 500 and 1300 ppm was 2.8 ± 0.9 fmol, 11 ± 2.0 fmol and 30 ± 6.7 fmol in 10 μg DNA, respectively. The level of N^6-dAMP adducts in lung samples after 500 ppm and 1300 ppm exposure was 0.09 ± 0.06 fmol and 0.11 ± 0.05 fmol in 10 μg DNA. At 200 ppm the adduct level was below the detection limit. A sub-group of animals exposed to 1300 ppm was killed 3 weeks after the last exposure. N7-dGMP adducts were not detected but the level of N^6-dAMP adducts was not affected. N7-dGMP adducts were formed in a clear stereospecific manner *in vivo*. *S*-BMO adducts were the main product and represented 77 % ($n = 4$, SD = 2%) of total BMO adducts. No clear conclusion can be drawn about the enantiospecific DNA binding at the N^6 position of dAMP, because of the poor separation of the enantiomers. However, we could separate regioisomeric adducts which indicated that C-2 adducts represented 69 ± 3 % of the total N^6 adducts formed in mice lung DNA. This observation is supported by the data derived from *in vitro* DNA experiments but is different to our previously published data, which indicates the 2:1 (C-1:C-2) ratio in regioisomer formation in nucleotides or nucleosides. We suggest that the data presented in this communication indicate a different mechanism between nucleotides and DNA in BMO-derived adduct formation – Dimroth rearrangement dominates in nucleotides, but in double stranded DNA a direct alkylation is probably the major mechanism of adduct formation.

Keywords: 1,3-butadiene, butadiene monoepoxide, DNA, adduct, postlabelling, HPLC.

Abbreviations: BD, 1,3-butadiene; BMO, 3,4-epoxy-1-butene; Ct-DNA, calf thymus DNA; dR, deoxyribose; NN, normal nucleotides; TLC, thin layer chromatography; PEI, polyethyleneimine.

Introduction

1,3-Butadiene (BD) is a high production volume chemical and it is widely used in the polymer industry. The major use of BD is the production of butadiene–

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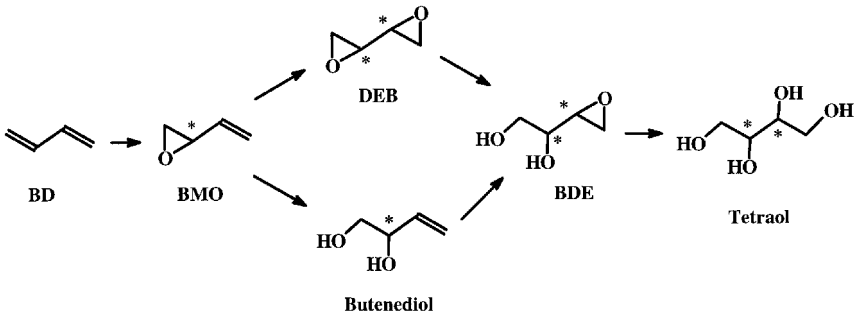

Figure 1. The metabolism of 1,3-butadiene. An asterisk indicates a chiral carbon.

styrene plastics and polybutadiene. The high use of BD is therefore a risk for a number of workers in the petrochemical or related industries (IARC 1992). Besides its industrial use BD is a common environmental pollutant. It has been estimated that most BD emissions derive from car exhausts (Lofgren and Petersson 1992), but combustion of organic material also produces BD emissions (Ehrenberg and Toernqvist 1993). BD has been detected in main and sidestream tobacco smoke (Brunnemann *et al.* 1990).

BD is not very toxic *per se* (Carpenter *et al.* 1944). It is metabolized by cytochrome P450-dependent monooxygenases 2E1 and 2A6 to butadiene monoepoxide (BMO) – BMO can then be further oxidized to diepoxide (DEB), or after hydrolysis and further oxidizing to butadiene diolepoxide (BDE) (Malvoisin and Roberfroid 1982). BMO exists as two enantiomers, a fact that has often been overlooked, even though enantiomers have been shown to have a different genotoxic activity (Sinsheimer *et al.* 1993). BD epoxy metabolites have been shown to be mutagenic in *in vitro* and in *in vivo* assays (Adler *et al.* 1997). BD exposure causes tumours in several organs in rodents, and a clear difference in tumour susceptibility has been observed between mice and rats (Melnick and Huff 1992). BMO and DEB have been detected in mouse and rat tissues, mice having a higher level of the metabolites (Himmelstein *et al.* 1995). The metabolism of BD is shown in figure 1.

BMO is an unsaturated alkylepoxide whose reaction chemistry differs from saturated alkylepoxides. Saturated alkylepoxides produce almost exclusively adducts in which the substitution has occurred at the terminal carbon of the epoxide (Solomon *et al.* 1988, Li *et al.* 1992, Hemminki *et al.* 1994). BMO forms adducts differently by producing guanine N7 alkylation products with an almost 1:1 ratio between the terminal carbon (C-1 carbon) and the second carbon of the epoxide (C-2) (Citti *et al.* 1984, Neagu *et al.* 1995) (figure 2). In that respect BMO shares some character of styrene oxide, which also produces adducts to both carbons of the epoxide (Hemminki and Hesso 1984). DNA alkylation by the butadiene-derived epoxides has gained a strong research activity and various alkylation sites in purine bases have already been detected. The main site of alkylation in deoxyguanosine is the N7 position and recently all four BMO-derived isomers have been separated, characterized and detected both *in vitro* and *in vivo* (Citti *et al.* 1984, Jelitto *et al.* 1989, Neagu *et al.* 1995, Kumar *et al.* 1996, Koivisto *et al.* 1997, Tretyakova *et al.* 1997). *In vitro* a minor amount of alkylation at the N^2 and N1 positions of guanosine have also been reported (Selzer and Elfarra 1996b).

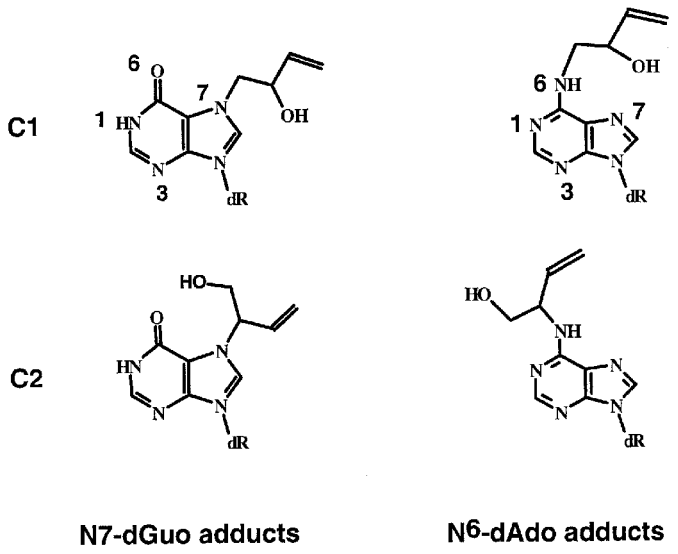

Figure 2. The structures of the BMO-derived purine adducts formed in *vitro* and *in vivo*.

Alkylation products at the N^6 position of adenosine are formed after exposure to alkylepoxides in neutral aqueous solutions (Fujii *et al.* 1986, Solomon *et al.* 1988). The path of formation is an initial binding of a particular epoxide at the N1 position of adenine followed by intramolecular Dimroth rearrangement (Engel 1975). Recently, Koivisto *et al.* suggested that a direct alkylation of the exocyclic N^6 amino group of deoxyadenosine could take place in DNA, even though Dimroth rearrangement is apparent if adenine nucleotides or nucleosides are exposed to BMO (Koivisto *et al.* 1995). Later on alkylation at the N1 and N3 positions of adenine was detected in DNA exposed to BMO *in vitro* (Tretyakova *et al.* 1997). Other products that can be derived after alkylation of adenosine at the N1 position are adducted inosine derivatives resulting from deamination (Fujii *et al.* 1986). C-2 adducts of BMO and styrene oxide in dAdo are reported to partially deaminate while C-1 adducts tend to rearrange through the Dimroth mechanism (Qian and Dipple 1995, Selzer and Elfarra 1996a). The N1/N^6 ratio is influenced by the BMO concentration in a way that at high epoxide concentration the initial formation of N1 adducts is much higher than the corresponding N^6 adducts (Selzer and Elfarra 1996a).

Guanine N7 alkylation products are chemically labile which leads to depurination. The number of depurinations caused by alkylating agents has a poor correlation to mutagenic activity, although the lesion is mutagenic (Drinkwater *et al.* 1980, Loeb and Preston 1986). It has also been estimated that 10^4 spontaneous depurinations occur in each cell per day (Lindahl and Nyberg 1972). Besides spontaneous depurination guanine N7 adducts have an active DNA repair mechanism. 3-Methyladenine-DNA glycosylase has been found to excise several structurally unrelated adducts, but there seems to be preferential excision of charged bases. Therefore N7 adducts of dGMP are substrates for this particular DNA repair enzyme system (Singer and Hang 1997). The activity of glycosylase, however, has been shown to be assosiated with the structure of the adduct. Methyl adducts are repaired more efficiently than ethyl adducts. Also the 3D structure of

the adduct moiety may have an effect on the repair rate and fidelity of repair (Singer and Brent 1981, Koivisto *et al.* 1998).

In general N^6 adducts are chemically stable and the disappearance of these lesions is mainly affected by an active DNA repair. Also, a few studies exist which demonstrate the high stability of adducts at exocyclic amino groups of purine bases *in vivo* (Beland *et al.* 1980, Tillis *et al.* 1981). Huang *et. al.* have reported that human nucleotide excision repair can recognize N^6 methyl adducts (Huang *et al.* 1994).

DNA adducts derived from BD exposure *in vivo* have only been the subject of a few studies. Jelitto *et al.* exposed mice and rats to ^{14}C-labelled BD, which resulted in BMO- and DEB-derived DNA adduct formation in guanine residues only in mouse liver (Jelitto *et al.* 1989). These data are in contradiction to Kreiling's observation that total radioactivity in mouse and rat liver DNA after ^{14}C-labelled BD exposure was about the same (Kreiling *et al.* 1986). Kreiling's observation was supported by recent data of Koivisto *et al.* which demonstrate the N7 adducts of BMO in rat liver DNA detected by the modified ^{32}P-postlabelling analysis (Koivisto *et al.* 1997).

We have studied the capability of BMO to alkylate purine bases and have used the formed DNA adducts to estimate the target organ dose. Previously we reported the identification of dAdo alkylation products of BMO and showed that these adducts are formed in mice and rats exposed to BD (Koivisto *et al.* 1996). Recently we reported an analytical method which allows the separation of all four BMO-derived isomeric N7 products of dGMP (Koivisto *et al.* 1997). We now report the results of animal experiments in which BMO-derived DNA adducts are determined in mouse lung at N^6 in dAMP and at N7 in dGMP. The adducts were determined immediately after the exposure and 3 weeks after the last exposure.

Materials and methods

Chemicals

BMO, *N*^{6}-methyl deoxyadenosine, *N*7-methyl-guanine, micrococcal nuclease (MN), and RNAse A were from Sigma Chemical Co. (St Louis, MO). HPLC grade methanol was purchased from LAB-SCAN (Dublin, Ireland). Spleen phosphodiesterase (SPD), nuclease P1 and alkaline phosphatase from calf intestine were from Boehringer-Mannheim (Indianapolis, IN). Proteinase K and calf thymus DNA (Ct-DNA) were from Merck (Germany). Crude γ-^{32}P-ATP with 7000 Ci mmol^{-1} specific activity was from ICN (Belgium). TLC plates were 0.1 mm CEL 300 PEI (polyethyleneimine) from Macherey Nagel. Scintillation liquid was Ultima Flo AF (Packard, Holland).

Preparation of BMO-modified nucleotides and DNA

3′ and 5′dAMP (2 mg ml^{-1}) were exposed with BMO (10 µl ml^{-1} day^{-1}) in 100 mM AF at pH 10.5. Nucleotides were first purified with a 1cc SAX cartridge (Bond Elut, Varian USA) followed by an HPLC purification. Aliquots of the products were dephosphorylated by alkaline phosphatase (AP) and HPLC retention times were compared with fully characterized nucleoside standards (Koivisto *et al.* 1995). Quantitation of adducted 3′dAMP standards was carried out using a calibration graph based on commercially available *N*^{6}-methyl-dAdo and generated with an HPLC. dGMP nucleotides were exposed at pH 7.4 in 30 mM TRIS–HCl overnight at room temperature. The adducts formed were purified with an SAX HPLC column (Pharmacia Mono Q HR 5/5) by collecting a 3 min fraction started from the void volume. The products were depurinated with neutral thermal hydrolysis (30 min at 100 °C) and identified by comparing the RP-HPLC retention time of characterized N7 guanine BMO adducts (Neagu *et al.* 1995, Koivisto *et al.* 1997). The quantitation is based on the calibration graph of *N*7-methyl-guanine and HPLC analysis. Calf thymus DNA (1 mg ml^{-1}) was exposed for 24 h in 10 mM TRIS–HCl pH 7.4 at 37 °C with 44, 174 and 870 nmol ml^{-1} with BMO. After exposure DNA was precipitated with ethanol/NaCl and diluted with water.

Equipment

Normal nucleotides were analysed by using a Shimadzu LC-6A HPLC pump with an SPD 6AV UV-detector at 254 nm. Samples were injected using a Gilson 2324 Autoinjector. A biocompatible guard column (Upchurch ODS, 2 ×10 mm) and a reversed phase analytical column were used (Inertsil ODS-2, 2.1 ×150 mm). N^6-dAMP adducts were enriched with an LKB 2152 controller, an LKB 2150 pump and a 2140 rapid spectral detector (254 nm). Eluent was mixed prior to the pump by an LKB low pressure mixer. Samples were introduced by using a Rheodyne 9125 injector and a Rheodyne 7000 switching valve. The first column was a 2 ×20 mm Inertsil ODS-2 and the second column was an Upchurch α-chrom 2 ×100 mm (C-18, 300 Å). Postlabelled samples were analysed with a Waters 717 autosampler, a 600 controller and a 486 Tunable absorbance detector (254 nm). A UV detector was connected to a Radiomatic Flo-One β 150 TR flow scintillation analyser. The loop used was either LQ TR 500 µl or 2000 µl and the measured energy range was set to 0–1710 KeV. The column used was an Inertsil ODS-3 (4 ×150 mm).

Animal samples

CD-1 mice were exposed for 5 days and 6 h per day to 200, 500 and 1300 ppm of BD (GSF laboratories Germany, Dr I-D Adler). Control animals were sham exposed. The animals were originally used for germ cell mutation studies and therefore a group of 1300 ppm exposed animals was killed 21 days after exposure. Lungs were frozen with liquid nitrogen and powdered in a cooled mortar. DNA was extracted using Qiagen extraction columns by following the instructions of the manufacturer except that proteinase treatment was extended from two to three hours at 37 °C. An approximate DNA concentration was measured spectrophotometrically.

DNA digestion and adduct enrichment

DNA (10–100 µg) was digested to nucleotides by using MN (1U per 10 µg DNA, 2 mM TRIS–HCl /0.2 mM $CaCl_2$ at pH 8.8, 2 h) followed by SPD digestion (10 mU per 10 µg DNA, 5 mM ammonium acetate pH 4, 2 h) (Kumar *et al.* 1996). All digested samples were stored in a freezer (max. 3 h at –20 °C). The sample volume was adjusted to 200 µl and immediately loaded into an SAX column (1cc, Bond Elut, Varian, USA) and subsequently followed by buffer wash (500 µl ammonium formate (AF) 2 mM, pH 8). Effluent was transferred to a second cartridge, eluted and washed with 200 µl of AF buffer. Retained nucleotides in the SAX column were eluted by using AF buffer (0.2 M at pH 4) and transferred to a C-18 column (1 cc, Bond Elut, Varian, USA) and eluted with 4 % MeOH in 50 mM AF pH4. Retained N^6-dAMP adducts were eluted with 50 % MeOH in water. Three fractions were collected: (1) N7-dGPM adducts, (2) normal nucleotides, (3) N^6-dAMP adducts, and lyophilized. The normal nucleotide fraction was diluted with water (1 ml) and NN were quantitated by using HPLC. NN concentration was used to determine the amount of DNA which was digested to nucleotides during enzymatic DNA hydrolysis (Koivisto *et al.* 1997).

After a solid phase extraction N^6-dAMP adducts were further purified by HPLC using a column switching technique. A sample (30 µl) which was spiked with dAdo (1 µg) and 6-Me dAdo (1 µg), was injected to the first column and a UV response at 254 nm was monitored. Effluent was conducted to waste until switched to the second column at the top of the dAdo peak. After column switching the elution of N^6-Me dAdo was monitored. Fraction collection started from 3 min of the retention time of N^6-Me-dAdo and was continued for 5 min (figure 3). The HPLC purification step was repeated once and samples were stored in a freezer until postlabelled. A linear methanol gradient, in a buffer (100 mM AF at pH 2.8) was used with a flow rate of 0.25 ml min^{-1}. The gradient started from 1 % of methanol in the buffer and reached the final concentration (21 %) in 20 min. Between each sample the column was washed for 10 min using 70 % methanol. When 10 samples were analysed the columns were washed for 4 h with ascending and descending methanol gradients.

Postlabelling

An aliquot of a purified sample was evaporated to dryness. N7-dGMP adducts were labelled using 5 U of phosphatase free T4 kinase (Boehringer-Mannheim, 50 mM TRIS–HCl at pH 9.0, 10 mM $MgCl_2$, 10 mM mercaptoethanol) followed by nuclease P1 treatment (1 µg in 1 µl of 0.4 M Na-acetate and 20 mM $ZnCl_2$ pH 4.5, 15 min). N^6-dAMP adducts were postlabelled using 8 U of cloned T4 kinase (USB, 50 mM TRIS–HCl at pH 8.0, 10 mM $MgCl_2$, 10 mM mercaptoethanol) followed by nuclease P1 treatment (1 µg in 1 µl of 0.4 M Na-acetate and 20 mM $ZnCl_2$ pH 4.5, 15 min). 3´dGMP standards were postlabelled at the same time. By doing this we were able to control radioactive decay and variation of T4 kinase activity. Corresponding non-radioactive adducted 5´ purine nucleotides were applied on a T LC plate followed by addition of radioactive sample. The adducted non-radioactive 5´purines on TLC worked as UV markers on HPLC, and they were also used to quantitate extraction efficiences from TLC and depurination of N7-dGMP adducts occurring during the analysis. Labelled samples (4.5 µl) were analysed on a TLC plate by eluting about 18 cm with 0.2 M AF pH 8 (N7-dGMP adducts ~ 25 %

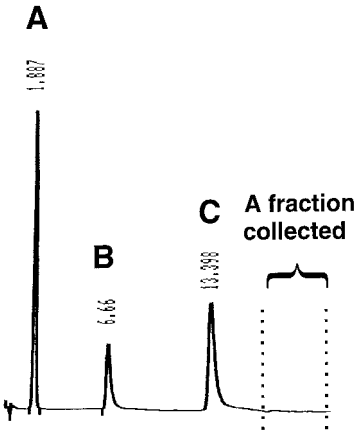

Figure 3. An HPLC chromatogram which demonstrates N^6-3´dAMP BMO adduct enrichment method. (A) indicates the retention time of dAdo, the marker compound used in column switching, (B) the retention time of dAdo in an analytical column, (C) the retention time N^6-Me-dAdo. The fraction was collected from 3 to 8 min after the elution of N^6-Me-dAdo.

HCOOH in MeOH (N^6-dAMP adducts). TLC plates were dried and adducts were localized by exposing a film for 1 h (Kodak, X-OMAT AR). N7-dGMP adducts had an *Rf* value of 0.3 and N^6-dAMP adducts had an *Rf* value of 0.6. Adducts were scraped from the plate and extracted two times with 85 µl of 1 M ammonium formate (pH 6.5 for N7 adducts and pH 4 for N^6 adducts).

HPLC with radioactivity detector

Three different HPLC conditions were applied on the analysis of N7 and N^6 adducts:

1. N7-dGMP adducts: A linear methanol gradient at a flow rate of 0.45 ml min^{-1} was used, starting from 2 % of methanol and reaching 42 % in 40 min (buffer was AF 0.6 M at pH 4.5). Scintillation liquid was pumped to a 500 µl loop at a flow rate of 1.2 ml min^{-1}. The UV detector was set to 254 nm.

2. N^6-dAMP adduct analysis with a high sensitivity: Linear methanol gradient from 2% to 42% methanol in 0.6 M AF at pH 3.5. The radioactivity detector was equipped with a 2 ml teflon loop. Other parameters were the same as above.

3. N^6-dAMP adduct analysis with a high resolution: A linear methanol gradient from 1% to 21% for 40 min with 1 M AF buffer at pH 5.2. Scintillation liquid flow rate was set to 1.8 ml min^{-1} and a 500 µl loop was used. Other parameters were identical to method No. 1.

Results

Adduct formation in vitro

The recovery of N7-dGMP and N^6-dAMP adducts were 31 % and 9.5 % respectively. The effect of standard deviation to the results was controlled with the aid of an internal standard (i.e. the corresponding adducted non-radioactive 5´ monophosphate). On the postlabelling conditions applied the adducts formed to C-2 carbon of the epoxide had a lower labelling efficiency than the adduct formed to C-1 carbon of the epoxide in both adducted purine standards. This property of the T4 kinase would have resulted in a systematic error of the C-1 to C-2 ratio but was avoided by measuring the labelling efficiency of each isomer (Kumar *et al.* 1996).

Exposure of Ct-DNA (1 mg ml^{-1} 24 h) at various concentrations of racemic BMO produced a linear increase of N^6-dAMP and N7-dGMP adducts, respectively. The dose response is demonstrated in table 1 in which the total levels of the adducts are presented. The elution orders in relation to stereochemistry of the adducts have been determined in our previous papers [illegible] the BMO

Table 1. The amount of N^6-dAMP and N7-dGMP adducts formed in Ct-DNA after BMO exposure (24 h, 37 °C, pH 7.4).

Exposure (nmol mg^{-1} DNA)	N7 adduct level (fmol per 10 μg DNA)	N^6 adduct level (fmol per 10 μg DNA)
0	0	0
44	140	0.5
174	630	2.3
870	2500	18

enantiomers in adduct preparation (Koivisto *et al.* 1995, 1997). These markers were utilized in this study. The formation of N7 regioisomers was 52 and 48 % (C-1:C-2) and the corresponding amounts of N^6 adducts was 14 and 86 % (C-1:C-2) (figure 4). From an analytical point of view the pH of the eluting buffer considerably affected resolution of the isomers of N^6 adducts. At pH 5.2 we were able to quantitate the regioisomers (C-1 and C-2) of N^6 adducts. To increase the sensitivity pH was adjusted to pH 3.5 – the pH value in which all isomers of N^6-dAMP adducts eluted as one peak (figure 5).

Adduct formation in vivo

Five days of inhalation exposure to BD caused a clear purine alkylation in mouse lung. Table 2 presents the total level of N^6-dAMP and N7-dGMP adducts. The amounts of C-1 and C-2 regioisomers of N^6 adducts was 31 and 69 %±3% and was independent of exposure level. Unfortunately, an acceptable separation between the four N^6 diastereomers was not achieved. In contrast, all regio- and diastereomers of N7-dGMP adducts were separated on HPLC. Again, the ratio of the isomers was independent of the exposure level, for example exposure at 500 ppm resulted in 60 % of C-1 adducts and 40 % of C-2 adducts. The diastereomeric *S* to *R* ratio of the C-1 adducts was 5:1 and the corresponding *S* to *R* ratio of C-2

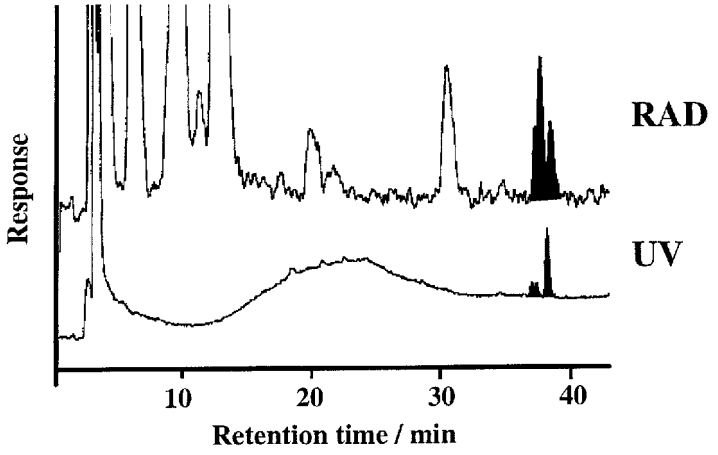

Figure 4. HPLC analysis of N^6-dAMP adducts formed in Ct-DNA after BMO exposure. The adducts were detected after postlabelling using a radioactivity detector (RAD). The internal standard (non-radioactive N^6-BMO-5′dAMP adducts) were detected with UV detection (UV).

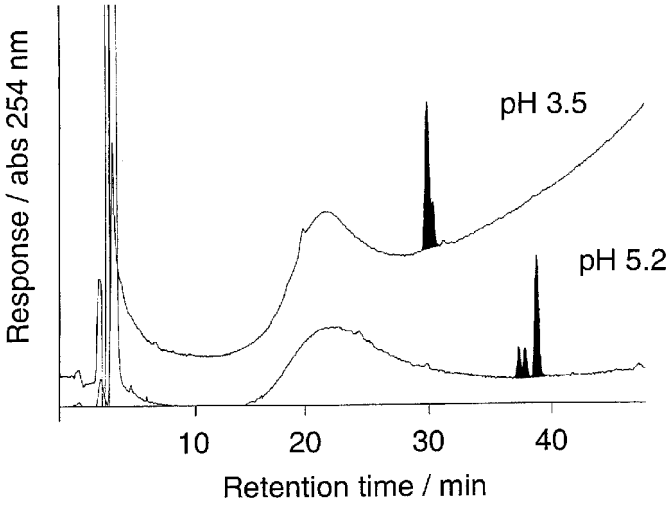

Figure 5. Reversed phase HPLC chromatograms of N^6-BMO adducts of 5′dGMP eluted at two different pH values of the eluent.

adducts was 3:1. The adducts formed to N7-dGMP from *S*-BMO represented 77 % of the total adducts. Figure 6 shows a typical chromatogram of the N7 adducts formed in mouse lung.

We had an opportunity to analyse mouse lung samples killed 21 days after inhalation exposure to 1300 ppm of BD. No N7-BMO adducts were detected, but the N^6 lesion was present at about the same level as in animals sacrified immediately after the last exposure (figure 7).

Discussion

Adduct analysis

One of the difficulties in analysing aliphatic DNA adducts by using the ^{32}P-postlabelling technique is the enrichment of adducts. We did not apply the nuclease P1 technique because we could not confirm that N^6 adducts of BMO would be nuclease P1 resistant, even though P1 treatment has recently been used to enrich N^6-dAMP adducts of allylglycidylether (Plna and Segerback 1997). The other adduct enrichment method in use is a butanol extraction technique which can be

Table 2. DNA adduct levels in purine bases in mouse lung after inhalation exposure to 1,3-butadiene (5 days, 6 h per day). The number of analyses is 4.

Exposure (ppm)	N7 adduct level (fmol ± SD per 10 μg DNA)	N^6 adduct level (fmol ± SD per 10 μg DNA)
0	0	0
200	2.8 ± 0.9	nd[a]
500	11 ± 2.0	0.09 ± 0.06
1300	30 ± 6.7	0.11 ± 0.05
1300[b]	nd[a]	0.20 ± 0.15

[a] nd, not detected.
[b] Animals killed 21 days after exposure.

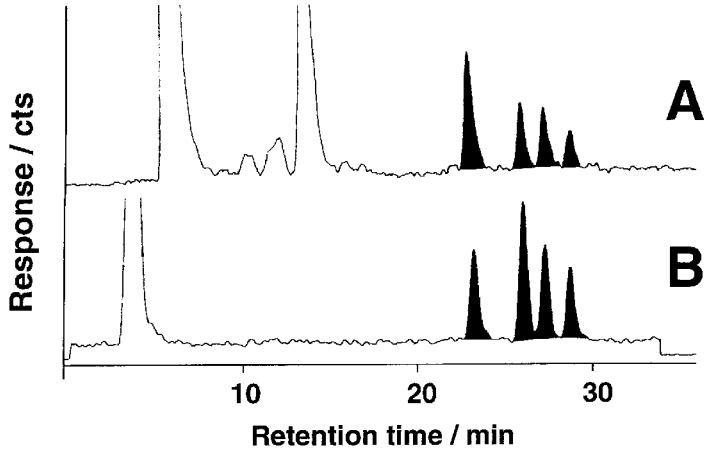

Figure 6. N7-dGMP BMO adducts detected in mouse lung after postlabelling. (A) Radiochromatogram derived from mouse lung and (B) the synthesized standards.

used for large lipophilic adducts – presumably not applicable to butadiene-derived adducts. HPLC separation combined with fraction collection has previously been applied to various types of adducts (Wilson *et al.* 1988, Kato *et al.* 1993). An alternative method for HPLC enrichment would be the use of monoclonal antibodies, but unfortunately specifically raised antibodies against BMO adducts were not available (Guichard *et al.* 1993). N7-dGMP adducts are typically enriched by solid phase extraction with ion exchange columns (Kumar *et al.* 1995).

We have found that HPLC-based adduct enrichment is error prone, because of column loading. Column loading is indicated by the presence of normal nucleotides in effluent and seen as normal nucleotide 'spots' in postlabelling analysis. Our experience indicates that column washing between the two analyses is of critical

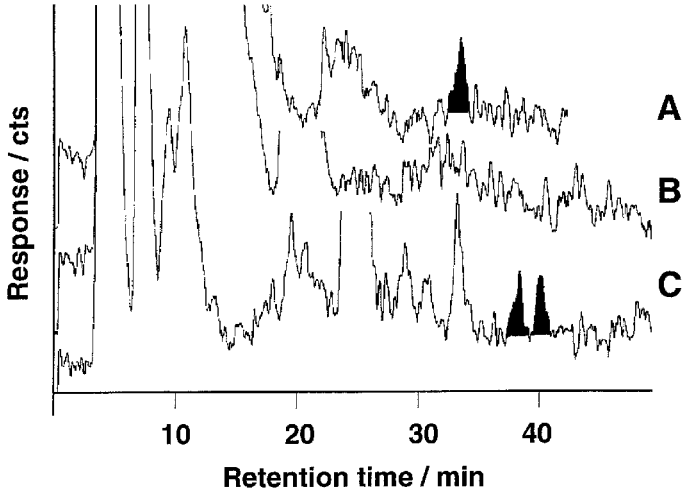

Figure 7. N^6-5′dAMP BMO adducts in mouse lung 3 weeks after exposure. (A) An analysis carried out at pH 3.5 (exposed 1300 ppm), (B) a control lung sample analysed at pH 3.5, (C) same sample as in chromatogram (A) but analysed at pH 5.2 (exposed to 1300 p

importance. Enough washing can only be confirmed by collecting HPLC effluent and detect normal nucleotides by postlabelling. If high amounts of normal nucleotides are detected a full check of the instrumentation is needed. The most probable reasons are 'dead volume' in the HPLC system or improper packing material in the HPLC column. We have tested several C-18 columns and ended up using a narrow bore (2 ×100 mm) column with a large pore size packing material (300 Å) to diminish column loading. Column switching also effectively decreases the amount of NN entering the analytical column.

Adducts in vitro

BMO concentrations used in *in vitro* experiments are close to those found in animal tissues, assuming that 1 g of lung tissue contains about 1 mg DNA (Qiagen 1995). The formation of BMO N7-dGMP adducts in DNA reaches its maximum value after 5 h of exposure and the adducts have a half-life of 48 h in DNA (Neagu *et al.* 1995). The kinetics of N^6 adduct formation is somewhat problematic because of Dimroth rearrangement. Dimroth rearrangement involves the initial adduct formation at the N1 position followed by the intramolecular ring opening and reclosure. In adenosine BMO adduct formation at the N1 position is reported to be fast and the rearrangement rate about 7 h. N1 adducted dAdo can neutralize the positive charge by deamination or depurination (Selzer and Elfarra 1996a, Tretyakova *et al.* 1997). It is important to notice that the nucleotide/nucleoside chemistry is very different to the chemistry involved in adduct formation in DNA and indeed allyl glycidyl ether N1 adducts have been recently reported to slowly disappear in DNA (Plna *et al.* 1996). Our value for the N^6-dAdo and N7-dGuo ratio is about four times less than has been reported for the N1-dAdo to N7-dGuo ratio (Tretyakova *et al.* 1997). We interprete these data to mean that N1 adducts of BMO in DNA do not rearrange as readily as nucleoside/nucleotide adducts. However, the observed difference may also be due to the different analytical methods used.

The regioisomeric adenine N^6 alkylation products formed in Ct-DNA *in vitro* at physiological conditions contained mainly C-2 adducts (86 %). In a recent paper by Tretjakova *et al.* an equimolar amount of regioisomeric alkylation products (C-1 to C-2 ratio 1:1) were detected at the N1 position of adenine residue in DNA (Tretyakova *et al.* 1997). Deamination has been reported to be more common for C-2 adducts of styrene oxide and BMO than for C-1 adducts (Qian and Dipple 1995, Selzer and Elfarra 1996a). Based on the observation that C-2 adducts tend to deaminate this could indicate that the high percentage of the C-2 N^6 adducts we have detected *in vitro* may have derived from the direct alkylation of the N^6 position in dAdo. If a direct reaction does not play a role in DNA alkylation the detected N^6 adducts of BMO should be C-1 rich rather than C-2 rich (Qian and Dipple 1995, Selzer and Elfarra 1996a).

DNA alkylation in vivo

If the depurination rates of N7 adducts are assumed to be the same as measured *in vitro* the level of adducts measured *in vivo* is mainly a reflection of the exposure of the last 2 days. In our recent communication we have measured N7-dGMP BMO adducts in mouse testis, and interestingly the one N7 adduct of each

enantiomer had disappeared, which indicates an active structure specific DNA repair (Koivisto *et al.* 1998). If we assume that active repair of N7 adducts takes place *in vivo* we can conclude that the repair is linear because a dose response was achieved. The half-lives of ethylene oxide adducts in mouse liver were observed to vary from 12 h to 24 h, which is a substantially shorter time than was observed *in vitro* (Segerback 1983, Walker *et al.* 1992). A short half-life time can be explained by an active DNA repair of the lesion (Singer and Hang 1997), but on the other hand clearly longer half-lives have been detected in rats (Walker *et al.* 1992).

An interesting observation is that the adducts are formed and/or repaired regio- and enantioselectively *in vivo*. Of the N7 adducts, 60 % had a C-1 configuration which is higher than observed *in vitro*, in which C-1 and C-2 regioisomers are found in equimolar ratio. The amount of N7 adducts from the *S* and *R* isomers of BMO in mouse lung were 77 and 23%. Because the DNA adduct formation is not stereospecific *in vitro*, indicating that the chemistry involved in adduct formation is not stereoselective, it is reasonable to assume that it is not stereospecific *in vivo* either (Kumar *et al.* 1996). If this hypothesis is accepted the data suggest that there is a stereospecific activation of BD and/or stereospecific detoxification of epoxides involved, and/or DNA repair acting stereoselectively or presumably the data are a combination of all these factors.

Stereospecific metabolism of BD is poorly studied. Mouse liver microsomes have been used to activate BD and 54% of the formed BMO had an *S* configuration and 46% an *R* configuration (Wistuba *et al.* 1989). In the same study the phenobarbital treatment induced the formation of the *R* enantiomer, resulting in an *S* to *R* ratio of 39 and 61%. These observations were supported in a recent paper in which mouse liver microsomes were found to synthesize a slight excess of *S*-BMO enantiomer. In that study the enantiomeric ratios of BMO were not significantly affected by TCPO, an inhibitor of epoxy hydrolase (Nieusma *et al.* 1997). However, the role of stereospecific glutathione conjugation to the persistence of BMO was not studied.

By chance we got an opportunity to analyse purine alkylation in animals killed 3 weeks after exposure. N7-dGMP adducts were not found in these animals, but N^6-dAMP adducts were present at about the same level as just after exposure. This indicates that N^6 adducts are chemically stable and that there is not an efficient DNA repair machinery for these adducts in mouse lung. This observation also indicates that N^6-dAMP adducts can accumulate during a long term exposure, which could be a valuable observation keeping in mind the adverse effects of BD. A slightly higher, but not statistically significant, level of N^6 adducts could have been accumulated as BMO in fat tissue (Thornton-Manning *et al.* 1997). The role of chemoselectivity in purine alkylation is not clear, but it has been demonstrated that the capability of PAH diolepoxides to aralkylate of adenine residue in DNA correlates with the carcinogenic potency (Dipple *et al.* 1987).

To understand better the chemical carcinogenesis of butadiene it is important to measure several markers of exposure such as epoxides and their hydrolysis products in tissues and in blood as well as protein or DNA adducts. These studies should include stereochemical aspects of metabolism, adduct formation and repair.

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